# Supplementary Appendix

Increasing Artemisinin-Resistant HRP2-Negative Malaria in Eritrea

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This appendix has been provided by the authors to give readers additional information about their work.

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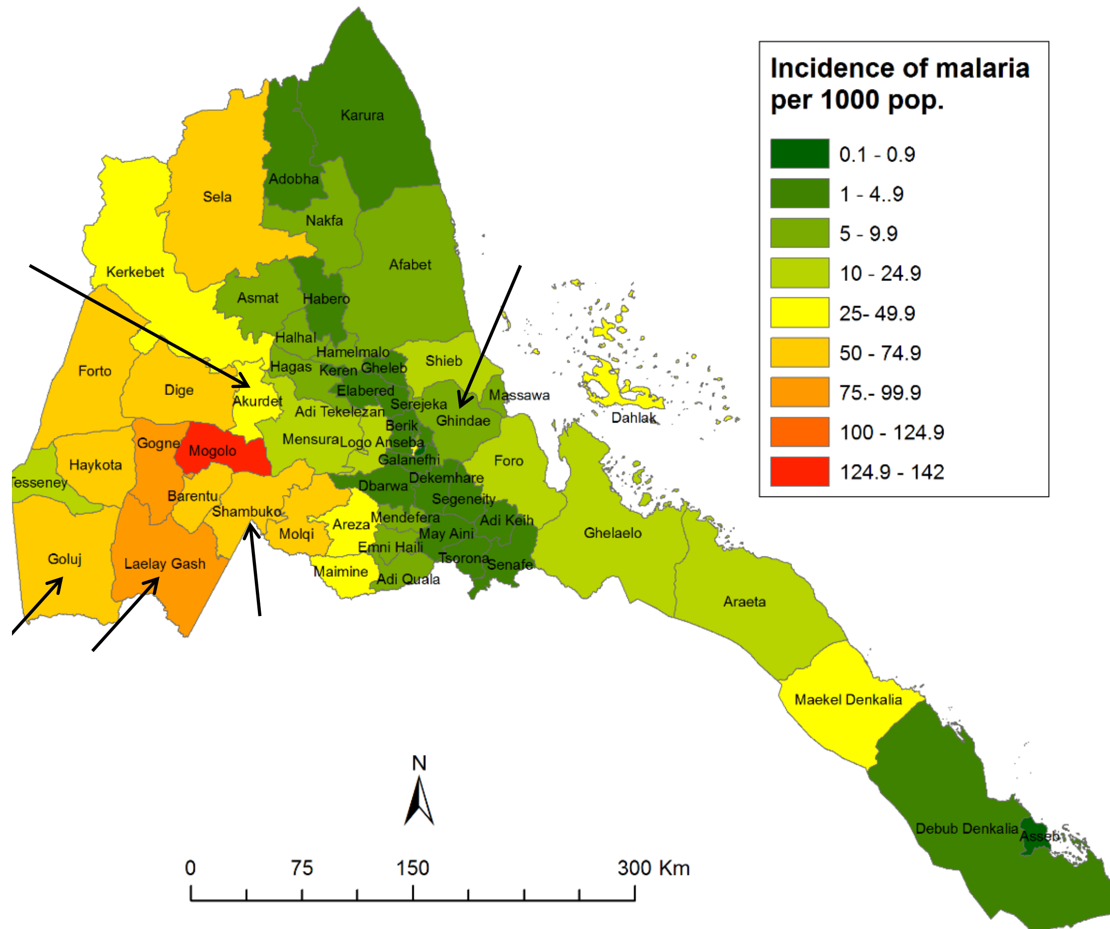
# 1. Contributions

S.M., A.B., A.Z.K., P.R., D.A.F. and D.M. contributed to study design. SM, AB and AZK, M.W. supervised the clinical drug studies, collected clinical samples and data. L.P., N.P., E.L., C. D-L, and D.M. prepared the DNA. C. D-L, N.P., L.M., P.C., A.C. and D.M. performed the sequencing and sequence analyses. B.H.S., K.E.W, D.A.F., L.P., E.L. and D.M. performed genome-editing and in vitro assays. S.M., A.B., A.Z.K., P.C., A.C., P.R., D.A.F. and D.M. analyzed data. D.M. wrote the first draft of the manuscript, with contributions from D.A.F, B.H.S., K.E.W., P.R., M.W., S.M. and A.B. All authors reviewed and approved the manuscript. S.M., A.B., A.Z.K., D.A.F, P.R., and D.M. decided to publish the paper

## 2. Supplementary Information

### 2.1. Malaria incidence in the study sites.

The malaria incidence in the study sites is presented in the map below, published in the National Malaria Strategic Plan 2021-2026, Ministry of Health, State of Eritrea in March 2020.[1]




The study sites are presented by the arrows. They were Ghindae city (located in the Ghindae subzoba/district), Goluj (or Guluj) city (in the Goluj/Guluj subzoba), Akordat city (in the Akordat/Akurdet subzoba), Shambuko town (in the Shambuko subzoba) and Tekombi town (in the Laelay Gash subzoba).

### 2.2 Clinical drug efficacy trial oversight and blood sample collection

***2.2.1. Screening and recruitment of study participants.*** Eligible patients were at least 6 months of age, presenting with suspected uncomplicated *P. falciparum* infection with fever (axillary temperature ≥37.5°C) and/or history of fever in the past 24 h. Participants were enrolled if they were subsequently confirmed to have parasitemia ranging from 250 to 200,000 parasites per microliter and were able to attend follow-up visits until day28 post initiation of treatment.[2] Enrolled patients were assigned to receive a supervised standard 3-day course of ASAQ [Sanofi, 25/67.5 (artesunate/amodiaquine) mg/day for 2-11 months (≥4.5kg to <9kg), 50/135 mg/day for 1-5 years (≥9kg to <18kg), 100/270 mg/day (≥18kg to <36kg), 150/405 mg/day (≥36kg to <50kg), 200/540 mg/day (≥50kg)] in 2016 and 2019, or AL (IPCA 20 mg artemether and 120 mg lumefantrine per tablet; 6 tablets for 5kg to <15kg;

12 tablets for 15 to <25kg; 18 tablets for 25 to <35kg; 24 tablets for ≥35kg) in 2017 according to the manufacturer's dosing schedule.

Informed written consent was obtained from the adult patient or parent/caretaker of a child prior to recruitment. If a patient or parent/caretaker was illiterate, consent was obtained in the presence of a witness (witness' signature and the thumbprint of the participant's parent/caretaker). Children aged 12 years and above also signed informed assent in addition to their parents' consent.

Studies were approved by the Health Research Proposal Review and the Ethical Committee in Eritrea (2016, 2017 and 2019) and by the WHO Ethical Review Committee (2019). Studies were registered on the Australian New Zealand Clinical Trials Registry (ANZCTR) (https://www.anzctr.org.au/, ANZCTR12618000353291, ANZCTR12618001223224 and ANZCTR12619000859189).

| **Trial registration** | ACTRN12618001223224 | ACTRN12618000353291 | ACTRN12619000859189 |
|---|---|---|---|
| Date of registration | 20/07/2018 | 08/03/2018 | 17/06/2019 |
| Date the first patient was enrolled | 15/09/2017 | 08/09/2016 | 01/08/2019 |
| Number of patients enrolled before registration | 280 | 211 | |
| Explanation of the delay | The first two studies (2016 & 2017) were not sponsored by the WHO. The country did not know that registration was a requirement as therapeutic efficacy studies were considered surveillance and not research by the national malaria programme. However, while negotiating the 2019 study, WHO informed the Eritrean National Malaria Programme about this requirement and supported retrospective registration of the two prior studies (2016 & 2017) and proactive registration of the last study (2019). | | |

***2.2.2. Clinical follow-up assessments.*** Patients were monitored clinically and parasitologically at recruitment (day0) and on days 1, 2, 3, 7, 14, 21, and 28 post initiation of treatment, and any other unscheduled days if symptoms occurred. Thick and thin smears were obtained by finger prick prior to the initiation of treatment (day0) and on scheduled and unscheduled follow up visits for detection of malaria parasites and estimation of parasite density. Patients were advised to come to the health facilities for unscheduled visits if symptoms developed. The health facilities were open 24/7 for such patients. Supervisory visits were conducted during the study period by the Zoba/province malaria and the National Malaria Control Programme teams to supervise the Study Registers, data collections forms, and laboratory procedures. Blood samples were spotted onto filter papers for molecular studies on days 0, 7, 14, 21 and 28.

***Primary outcome***

Artemisinin partial resistance (ART-R) is defined as delayed parasite clearance (persistent parasitemia detected by microscopy at day3 in patients enrolled in Therapeutic Efficacy Studies) after treatment with a drug containing an artemisinin derivative (i.e., Artemisinin-based Combination Therapies such as ASAQ and AL), of a parasite strain carrying a particular mutation or set of mutations that are validated as associated with this delayed clearance, despite the administration and absorption of the drug given in doses equal to or higher than those usually recommended. ART-R is confirmed in a site or a country when a quality-controlled study using an ACT or an artesunate monotherapy finds more than 5% of patients with delayed parasite clearance, as shown either by persistent parasitemia detected by microscopy at 72 hours (± 2 hours, i.e., day 3, D3+) or a parasite clearance half-life ≥ 5h, carrying *Pfkelch13* resistance-validated mutations. Therefore, **we evaluated as primary the day3 positivity rate (D3+),[3] defined as the proportion of patients who were still parasitemic on day3 after initiation of treatment as assessed by microscopic examination of thick blood smears.**
Validated *Pfkelch13* mutations are those that have been significantly associated with delayed parasite clearance and found to confer *in vitro* ART-R using the Ring-stage Survival Assay (RSA). Candidate

markers are *Pfkelch13* mutations significantly associated with delayed parasite clearance or identified to confer *in vitro* ART-R using RSA. The list of validated and candidate markers is kept up to date on the WHO Global Malaria Programme website (https://www.who.int/teams/global-malaria-programme/case-management/drug-efficacy-and-resistance/antimalarial-drug-efficacy-database, accessed March 3, 2023).

***Secondary outcome***

The WHO recommends that the national treatment guideline be changed if the treatment failure rate exceeds 10% in a study that complies with the WHO protocol. **We evaluated, as secondary outcome, the PCR-adjusted clinical response to the designated treatment on day28. PCR correction (see next section 1.2.3., Parasite genotyping for details), used to differentiate recrudescence from reinfection in recurrent infection samples, was carried out by genotyping the recommended genetic markers *msp1*, *msp2* and *polya* (a polymorphic microsatellite marker) in the paired pre-treatment and post-treatment samples.**

PCR-adjusted clinical efficacy rates for ASAQ and AL at day28 were calculated per site to evaluate ACT treatment outcomes using complete-case (proportional) and Kaplan-Meier (cumulative survival) estimates. Patients who were lost or withdrew or had new infection (defined by parasite genotyping) during the follow-up or missing outcome data were excluded from the complete-case estimates. To calculate Kaplan-Meier estimates, patients who were lost or withdrew or had new infection during the follow-up or missing outcome data were censored on the last day of the patient follow-up. Patients with indeterminate PCR results were excluded from both complete-case and Kaplan-Meier estimates.[4]

***Sample size***

According to the thresholds defined by the WHO for ART-R (5%) and ACT treatment failure (10%), we calculated the sample size (both addressing assessment of ART-R and ACT treatment efficacy) per site based on assumptions summarized in the table below:

| Outcomes | Confidence level | Expected proportion (%) | Confidence interval width (2-sided) | Minimum required participant size |
|---|---|---|---|---|
| Primary | 95.0 | 0.001 | 0.05 | 73 |
| Secondary | 95.0 | 0.95 | 0.1 | 73 |

With a 20% increase to allow loss to follow-up and withdrawals during the 28-day follow-up period, the target number of participants was estimated to 88 (73 x 1.2) per site. For the material described in the paper, the power calculations refer to the original protocols and are not directly relevant to the analyses presented herein.

***2.2.3. DNA extraction and molecular analysis.*** Parasite DNA was extracted from dried blood spots (DBS) collected pre and post treatment (the latter in case of recurrence) using the QIAamp DNA Blood Mini Kit (Qiagen).

***Parasite genotyping.*** Parasite genotyping was carried out by PCR amplification, according to the recent WHO recommendation (2021).[5] We amplified paired DNA samples (day0 and day of recurrence - dayX) of recurrent patients using the recommended genetic markers *msp1*, *msp2* and *polya* (a polymorphic microsatellite marker). Band sizes of amplicons were estimated by capillary electrophoresis (Agilent). The cut-off settings for PCR artefacts and stutter peaks were defined for peaks <10% of the low and upper control bands. The bins used to define a match were ± 10bp for *msp1/msp2* and ± 5bp for *polya*. The genotypes of parasites on day0 and on the day of recurrence were compared to classify infections as recrudescence (same genotype) or new infection (different genotype).

The WHO/Medicines for Malaria Venture decision algorithm was used to define PCR-adjusted clinical efficacy rates. Recrudescence was defined as a genotype that had already been detected in the blood sample taken before treatment (i.e., at least one allele was shared at day0 and dayX for all three loci). A new infection was defined as the absence of a shared allele between day0 and dayX at any of the three loci. We also performed comparative analysis using the *msp1, msp2* and *glurp* markers (the

previous WHO recommendation for genotyping). The bins used to define a match were ± 20bp for *glurp*. The 2/3 decision algorithm was also used for research purposes. Thereby, recrudescence was defined as a genotype that had already been detected in the blood sample taken before treatment (i.e. at least one allele was shared at day0 and dayX at the *msp1* and *msp2* loci). In case of discordance between *msp1* and *msp2*, a third genotyping marker (*polyα* or *glurp*) was used as the deciding factor.

***Molecular signatures associated with antimalarial drug resistance.*** Parasite DNA extracted from blood obtained at day0 and on the day of recurrence – dayX - was analysed for the presence of point mutations in the *Pfkelch13* propeller domain (codons 430-720) associated with ART-R, and in *pfcrt* (at codons 72-76, 93, 97, 145, 218, 343, 350, 353 and 356), *pfmdr-1* (at codons 86, 184, 1034, 1042 and 1246), *dhfr* (at codons 16, 50, 51, 59, 108, 164), and *dhps* (at codons 431, 436, 437, 540, 581, 613) genes associated with 4-aminoquinoline, amino-alcohol, pyrimethamine and sulfadoxine resistance, respectively.[3] We also tested for deletions in *hrp2* and *hrp3*, which results in variable performance of HRP2-based malaria RDTs.[6]

Amplicons from targeted regions (presented in the table below) were generated using multiplexing nested PCR assays with indexed primers containing specific tags (barcodes). A total of 4 µl of PCR reactions from each sample were mixed in one pool (96 samples) to increase the sample volume and minimize sample usage for downstream protocol steps. For each pool, amplicons were purified with AMPure XP beads (Beckman Coulter) according to manufacturer's protocol to eliminate dNTPs, salts, primers, and primer dimers. The quality of purified PCR products was assessed by analysing eluates containing the purified amplicons on a Fragment Analyzer (Agilent). DNA concentrations of pooled fragments were assessed by fluorometric quantitation (Qubit, Thermo Fischer). The pooled libraries were denatured with NaOH to a final concentration of 0.1 N and diluted with hybridization buffer before sequencing. Sequencing was performed using the MiSeq v2 reagents using the 300-cycle kit (Illumina) according to the manufacturer's recommendations.

***List of the targeted regions and number of amplicons generated by multiplexing nested PCR assays.***

| Gene Name | Gene ID | Chr. | Gene location | SNPs/CNVs detection | No. of amplicons |
|---|---|---|---|---|---|
| ***Pfkelch13*** | PF3D7_1343700 | 13 | 1,724,817 - 1,726,997 (-) | SNP from codon 440 to 727 | 12 |
| ***chloroquine resistance transporter*** | PF3D7_0709000 | 7 | 403,222 - 406,317 (+) | Codons 72-76, 93, 97, 145, 218, 343, 350, 353 | 5 |
| ***multidrug resistance protein 1*** | PF3D7_0523000 | 5 | 957,890 - 962,149 (+) | Codons 86, 184, 1034, 1042, 1246 | 5 |
| ***bifunctional dihydrofolate reductase-thymidylate synthase*** | PF3D7_0417200 | 4 | 748,088 - 749,914 (+) | Codons 16, 50, 51, 59, 108, 164 | 5 |
| ***hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase*** | PF3D7_0810800 | 8 | 548,200 - 550,616 (+) | Codons 436, 437, 540, 581, 613 | 4 |
| ***histidine-rich protein 2*** | PF3D7_0831800 | 8 | 1,373,212 – 1,376,988 (-) | Deletion | 2 |
| ***histidine-rich protein 3*** | PF3D7_1372200 | 13 | 2,840,236 – 2,842,840 (-) | Deletion | 2 |

Raw sequences were demultiplexed and quality trimmed at a phred score of 30. Primer sequences were trimmed from the 5′-end of the sequences to avoid primer bias in the sequenced fragments. Base calling was performed by comparing reads with a custom database consisting of the 3D7 reference sequence (v45). Bioinformatic analyses were performed using the CLC Genomics Workbench 22 software (Qiagen). Laboratory reference parasite strains (Dd2, 7G8, HB3 and the 3601 strain, a Cambodian culture-adapted parasite) with known alleles of each gene were used as controls, as presented in the table below.

*List of the alleles of the parasite strains used as controls*

| Gene name | Dd2 allele | 7G8 allele | HB3 allele | 3601 strain |
|---|---|---|---|---|
| ***Pfkelch13*** | Wild-type | Wild-type | Wild-type | C580Y |
| ***chloroquine resistance transporter*** | CVIETTHFIMCGT | SVMNTTHFIMCGT | CVMNKTHFIMCGI | CVIETTHFIMCGT |
| ***multidrug resistance protein 1*** | YYSND | NFCDY | NFSDD | NFSND |
| ***bifunctional dihydrofolate reductase-thymidylate synthase*** | ACIRNI | ACICNI | ACNCNI | ACIRNI |
| ***hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase*** | IFGKAS | ISGKAA | ISAKAA | IAGEAA |
| ***histidine-rich protein 2*** | Deletion | - | - | - |
| ***histidine-rich protein 3*** | - | - | Deletion | - |

*Position of amino acid corresponds to codons 72, 73, 74, 76, 93, 97, 145, 218, 343, 350, 353 and 356 for pfcrt, to codons 86, 184, 1034, 1042 and 1246 for pfmdr-1, to codons 16, 50, 51, 59, 108, 164 for dhfr and to codons 431, 436, 437, 540, 581, 613 for dhps.*

***Parasite whole-genome sequencing, phylogenetic and haplotype analyses.*** We performed selective whole-genome amplification (sWGA) using short oligonucleotides of 8–12 mers as primers as previously described,[7] on 285 DNA extracts from day0 dried blood samples. The choice of samples and the mutant vs wild-type ratio was determined by selecting all *Pfkelch13* 622I mutants (109, of which 107 were successfully sequenced) and including a selection of *Pfkelch13* wild-type parasites paired with *Pfkech13* 622I mutant by site and year. According to the availability of the *Pfkelch13* wild-type parasites and the cost for the SWGA-based sequencing, we selected a range of mutant to wild-type of between 2:1 and 6:1, as described in the following table.

| Year | *Pfkelch13* variant | Site | | | | **Total** |
|---|---|---|---|---|---|---|
| | | Akordat | Guluj | Shambuko | Tokombia | |
| 2016 | *Pfkelch13 622I* | 5 | 4 | 4 | 7 | **20** |
| | *Pfkelch13 wild-type* | 12 | 12 | 12 | 17 | **53** |
| 2017 | *Pfkelch13 622I* | | 0 | 3 | 6 | **9** |
| | *Pfkelch13 wild-type* | | 11 | 17 | 16 | **44** |
| 2019 | *Pfkelch13 622I* | 10 | 8 | 8 | 8 | **34** |
| | *Pfkelch13 wild-type* | 34 | 35 | 8 | 48 | **125** |
| **Total** | | **61** | **70** | **52** | **102** | **285** |

The sWGA reaction was performed in a volume of 50 µl containing >5 ng of template DNA, BSA (New England Biolabs), 1 mM dNTPs (New England Biolabs), 2.5 µM of each amplification primer, 1× Phi29 reaction buffer (New England Biolabs), and 30 units of Phi29 polymerase (New England Biolabs). Samples were amplified on a C1000 Touch Thermal Cycler (Bio-Rad) programmed to run a "stepdown" protocol consisting of 35 °C for 5 min, 34 °C for 10 min, 33 °C for 15 min, 32 °C for 20 min, 31 °C for 30 min, 30 °C for 16 h, then heating at 65 °C for 15 min to inactivate the enzymes prior to cooling to 4 °C. Amplicons were then quantified using the Qubit® dsDNA high sensitivity assay (Thermo Fisher Scientific) to determine whether there was enough material for sequencing (minimum required was 500 ng of product). Library preparations of amplified samples and short-read high-throughput sequencing sWGA products (≥500 ng total DNA) were purified using AMPure XP beads (Beckman Coulter) according to the manufacturer's instructions. Purified amplicons were used to prepare a PCR-free Illumina library using the NEBNext DNA sample preparation kit (New England Biolabs) for high-throughput sequencing. DNA libraries were sequenced at the Genomic Platform at Institut Pasteur Paris using Illumina NextSeq 2000 instruments and Illumina V.3 chemistry. For each sequenced sample, read alignments against the chromosome sequences of *P. falciparum* 3D7 v45 (done with bwa mem) were processed to infer consensus sequences.

For phylogenetic analyses, each of the 290 read alignments (128/285 Eritrean *P. falciparum* sequences generated for this study, 162 publicly available sequences) were processed using SAM2MSA (https://gitlab.pasteur.fr/GIPhy/SAM2MSA) to infer the pseudo-sequences of the 14 chromosomes (see details in Uwimana et al. 2020).[8] All inferred pseudo-chromosomes were pooled into 14 matrices of aligned nucleotide characters, and every aligned character containing > 15% degenerated character states was discarded. The 14 resulting multiple sequence alignments were concatenated into a supermatrix of 8,468,008 nucleotide characters. Phylogenetic analysis was carried out using IQ-TREE v2.1.3 with evolutionary model TVM+F+R6 (assessed by minimizing the BIC criterion).[9,10]

The Genome Analysis Toolkit (GATK) was used to identify single nucleotide polymorphisms (SNPs) in isolates following GATK best practices.[11] Low-quality sites were excluded based primarily on two parameters: GQ ≥ 30 and QUAL ≥ 40. Lastly, calls from sites exhibiting a low call rate across the whole dataset were discarded.
Based on genotyped data, pairwise Euclidean genetic distances were computed between samples in extended 200-kb and 1Mb windows around the mutation, to assess genetic structure at finer genomic scale. Principal Coordinate Analysis (PCoA), hierarchical clustering as well as AMOVA (Analysis of Molecular Variance) were then run. Principal Coordinate Analysis (PCoA) and hierarchical clustering were used to evaluate whether *P. falciparum* isolates tended to cluster together depending on (i) their *Pfkelch13* genotype (wild-type or R662I mutation); (ii) their geographical origin (site); (iii) their sampling date (year). Then, AMOVA (Analysis of Molecular Variance) was run to estimate the amount of genetic differentiation among groups (at the same 1Mb genomic window): *P. falciparum* Eritrean isolates were split into three groups (wild-types, R622I mutants, unknown *Pfkelch13* genotype), while other isolates were grouped depending on the country they originated from. A matrix of pairwise indices of genetic differentiation ($0 < \Phi ST < 1$) thus calculated among groups. Further analysis of extended haplotype homozygosity was performed around mutated positions to compare both wild-type and mutant haplotypes.

***2.2.4. Generation of gene-edited lines and in vitro susceptibility assays.*** To evaluate the *in vitro* susceptibilities of *Pfkelch13* R622I mutant parasites to dihydroartemisinin, CRISPR-Cas9 was used to genetically modify the *Pfkelch13* locus in the NF54 and Dd2 reference lines, resulting in the $NF54^{R622I}$ and $Dd2^{R622I}$ lines. To achieve this, a *Pfkelch13*-propeller domain-specific guide RNA (gRNA) was cloned into an all-in-one CRISPR-Cas9 vector at the BbsI restriction sites using the primer pair p1/p2 (see list below) to generate the pDC2-cam-coSpCas9-U6-gRNA-hdhfr plasmid.[12] A *Pfkelch13* donor sequence harboring the mutations of interest was generated by site-directed mutagenesis of a 1.5 kb region of the *Pfkelch13*-propeller domain, which was amplified using the primer pair p3/p4 and cloned into the pGEM T-easy vector system (Promega). Silent shield mutations were introduced at the Cas9 cleavage site using the primer pair p5/p6, and allele-specific mutations were introduced using the primer pairs p7/p8 for the R622I mutation. *Pfkelch13* donor sequence was amplified from pGEM using the primer pair p9/p10 and sub-cloned into the pDC2-cam-coSpCas9-U6-gRNA-hdhfr vector at the EcoRI and AatII restriction sites by In-Fusion® Cloning (Takara). The final plasmid (pDC2-cam-coSpCas9-U6-gRNA-*Pfkelch13*$^{R622I}$-hdhfr) was sequenced using primers p11, p12 and p13.

***List of oligonucleotides used for the generation of gene-edited lines.***

| Name | Nucleotide sequence (5'-3') | Description | Fidock Lab Name |
|---|---|---|---|
| p1 | TATTACACATAGCTGATGATCTAG | *Pfkelch13* gRNA fwd | p6558 |
| p2 | AAACCTAGATCATCAGCTATGTGT | *Pfkelch13* gRNA rev | p6287 |
| p3 | GTGACGTCGATTGATATTAATGTTGGTGGAGC | *Pfkelch13* CRISPR/Cas9 donor amplification fwd | p3984 |
| p4 | CCGCATATGGTGCAAACGGAGTGACCAAATCTGGG | *Pfkelch13* CRISPR/Cas9 donor amplification rev | p3986 |

| | | | |
|---|---|---|---|
| p5 | GAATACGCCAAGATCATCAGCTATGTGTGTTGCTTTTGATAATAAAATTTATGTCATTGG | SDM *Pfkelch13* shield mutations fwd | p6090 |
| p6 | GCAACACACATAGCTGATGATCTTGGCGTATTCAAAGGTGCCACCTCTACCC | SDM *Pfkelch13* shield mutations rev | p6091 |
| p7 | CCATATGCCTTATTAGAAGCTAtAAGTTCAGGAGCAGCTTTTAATTACC | SDM *Pfkelch13* R622I fwd | p7912 |
| p8 | GGTAATTAAAAGCTGCTCCTGAACTTaTAGCTTCTAATAAGGCATATGG | SDM *Pfkelch13* R622I rev | p7913 |
| p9 | GAGGTACCGAGCTCGAATTCGAAACGGAATTAAGTGATGCTAG | *Pfkelch13* CRISPR/Cas9 donor EcoRI In-Fusion fwd | p6655 |
| p10 | CGAAAAGTGCCACCTGACGTCAAACGGAGTGACCAAATCTGGG | *Pfkelch13* CRISPR/Cas9 donor AatII In-Fusion rev | p6656 |
| p11 | AACATATGTTAAATATTTATTTCTC | CRISPR/Cas9 donor sequencing fwd | p282 |
| p12 | AGGGTTATTGTCTCATGAGCGG | CRISPR/Cas9 donor sequencing fwd | p283 |
| p13 | AAGCACCGACTCGGTGCCAC | gRNA sequencing rev | p35 |
| p14 | GGGAATCTGGTGGTAACAGC | *Pfkelch13* integration primer fwd (5' end) | p6176 |
| p15 | CGGAGTGACCAAATCTGGGA | *Pfkelch13* integration primer rev (3' end) | p6175 |
| p16 | GGTATTAAATTTTTACCATTCCCATTAGTATTTTGTATAGG | *Pfkelch13* sequencing fwd (internal) | p4186 |

*fwd, forward; rev, reverse; SDM, site-directed mutagenesis*

*P. falciparum* asexual blood-stage parasites were cultured in human erythrocytes (at 3% hematocrit) and RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 mg/L hypoxanthine, 25 mM HEPES, 0.225% $NaHCO_3$, 10 mg/L gentamycin and 0.5% w/v Albumax II (Invitrogen). At Institut Pasteur, Paris, human red blood cells were extracted from human peripheral blood sampled on healthy volunteers through the ICAReB platform (Clinical Investigation & Access to Research Bioresources) from the center for translational science, Institut Pasteur.[9] All participants received an oral and written information about the research and gave written informed consent in the frame of the healthy volunteers Diagmicoll cohort (Clinical trials NCT 03912246) after approval of the CPP Ile-de-France I Ethics Committee (April 30$^{th}$, 2009) and CoSImmGEn cohort (Clinical trials NCT 03925272). At Columbia University, Irving Medical Center, human red blood cells were obtained as anonymized, de-identified samples from a commercial blood bank (Interstate Blood Bank, Memphis, TN, USA) and washed to remove leukocytes. Parasites were maintained at 37ºC in 5% $O_2$, 5% $CO_2$, and 90% $N_2$. Cultures were monitored by blood smears fixed in methanol, stained with Giemsa, and viewed by light microscopy.

Transfections were performed by electroporating ring-stage parasites at 5–10% parasitemia with 50 µg of purified circular plasmid DNA resuspended in Cytomix. Transfected parasites were maintained under 2.5 nM WR99210 (Jacobus Pharmaceuticals, Princeton, NJ, USA) to select for editing events. Parasite cultures were monitored for recrudescence microscopically for up six weeks post electroporation. To test for successful editing, the *Pfkelch13* locus was amplified directly from whole blood using the primer pair p14/p15 and the MyTaq™ Blood-PCR Kit (Bioline Meridian Bioscience, Cincinnati, OH, USA). PCR products were submitted for Sanger sequencing using primer p16. Bulk-edited cultures were cloned via limiting dilution and flow cytometry was used to screen for positive wells after 17-20 days. Parasites were stained with 1× SYBR Green (Thermo Fisher, Waltham, MA, USA) and 100 nM MitoTracker Deep Red (Invitrogen, Grand Island, USA), and positive wells were detected by flow cytometry using an iQue flow cytometer (IntelliCyt, Albuquerque, NM, USA).

*In vitro* ring-stage survival assays ($RSA_{0\text{-}3h}$) were conducted on early ring-stage parasites (0-3 hours post-invasion; hpi).[13] These assays included the $NF54^{R622I}$ and $Dd2^{R622I}$ CRISPR/Cas9-edited lines, as well as the $NF54^{WT}$, $Dd2^{WT}$ and the $NF54^{C580Y}$ line as controls. Briefly, tightly synchronized 0-3 hpi rings were exposed to a pharmacologically-relevant dose of 700 nM dihydroartemisinin (DHA) or 0.1% dimethyl sulfoxide (DMSO; vehicle control) for 6h, washed three times with RPMI to remove drug, transferred, and cultured for an addition 66h in drug-free medium. Parasitemia were measured at 72 h by microscopy (D. Ménard lab, Institut Pasteur) as previously described[13] or by flow cytometry (D. Fidock Lab, Columbia University) with 50,000-100,000 events captured per sample.[7] Parasite survival rates are expressed as ratios of viable parasites in DHA- to DMSO-treated samples. The threshold for resistance in the $RSA_{0\text{-}3h}$ was considered to be a survival rate of >1%.

***2.2.5. Outcome classification, molecular and statistical analysis.*** Data were reported in Microsoft Excel and analysed with MedCalc 20.027 (MedCalc Software) and GraphPad Prism 9.3.1 (GraphPad Software). The primary analysis used complete cases only and ignored data from subjects with missing outcome data. The Kaplan-Meier analyses were conducted as an alternative to the complete-case analysis. Because the analyses presented here were not originally specified in the protocols for the three component studies, all analyses are descriptive. 95% confidence intervals [CI] are provided for all estimates, but these have not been adjusted for multiple comparisons and were not used in place of hypothesis tests.

## 3. Supplementary Figures

**Fig. S1. Patient workflow: enrollment and outcomes.**

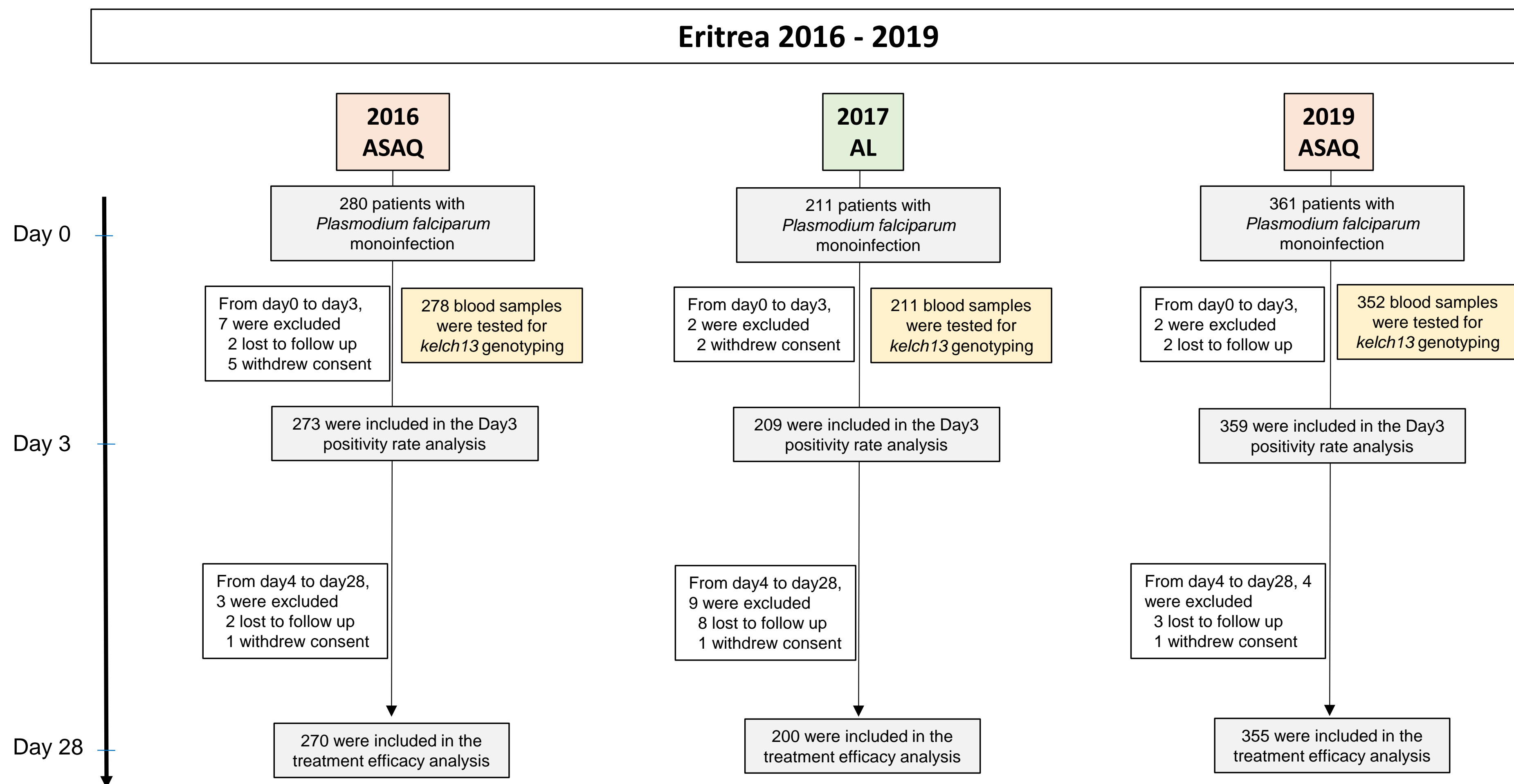
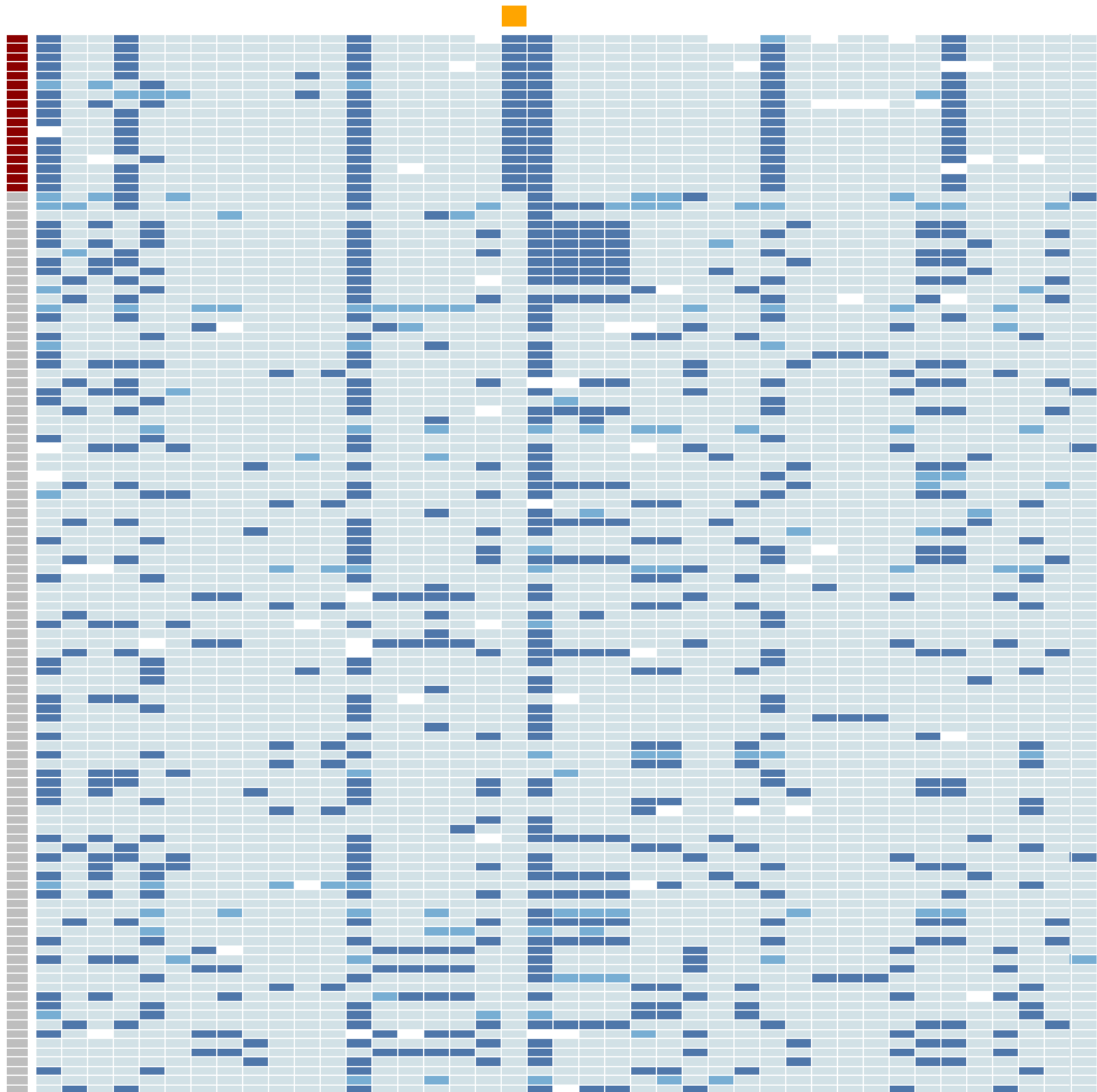
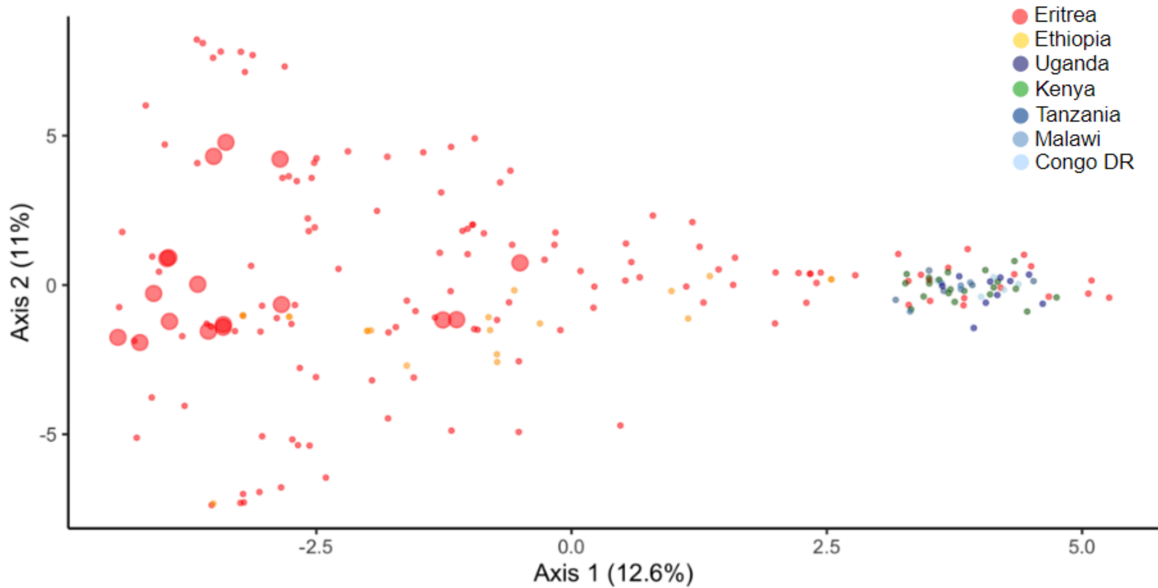


**Fig. S2. Comparison of pseudo-haplotypes in a 200 kb window on chromosome 13 around the R622I mutation (100 kb on both sides of the mutation).**



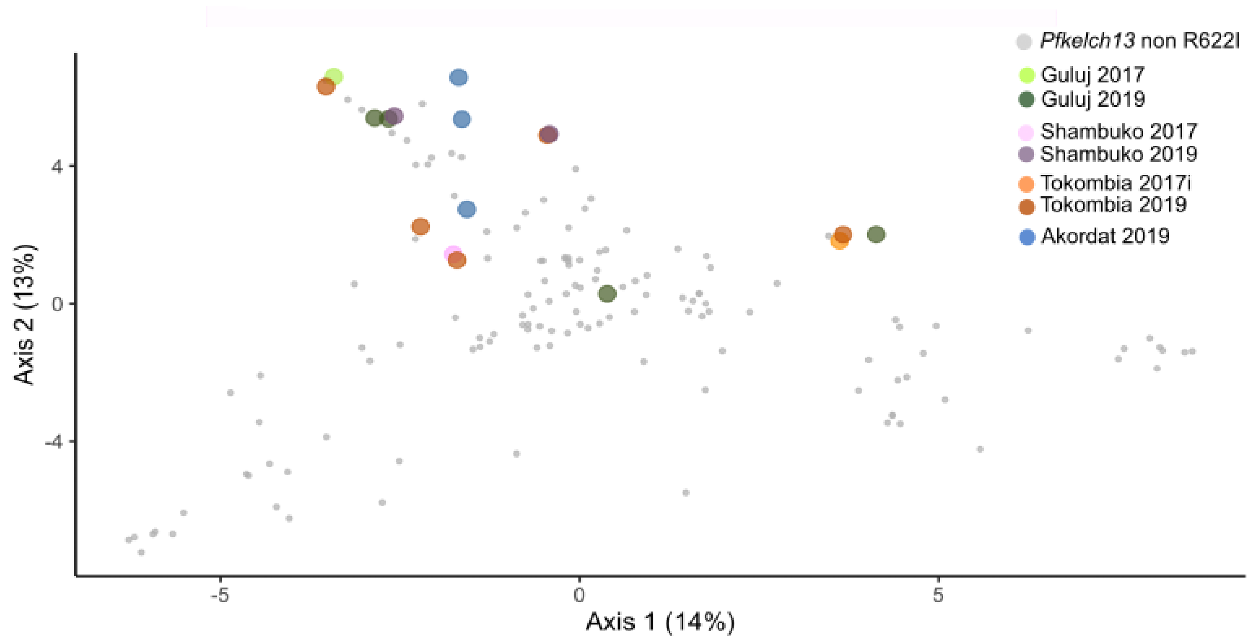
Each cell represents a single position. The *Pfkelch13* 622I mutation is flagged with the orange cell at the top. Light blue cells correspond to the reference allele (from the 3D7 genome), dark blue cells correspond to the alternate allele, medium-blue cells correspond to ambiguous non-haploid type at this position, and empty cells to missing values. Each row represents one isolate, with isolates color-coded according to their wild-type (grey) or mutant (red) status. The presence of a single shared haplotype surrounding the 622I mutation in Eritrean isolates of *P. falciparum* is consistent with a single origin of the genetic background on which the mutation arose. Further hierarchical clustering and bifurcation analyses (***Fig. S5 & S6***) provide evidence that this origin of the 622I mutation in Eritrean isolates was not a recent event.

**Fig. S3. Principal Coordinate analysis (PCO) based on pairwise genetic distances in a 1 Mb window) at regional level.**



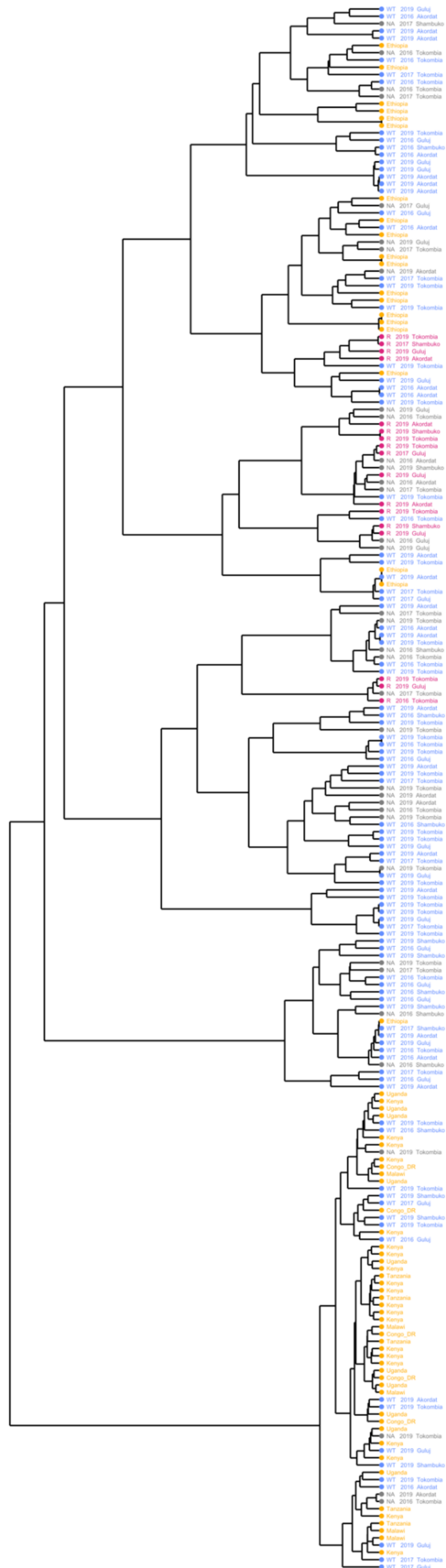

*Pfkelch13* 622I mutants are represented by large dots, and *Pfkelch13* wild-type isolates from Eritrea as well as isolates originating from various other East African countries sourced from a public database (the MalariaGEN Plasmodium falciparum Community Project; https://www.malariagen.net/apps/pf/4.0) are represented by small dots color coded according to their origin.

**Fig. S4. Principal Coordinate analysis (PCO) based on pairwise genetic distances in a 1 Mb window at country level.**



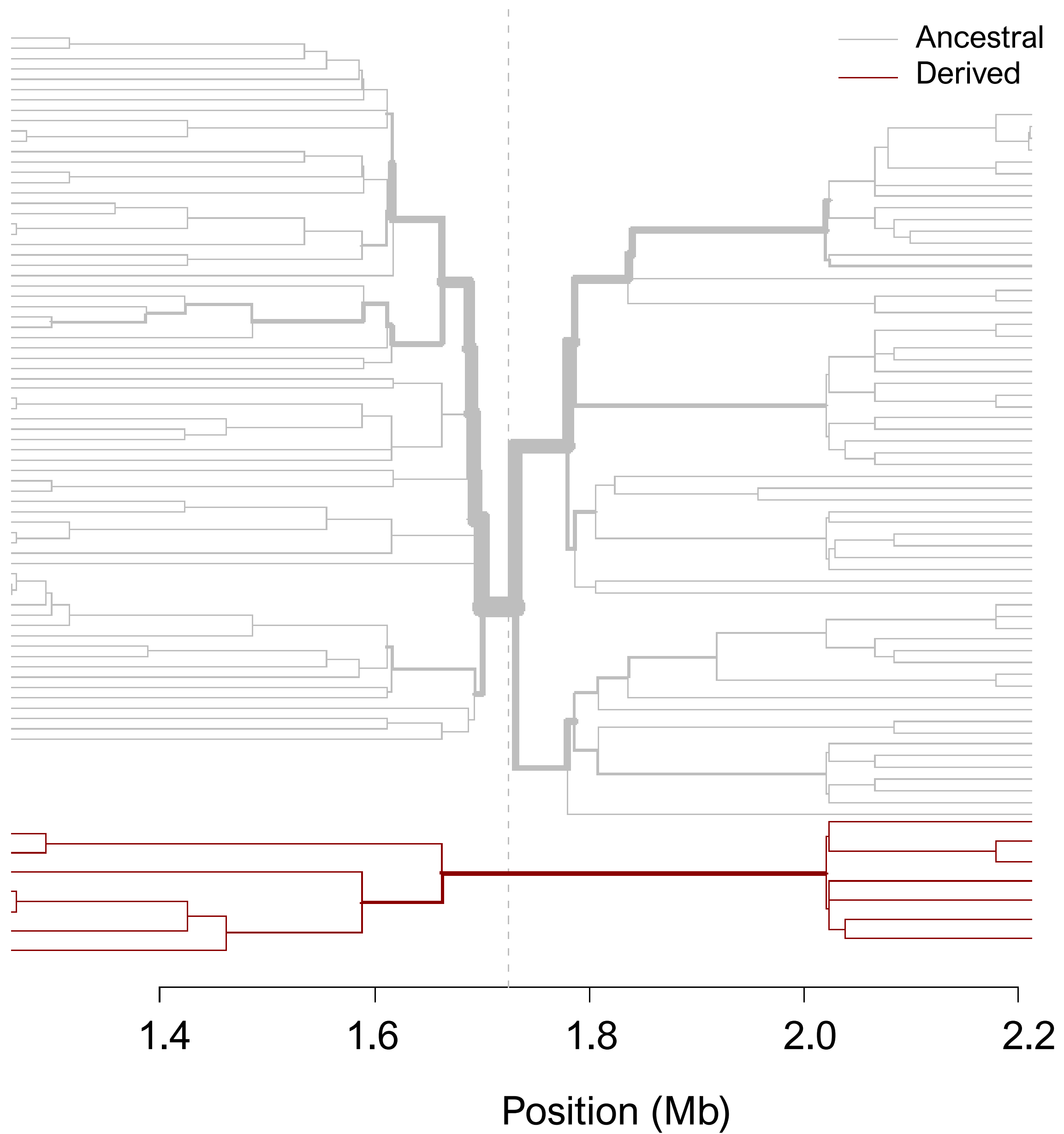

*Pfkelch13* 622I isolates are represented by large dots and the *Pfkelch13* wild-type isolates from Eritrea by grey small dots. Dot color represents the year and site at which the mutant isolate was collected.

**Fig. S5. Hierarchical clustering (using Ward's algorithm) based on pairwise genetic distances in a 1Mb window.**



*Pfkelch13* 622I mutants are represented in red and *Pfkelch13* wild-type isolates from Eritrea in blue. Isolates for which mutational status could not be properly determined are represented in grey. Orange labels correspond to individuals originating from various East African countries, sourced from a public database (the MalariaGEN *Plasmodium falciparum* Community Project; https://www.malariagen.net/apps/pf/4.0). The labels of Eritrean isolates display the following information: type WT or M (wild-type or mutant), sampling year, site of origin. NA, missing information.

**Fig. S6. Bifurcation diagram of haplotypes around the R622I mutation**




The genomic position at which the 622I mutation was observed is indicated by the dashed vertical line. Wild-type (ancestral) individuals are in grey and *Pfkelch13* 622I mutant isolates are in red.

## 4. Supplementary Tables

**Table S1. D3+ rate per year and site, Eritrea, 2016-2019**

| Site | Year | | | | | |
|---|---|---|---|---|---|---|
| | 2016 | | 2017 | | 2019 | |
| | N | % D3 + | N | % D3 + | N | % D3 + |
| Akordat | 54 | 0 | 19 | 0 | 88 | 0 |
| Ghindae | - | - | 15 | 0 | - | - |
| Guluj | 71 | 0 | 23 | 0 | 95 | 1.1 |
| Shambuko | 73 | 1.4 | 64 | 6.2 | 88 | 5.7 |
| Tokombia | 75 | 0 | 88 | 0 | 88 | 10.2 |
| **Total** | **273** | **0.4** | **209** | **1.9** | **359** | **4.2** |

% D3+: Percentage of patients who were still parasitemic on day3 after initiation of treatment as assessed by microscopic examination of thick blood smears, (D3+). N: Sample size.

**Table S2. Clinical outcomes of the participants per year and treatment, Eritrea, 2016-2019.**

| Year | 2016 | | | | 2019 | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | ASAQ | | | | ASAQ | | | |
| Site | Akordat | Guluj | Shambuko | Tokombia | Akordat | Guluj | Shambuko | Tokombia |
| Sample size | 58 | 73 | 73 | 76 | 88 | 97 | 88 | 88 |
| Early treatment failure | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Recurrent infections | 0 | 6 | 3 | 5 | 0 | 2 | 6 | 5 |
| Days 15-21 | - | 3 | 2 | 3 | - | - | 3 | 3 |
| Days 22-28 | - | 3 | 1 | 2 | - | 2 | 3 | 2 |
| ACPR | 54 | 64 | 70 | 68 | 88 | 92 | 80 | 82 |
| Withdrawn | 3 | 2 | 0 | 1 | 0 | 0 | 1 | 0 |
| Lost to follow-up | 1 | 1 | 0 | 2 | 0 | 3 | 1 | 1 |

ASAQ: Artesunate-Amodiaquine; ACPR: Adequate Clinical and Parasitological Response.

| Year | 2017 | | | | |
|---|---|---|---|---|---|
| Treatment | AL | | | | |
| Site | Akordat | Ghindae | Guluj | Shambuko | Tokombia |
| Sample size | 19 | 15 | 25 | 64 | 88 |
| Early treatment failure | 0 | 0 | 0 | 0 | 0 |
| Recurrent infections | 0 | 0 | 0 | 0 | 0 |
| Days 15-21 | - | - | - | - | - |
| Days 22-28 | - | - | - | - | - |
| ACPR | 16 | 14 | 23 | 61 | 86 |
| Withdrawn | 0 | 0 | 2 | 1 | 0 |
| Lost to follow-up | 3 | 1 | 0 | 2 | 2 |

AL: Artemether-Lumefantrine; ACPR: Adequate Clinical and Parasitological Response.

**Table S3. Raw data of *msp-1*, *msp-2* and *poly α* polymorphisms (band size in bp) detected on day0 and day of recurrence (dayX) in isolates from recurrent infections, and classification of treatment outcomes, Eritrea, 2016-2019.**

| ID Patient | Site | Year | Treatment | Uncorrected Outcome | Day of recrudescence (DayX) | *msp-1* gene (allele) | | | | | | | *msp-2* gene (allele) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Mad20 | | K1 | | RO33 | | Conclusion *msp-1* | 3D7 | | FC27 | | Conclusion *msp-2* |
| | | | | | | Day0 | DayX | Day0 | DayX | Day0 | DayX | | Day0 | DayX | Day0 | DayX | |
| 2016_01_113 | Guluj | 2016 | AS+AQ | TF | Day28 | 180 | 220 | 250 | 580 | - | - | Reinfection | 250 | 250 | 900 | 800 | Recrudescence |
| 2016_01_15 | Guluj | 2016 | AS+AQ | TF | Day28 | - | 180 | 120/420 | - | 135 | 135 | Recrudescence | - | 250 | - | 550 | Indeterminate |
| 2016_01_50 | Guluj | 2016 | AS+AQ | TF | Day28 | - | - | - | - | 135 | 135 | Recrudescence | 250 | 250 | 800 | 800 | Recrudescence |
| 2016_01_54 | Guluj | 2016 | AS+AQ | TF | Day21 | - | - | - | - | 135 | 135 | Recrudescence | 250/300 | 250/300 | 850 | - | Recrudescence |
| 2016_01_68 | Guluj | 2016 | AS+AQ | TF | Day21 | - | - | 200 | - | 135 | 135 | Recrudescence | 270 | - | 950 | 650 | Reinfection |
| 2016_01_79 | Guluj | 2016 | AS+AQ | TF | Day21 | 180 | - | 500/580 | - | 135 | 135 | Recrudescence | 270 | 270 | - | - | Recrudescence |
| 2016_21_175 | Shambuko | 2016 | AS+AQ | TF | Day21 | 180 | 180 | - | - | - | - | Recrudescence | 350 | 350 | - | - | Recrudescence |
| 2016_32_192 | Shambuko | 2016 | AS+AQ | TF | Day28 | - | 220/280 | - | - | 135 | 135 | Recrudescence | 250 | 250 | - | - | Recrudescence |
| 2016_55_227 | Shambuko | 2016 | AS+AQ | TF | Day21 | 180 | 220/280 | 500/580 | 580 | 135 | 135 | Recrudescence | 250 | - | - | 750 | Reinfection |
| 2016_03_07 | Tokombia | 2016 | AS+AQ | TF | Day26 | 220 | - | - | - | 135 | 135 | Recrudescence | 250 | 250 | - | 750 | Recrudescence |
| 2016_03_108 | Tokombia | 2016 | AS+AQ | TF | Day21 | 220 | - | - | - | 135 | 135 | Recrudescence | 250 | - | 750 | 750 | Recrudescence |
| 2016_03_15 | Tokombia | 2016 | AS+AQ | TF | Day21 | 180 | 220 | 500/580 | 500/580 | 135 | 135 | Recrudescence | 250 | 250 | 700 | 900 | Recrudescence |
| 2016_03_69 | Tokombia | 2016 | AS+AQ | TF | Day25 | - | 220 | 220 | - | 135 | 135 | Recrudescence | - | 200/350 | 700 | 900 | Reinfection |
| 2016_03_98 | Tokombia | 2016 | AS+AQ | TF | Day21 | 180 | 180 | - | - | 135 | 135 | Recrudescence | - | - | 700 | 700 | Recrudescence |
| 01_259_19 | Guluj | 2019 | AS+AQ | TF | Day28 | 190 | 190 | 232 | - | - | - | Recrudescence | 280/410 | 280/410 | - | - | Recrudescence |
| 01_85_19 | Guluj | 2019 | AS+AQ | TF | Day21 | - | - | - | - | 135 | 135 | Recrudescence | 280 | 280 | - | 500 | Recrudescence |
| 05_208_19 | Shambuko | 2019 | AS+AQ | TF | Day21 | - | - | - | - | 135 | 135 | Recrudescence | - | - | 500 | 500 | Recrudescence |
| 05_246_19 | Shambuko | 2019 | AS+AQ | TF | Day28 | - | - | 295 | - | - | 135 | Reinfection | 423 | - | 501 | - | Indeterminate |
| 05_356_19 | Shambuko | 2019 | AS+AQ | TF | Day28 | 266 | - | 293 | - | 135 | 135 | Recrudescence | 280 | 280 | 340 | - | Recrudescence |
| 05_371_19 | Shambuko | 2019 | AS+AQ | TF | Day21 | 216/253 | 268 | - | 295 | 135 | 135 | Recrudescence | 270 | 270 | - | 500 | Recrudescence |
| 05_512_19 | Shambuko | 2019 | AS+AQ | TF | Day28 | 230 | 232 | - | - | 135 | 135 | Recrudescence | 280/300 | 280/300 | 505 | - | Recrudescence |
| 05_544_19 | Shambuko | 2019 | AS+AQ | TF | Day21 | 214 | - | - | 223 | - | 135 | Reinfection | - | 280 | 480 | 340/500 | Reinfection |
| 03_01_19 | Tokombia | 2019 | AS+AQ | TF | Day21 | - | - | - | - | - | - | Indeterminate | 310 | 310 | - | - | Recrudescence |
| 03_155_19 | Tokombia | 2019 | AS+AQ | TF | Day28 | 264 | 264 | - | - | - | - | Recrudescence | 200/400 | 200/400 | - | - | Recrudescence |
| 03_183_19 | Tokombia | 2019 | AS+AQ | TF | Day21 | 248 | 232 | - | - | 135 | 135 | Recrudescence | 275 | 275 | - | - | Recrudescence |
| 03_26_19 | Tokombia | 2019 | AS+AQ | TF | Day28 | 220 | 220 | - | - | - | - | Recrudescence | 310 | 310 | - | - | Recrudescence |
| 03_84_19 | Tokombia | 2019 | AS+AQ | TF | Day21 | - | - | - | - | 135 | 135 | Recrudescence | - | - | 500 | 500 | Recrudescence |

| ID Patient | Site | Year | Treatment | Outcome | Day of recrudescence (DayX) | Poly α microsatellite | | | WHO/MMV interpretation |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Day0 | DayX | Conclusion | |
| 2016_01_113 | Guluj | 2016 | AS+AQ | TF | Day28 | 184 | 211 | Reinfection | **Reinfection** |
| 2016_01_15 | Guluj | 2016 | AS+AQ | TF | Day28 | 184 | 184/223 | Recrudescence | **Recrudescence** |
| 2016_01_50 | Guluj | 2016 | AS+AQ | TF | Day28 | 182/208 | 183 | Recrudescence | **Recrudescence** |
| 2016_01_54 | Guluj | 2016 | AS+AQ | TF | Day21 | 184 | 184/234 | Recrudescence | **Recrudescence** |
| 2016_01_68 | Guluj | 2016 | AS+AQ | TF | Day21 | 188 | 175 | Reinfection | **Reinfection** |
| 2016_01_79 | Guluj | 2016 | AS+AQ | TF | Day21 | 168 | 182/205 | Reinfection | **Reinfection** |
| 2016_21_175 | Shambuko | 2016 | AS+AQ | TF | Day21 | 184 | 184 | Recrudescence | **Recrudescence** |
| 2016_32_192 | Shambuko | 2016 | AS+AQ | TF | Day28 | 198 | 198 | Recrudescence | **Recrudescence** |
| 2016_55_227 | Shambuko | 2016 | AS+AQ | TF | Day21 | 182 | 200 | Reinfection | **Reinfection** |
| 2016_03_07 | Tokombia | 2016 | AS+AQ | TF | Day26 | 182 | 182 | Recrudescence | **Recrudescence** |
| 2016_03_108 | Tokombia | 2016 | AS+AQ | TF | Day21 | 192 | 192 | Recrudescence | **Recrudescence** |
| 2016_03_15 | Tokombia | 2016 | AS+AQ | TF | Day21 | 198 | 184 | Reinfection | **Reinfection** |
| 2016_03_69 | Tokombia | 2016 | AS+AQ | TF | Day25 | 201 | 211 | Reinfection | **Reinfection** |
| 2016_03_98 | Tokombia | 2016 | AS+AQ | TF | Day21 | 192 | 190 | Recrudescence | **Recrudescence** |
| 01_259_19 | Guluj | 2019 | AS+AQ | TF | Day28 | 186 | 183 | Recrudescence | **Recrudescence** |
| 01_85_19 | Guluj | 2019 | AS+AQ | TF | Day21 | 186 | 188 | Recrudescence | **Recrudescence** |
| 05_208_19 | Shambuko | 2019 | AS+AQ | TF | Day21 | 214/241 | 213 | Recrudescence | **Recrudescence** |
| 05_246_19 | Shambuko | 2019 | AS+AQ | TF | Day28 | 201 | 211 | Reinfection | **Reinfection** |
| 05_356_19 | Shambuko | 2019 | AS+AQ | TF | Day28 | 190 | 209 | Reinfection | **Reinfection** |
| 05_371_19 | Shambuko | 2019 | AS+AQ | TF | Day21 | 191 | 211/241 | Reinfection | **Reinfection** |
| 05_512_19 | Shambuko | 2019 | AS+AQ | TF | Day28 | 201/214/240 | 203 | Recrudescence | **Recrudescence** |
| 05_544_19 | Shambuko | 2019 | AS+AQ | TF | Day21 | 206 | 177 | Reinfection | **Reinfection** |
| 03_01_19 | Tokombia | 2019 | AS+AQ | TF | Day21 | 198 | 198 | Recrudescence | **Recrudescence** |
| 03_155_19 | Tokombia | 2019 | AS+AQ | TF | Day28 | 211 | 211 | Recrudescence | **Recrudescence** |
| 03_183_19 | Tokombia | 2019 | AS+AQ | TF | Day21 | 183/208 | 211 | Recrudescence | **Recrudescence** |
| 03_26_19 | Tokombia | 2019 | AS+AQ | TF | Day28 | 198 | 200 | Recrudescence | **Recrudescence** |
| 03_84_19 | Tokombia | 2019 | AS+AQ | TF | Day21 | 208 | 211 | Recrudescence | **Recrudescence** |

ASAQ: Artesunate-Amodiaquine; MMV: Medicines for Malaria Venture; TF: treatment failure, WHO: World Health Organization.

**Table S4. PCR-corrected Kaplan-Meier and complete-case estimates of ASAQ and AL efficacy by genotyping approach at day28, Eritrea, 2016-2019 (Panel A and Panel B according to the decision algorithm).**

Molecular genotyping (= PCR correction) was performed to distinguish new *P. falciparum* infections from recrudescence in patients in whom recurrent parasitemia appeared during follow-up.

***Panel A. Genotyping interpretation based on 3/3 decision (or WHO/MMV) algorithm*** *(Recrudescence was defined as a genotype that had already been detected in the blood sample taken before treatment; at least one allele was shared at day0 and day X at all three loci. A new infection was defined as the absence of a shared allele between day0 and dayX at any of the three loci).*

| | | Treatment efficacy (PCR-corrected), % (95%CI) | | | | | |
|---|---|---|---|---|---|---|---|
| Genotyping markers | | *msp1/msp2/Polyα* | | | *msp1/msp2/glurp* | | |
| Analysis | Site | ASAQ 2016 | AL 2017 | ASAQ 2019 | ASAQ 2016 | AL 2017 | ASAQ 2019 |
| Complete-case | Akordat | 100 (93.4-100) | 100 (79.4-100) | 100 (95.9-100) | 100 (93.4-100) | 100 (79.4-100) | 100 (96.4-100) |
| | Ghindae | - | 100 (76.8-100) | - | - | 100 (76.8-100) | - |
| | Guluj | 95.9 (88.5-99.1) | 100 (85.2-100) | 98.9 (94.1-99.9) | 94.1 (85.6-98.4) | 100 (85.2-100) | 97.9 (92.5-99.7) |
| | Shambuko | 97.3 (90.4-99.7) | 100 (94.1-100) | 97.6 (91.5-99.7) | 97.2 (90.3-99.7) | 100 (94.1-100) | 96.4 (89.8-99.2) |
| | Tokombia | 96.0 (88.8-99.2) | 100 (95.8-100) | 94.3 (87.1-98.1) | 95.8 (88.1-99.1) | 100 (95.8-100) | 95.3 (88.5-98.7) |
| Kaplan-Meier | Akordat | 100 (93.8-100) | 100 (82.3-100) | 100 (95.9-100) | 100 (93.4-100) | 100 (82.3-100) | 100 (95.9-100) |
| | Ghindae | - | 100 (78.2-100) | - | - | 100 (78.2-100) | - |
| | Guluj | 95.9 (88.5-99.1) | 100 (86.3-100) | 98.9 (94.4-99.9) | 94.2 (88.6-98.5) | 100 (86.3-100) | 97.9 (92.7-99.7) |
| | Shambuko | 97.3 (90.4-99.7) | 100 (94.4-100) | 97.7 (92.0-99.7) | 97.2 (90.4-99.7) | 100 (94.4-100) | 96.6 (90.4-99.3) |
| | Tokombia | 96.0 (88.9-99.2) | 100 (95.9-100) | 94.3 (87.2-98.1) | 96.0 (88.9-99.2) | 100 (95.9-100) | 95.4 (88.8-98.7) |

***Panel B. Genotyping interpretation based on 2/3 decision algorithm.*** *[Recrudescence was defined as a genotype that had already been detected in the blood sample taken before treatment; at least one allele was shared at day0 and dayX at msp1 and msp2 loci. In case of discordance between msp1 and msp2, a third genotyping marker (poly α or glurp) was used as the deciding factor].*

| | | Treatment efficacy (PCR-corrected), % (95%CI) | | | | | |
|---|---|---|---|---|---|---|---|
| Genotyping markers | | *msp1/msp2/Polyα* | | | *msp1/msp2/glurp* | | |
| Analysis | Site | 2016 | 2017 | 2019 | 2016 | 2017 | 2019 |
| Complete-case | Akordat | 100 (93.4-100) | 100 (79.4-100) | 100 (96.4-100) | 100 (93.4-100) | 100 (79.4-100) | 100 (96.4-100) |
| | Ghindae | - | 100 (76.8-100) | - | - | 100 (76.8-100) | - |
| | Guluj | 94.5 (86.6-98.5) | 100 (85.2-100) | 97.9 (92.5-99.7) | 94.1 (85.6-98.3) | 100 (85.2-100) | 97.9 (92.5-99.7) |
| | Shambuko | 97.3 (90.4-99.7) | 100 (94.1-100) | 95.2 (88.2-98.7) | 97.2 (90.3-99.6) | 100 (94.1-100) | 95.2 (88.2-98.7) |
| | Tokombia | 94.7 (87.1—98.5) | 100 (95.8-100) | 94.3 (87.1-98.1) | 94.4 (86.3-98.4) | 100 (95.8-100) | 94.3 (87.1-98.1) |
| Kaplan-Meier | Akordat | 100 (93.8-100) | 100 (82.3-100) | 100 (95.9-100) | 100 (93.8-100) | 100 (82.3-100) | 100 (95.9-100) |
| | Ghindae | - | 100 (78.2-100) | - | - | 100 (78.2-100) | - |
| | Guluj | 94.5 (86.5-98.5) | 100 (86.3-100) | 97.9 (92.7-99.7) | 94.5 (86.5-98.4) | 100 (86.3-100) | 97.9 (92.7-99.7) |
| | Shambuko | 97.3 (90.4-99.7) | 100 (94.4-100) | 95.4 (88.8-98.7) | 97.2 (90.4-99.6) | 100 (94.4-100) | 95.5 (88.8-98.7) |
| | Tokombia | 94.7 (87.1-98.5) | 100 (95.9-100) | 94.3 (87.2-98.1) | 94.7 (87.1-98.5) | 100 (95.9-100) | 94.3 (87.2-98.1) |

**Table S5. Comparison of the classification of treatment outcomes of recurrent infections according by genotyping approach, Eritrea, 2016-2019.**

| Patient | Site | Year | Treatment | Uncorrected Outcome | Day of recrudescence (DayX) | *msp1/msp2/glurp* | *msp1/msp2/Polyα* |
|---|---|---|---|---|---|---|---|
| 2016_01_113 | Guluj | 2016 | AS+AQ | TF | Day28 | **Reinfection** | **Reinfection** |
| 2016_01_15 | Guluj | 2016 | AS+AQ | TF | Day28 | **Recrudescence** | **Recrudescence** |
| 2016_01_50 | Guluj | 2016 | AS+AQ | TF | Day28 | **Recrudescence** | **Recrudescence** |
| 2016_01_54 | Guluj | 2016 | AS+AQ | TF | Day21 | **Recrudescence** | **Recrudescence** |
| 2016_01_68 | Guluj | 2016 | AS+AQ | TF | Day21 | **Reinfection** | **Reinfection** |
| 2016_01_79 | Guluj | 2016 | AS+AQ | TF | Day21 | **Recrudescence** | **Reinfection** |
| 2016_21_175 | Shambuko | 2016 | AS+AQ | TF | Day21 | **Recrudescence** | **Recrudescence** |
| 2016_32_192 | Shambuko | 2016 | AS+AQ | TF | Day28 | **Recrudescence** | **Recrudescence** |
| 2016_55_227 | Shambuko | 2016 | AS+AQ | TF | Day21 | **Reinfection** | **Reinfection** |
| 2016_03_07 | Tokombia | 2016 | AS+AQ | TF | Day26 | **Recrudescence** | **Recrudescence** |
| 2016_03_108 | Tokombia | 2016 | AS+AQ | TF | Day21 | **Recrudescence** | **Recrudescence** |
| 2016_03_15 | Tokombia | 2016 | AS+AQ | TF | Day21 | **Reinfection** | **Reinfection** |
| 2016_03_69 | Tokombia | 2016 | AS+AQ | TF | Day25 | **Reinfection** | **Reinfection** |
| 2016_03_98 | Tokombia | 2016 | AS+AQ | TF | Day21 | **Recrudescence** | **Recrudescence** |
| 01_259_19 | Guluj | 2019 | AS+AQ | TF | Day28 | **Recrudescence** | **Recrudescence** |
| 01_85_19 | Guluj | 2019 | AS+AQ | TF | Day21 | **Recrudescence** | **Recrudescence** |
| 05_208_19 | Shambuko | 2019 | AS+AQ | TF | Day21 | **Recrudescence** | **Recrudescence** |
| 05_246_19 | Shambuko | 2019 | AS+AQ | TF | Day28 | **Reinfection** | **Reinfection** |
| 05_356_19 | Shambuko | 2019 | AS+AQ | TF | Day28 | **Reinfection** | **Reinfection** |
| 05_371_19 | Shambuko | 2019 | AS+AQ | TF | Day21 | **Recrudescence** | **Reinfection** |
| 05_512_19 | Shambuko | 2019 | AS+AQ | TF | Day28 | **Recrudescence** | **Recrudescence** |
| 05_544_19 | Shambuko | 2019 | AS+AQ | TF | Day21 | **Reinfection** | **Reinfection** |
| 03_01_19 | Tokombia | 2019 | AS+AQ | TF | Day21 | **Recrudescence** | **Recrudescence** |
| 03_155_19 | Tokombia | 2019 | AS+AQ | TF | Day28 | **Recrudescence** | **Recrudescence** |
| 03_183_19 | Tokombia | 2019 | AS+AQ | TF | Day21 | **Reinfection** | **Recrudescence** |
| 03_26_19 | Tokombia | 2019 | AS+AQ | TF | Day28 | **Recrudescence** | **Recrudescence** |
| 03_84_19 | Tokombia | 2019 | AS+AQ | TF | Day21 | **Recrudescence** | **Recrudescence** |

ASAQ: Artesunate-Amodiaquine; TF: treatment failure. Discordance between the 2 genotyping approaches is shown in red font.

**Table S6. Distribution of *Pfkelch13* mutations in isolates collected prior treatment per site and per year, Eritrea, 2016-2019.**

| Site | Year | WT | *Pfkelch13* mutations | | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **622 (R>I)** | 503 (K>W) | 515 R>G | 520 V>A | 532 (C>W) | 533 G>N | 543 I>V | 548 G>C | 556 E>K | **561 R>H** | 591 G>N | 658 (K>E) | |
| Akordat | 2016 | 52 | **5** | | | | | | | | | | | | 57 |
| | 2017 | 19 | | | | | | | | | | | | | 19 |
| | 2019 | 45 | **16** | | | | | | | | | | | | 61 |
| Ghindae | 2017 | 15 | | | | | | | | | | | | | 15 |
| Guluj | 2016 | 67 | **5** | | | | | | | | | | | 1 | 73 |
| | 2017 | 21 | **4** | | | | | | | | | | | | 25 |
| | 2019 | 73 | **21** | | | | 1 | | | | 1 | | | | 96 |
| Shambuko | 2016 | 66 | **6** | | | | | | | | | | | | 72 |
| | 2017 | 59 | **5** | | | | | | | | | | | | 64 |
| | 2019 | 76 | **7** | | | | | | 1 | 1 | | **1** | | | 86 |
| Tokombia | 2016 | 66 | **8** | | | 1 | | | | | | | 1 | | 76 |
| | 2017 | 79 | **7** | 1 | 1 | | | | | | | | | | 88 |
| | 2019 | 60 | **25** | | | | | 1 | | | | | | | 86 |
| Total | | 698 | **109** | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | **1** | 1 | 1 | 818 |

**Table S7. Proportion of patients with detectable parasitemia on day3 (D3+) and harboring *Pfkelch13* 622I mutant parasites in day0 isolates, per age groups, year and site, Eritrea 2016-2019.**

| Site | Year | % of D3+ patients harboring *Pfkelch13* 622I mutant parasites at day0 | | | |
|---|---|---|---|---|---|
| | | All age | <5 years | <10 years | <15 years |
| Akordat | 2016 | 0% | - | - | - |
| | 2017 | 0% | - | - | - |
| | 2019 | 0% | - | - | - |
| Guluj | 2016 | 0% | - | - | - |
| | 2017 | 0% | - | - | - |
| | 2019 | 1.1% (1/95) | 0% (0/2) | 0% (0/20) | 2.4% (1/41) |
| Tokombia | 2016 | 0% | - | - | - |
| | 2017 | 0% | - | - | - |
| | 2019 | 4.5% (4/88) | **28.6% (2/7)** | **13% (3/23)** | **7.0% (3/43)** |
| Shambuko | 2016 | 0% | - | - | - |
| | 2017 | 3.1% (2/64) | 0% (0/4) | 0% (0/19) | **5.4% (2/37)** |
| | 2019 | 2.3% (2/88) | 0% (0/2) | 3.8% (1/26) | 2.0% (1/49) |
| Ghindae | 2017 | 0% | - | - | - |

Bold font represents location in Eritrea where endemic ART-R was observed according to the WHO definition.[13]

**Table S8.** ***Pfkelch13*** **genotypes in isolates collected at day0 and day of recrudescence (dayX) in 17 recrudescent infections, Eritrea 2016-2019.**

| ID Patient | Site | Year | Age | Sex | Treatment | Parasitemia (parasite/µL) | | PCR corrected outcome | Day of recrudescence | *Pfkelch13* genotype (Relative abundance of *Pfkelch13* 662I genotype) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Initial parasitemia (Day0) | Day3 parasitemia | | | At Day0 | At day of recrudescence (dayX) |
| 2016_03_98 | Tokombia | 2016 | 7 | M | AS+AQ | 81000 | 0 | Recrudescence | Day21 | WT (43.8%) 622I (66.2%) | WT (1.6%) 622I (98.4%) |
| 01_85_19 | Guluj | 2019 | 15 | M | AS+AQ | 86957 | 368 | Recrudescence | Day21 | WT (14.5%) 622I (85.5%) | WT (0.4%) 622I (99.6%) |
| 01_259_19 | Guluj | 2019 | 46 | M | AS+AQ | 3310 | 0 | Recrudescence | Day28 | WT (100%) 622I (0%) | WT (100%) 622I (0%) |
| 2016_01_15 | Guluj | 2016 | 13 | M | AS+AQ | 20270 | 0 | Recrudescence | Day28 | WT (97.6%) 622I (2.4%) | WT (62.4%) 622I (37.6%) |
| 2016_01_50 | Guluj | 2016 | 4 | M | AS+AQ | 21429 | 0 | Recrudescence | Day28 | WT (97.2%) 622I (2.8%) | WT (55.3%) 622I (44.7%) |
| 2016_01_54 | Guluj | 2016 | 12 | M | AS+AQ | 14340 | 0 | Recrudescence | Day21 | WT (96.8%) 622I (3.2%) | WT (38.0%) 622I (62.0%) |
| 2016_01_79 | Guluj | 2016 | 5 | F | AS+AQ | 1080 | 0 | Recrudescence | Day21 | WT (98.1%) 622I (1.9%) | WT (46.2%) 622I (54.8%) |
| 05_208_19 | Shambuko | 2019 | 11 | M | AS+AQ | 3000 | 0 | Recrudescence | Day21 | WT (100%) 622I (0%) | WT (100%) 622I (0%) |
| 05_371_19 | Shambuko | 2019 | 14 | M | AS+AQ | 11123 | 0 | Recrudescence | Day21 | WT (100%) 622I (0%) | WT (100%) 622I (0%) |
| 05_512_19 | Shambuko | 2019 | 19 | M | AS+AQ | 8380 | 0 | Recrudescence | Day28 | WT (100%) 622I (0%) | WT (100%) 622I (0%) |
| 2016_21_175 | Shambuko | 2016 | 8 | M | AS+AQ | 1320 | 0 | Recrudescence | Day21 | WT (100%) 622I (0%) | WT (100%) 622I (0%) |
| 2016_32_192 | Shambuko | 2016 | 8 | M | AS+AQ | 986 | 0 | Recrudescence | Day28 | WT (100%) 622I (0%) | WT (100%) 622I (0%) |
| 03_01_19 | Tokombia | 2019 | 10 | M | AS+AQ | 15127 | 0 | Recrudescence | Day21 | WT (100%) 622I (0%) | WT (100%) 622I (0%) |
| 03_155_19 | Tokombia | 2019 | 10 | M | AS+AQ | 17057 | 0 | Recrudescence | Day28 | WT (100%) 622I (0%) | WT (100%) 622I (0%) |
| 2016_03_07 | Tokombia | 2016 | 6 | M | AS+AQ | 32042 | 0 | Recrudescence | Day26 | WT (96.9%) 622I (3.1%) | WT (43.7%) 622I (56.3%) |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2016_03_108 | Tokombia | 2016 | 9 | M | AS+AQ | 28432 | 0 | Recrudescence | Day21 | WT (98.2%)<br>622I (1.8%) | WT (63.1%)<br>622I (36.9%) |
| 03_84_19 | Tokombia | 2019 | 6 | M | AS+AQ | 16541 | 127 | Recrudescence | Day21 | WT (98.6%)<br>622I (1.4%) | WT (56.1%)<br>622I (43.9%) |

WT: wild type; Cells color code: green (major proportion of *Pfkelch13* wild type), light red (proportion of *Pfkelch13* 622I<5%) and dark red (proportion of *Pfkelch13* 622I >30%).

**Table S9. Accession identifiers (IDs) of the *P. falciparum* sequences from African locations, Bangladesh, Southeast Asia, or South America, sourced from the MalariaGEN Plasmodium falciparum Community Project; https://www.malariagen.net/apps/pf/4.0**

| Location | Sample Accession ID | Location | Sample Accession ID | Location | Sample Accession ID |
|---|---|---|---|---|---|
| Bangladesh | ERR022851 | Gambia | ERR1172498 | Mali | ERR223046 |
| Bangladesh | ERR216531 | Gambia | ERR1081218 | Mali | ERR636271 |
| Benin | ERR924970 | Gambia | ERR1099203 | Mali | ERR216456 |
| Benin | ERR924976 | Ghana | ERR636031 | Mali | ERR1063590 |
| Benin | ERR924985 | Ghana | ERR636040 | Mauritania | ERR1081258 |
| Benin | ERR924989 | Ghana | ERR343119 | Mauritania | ERR1099210 |
| Benin | ERR924990 | Ghana | ERR343120 | Mauritania | ERR1106563 |
| Burkina_Faso | ERR018921 | Ghana | ERR590420 | Mauritania | ERR1081275 |
| Burkina_Faso | ERR211456 | Guinea | ERR403205 | Mauritania | ERR1081276 |
| Burkina_Faso | ERR211515 | Guinea | ERR590519 | Myanmar | ERR246541 |
| Burkina_Faso | ERR211519 | Guinea | ERR059406 | Myanmar | ERR126499 |
| Burkina_Faso | ERR216463 | Guinea | ERR063558 | Myanmar | ERR1274888 |
| Cambodia | ERR175515 | Guinea | ERR063604 | Myanmar | ERR1274860 |
| Cambodia | ERR123853 | Ivory_Coast | ERR636455 | Myanmar | ERR1215358 |
| Cambodia | ERR123830 | Ivory_Coast | ERR636016 | Nigeria | ERR1172593 |
| Cambodia | ERR114345 | Ivory_Coast | ERR636456 | Nigeria | ERR1172615 |
| Cambodia | ERR022854 | Ivory_Coast | ERR636015 | Nigeria | ERR1172616 |
| Cameroon | ERR562839 | Ivory_Coast | ERR636449 | Nigeria | ERR246574 |
| Cameroon | ERR562880 | Kenya | ERR126559 | Nigeria | ERR246576 |
| Cameroon | ERR568394 | Kenya | ERR205951 | Papua_New_Guinea | ERR527444 |
| Cameroon | ERR580477 | Kenya | ERR205952 | Papua_New_Guinea | ERR175537 |
| Cameroon | ERR580480 | Kenya | ERR205953 | Papua_New_Guinea | ERR175543 |
| Colombia | ERR042227 | Kenya | ERR223039 | Papua_New_Guinea | ERR376138 |
| Colombia | ERR042679 | Kenya | ERR223047 | Papua_New_Guinea | ERR376139 |
| Colombia | ERR042231 | Kenya | ERR701714 | Peru | ERR114340 |
| Colombia | ERR039903 | Kenya | ERR701715 | Peru | ERR123900 |
| Colombia | ERR042230 | Kenya | ERR701736 | Peru | ERR123902 |
| Congo_DR | ERR377578 | Kenya | ERR701738 | Peru | ERR590544 |

| Congo_DR | ERR377580 | Kenya | ERR701750 | Peru | ERR590545 |
|---|---|---|---|---|---|
| Congo_DR | ERR377582 | Kenya | ERR701758 | Senegal | ERR1081168 |
| Congo_DR | ERR404145 | Kenya | ERR701775 | Senegal | ERR1081170 |
| Congo_DR | ERR404159 | Kenya | ERR701716 | Senegal | ERR1081177 |
| Ethiopia | ERR1106575 | Kenya | ERR701726 | Senegal | ERR1081181 |
| Ethiopia | ERR1106576 | Kenya | ERR701727 | Senegal | ERR1081187 |
| Ethiopia | ERR1106577 | Kenya | ERR701731 | Tanzania | ERR676475 |
| Ethiopia | ERR1106579 | Kenya | ERR701739 | Tanzania | ERR171646 |
| Ethiopia | ERR1106582 | Kenya | ERR701763 | Tanzania | ERR449897 |
| Ethiopia | ERR1106586 | Kenya | ERR701764 | Tanzania | ERR676481 |
| Ethiopia | ERR1106587 | Laos | ERR223059 | Tanzania | ERR676488 |
| Ethiopia | ERR1106590 | Laos | ERR126405 | Thailand | ERR164705 |
| Ethiopia | ERR1106606 | Madagascar | ERR590536 | Thailand | ERR337564 |
| Ethiopia | ERR1035493 | Madagascar | ERR590541 | Thailand | ERR164704 |
| Ethiopia | ERR1035536 | Madagascar | ERR590538 | Uganda | ERR063571 |
| Ethiopia | ERR1045266 | Madagascar | ERR490354 | Uganda | ERR063572 |
| Ethiopia | ERR1045267 | Madagascar | ERR490355 | Uganda | ERR063573 |
| Ethiopia | ERR1045271 | Madagascar | ERR490357 | Uganda | ERR063574 |
| Ethiopia | ERR1045280 | Madagascar | ERR516397 | Uganda | ERR063576 |
| Ethiopia | ERR1045283 | Malawi | ERR069575 | Uganda | ERR063577 |
| Ethiopia | ERR1045286 | Malawi | ERR216655 | Uganda | ERR063579 |
| Ethiopia | ERR1045287 | Malawi | ERR226429 | Uganda | ERR063580 |
| Ethiopia | ERR1045288 | Malawi | ERR234536 | Uganda | ERR063581 |
| Ethiopia | ERR1045295 | Malawi | ERR248963 | Uganda | ERR216467 |
| Gambia | ERR1081227 | Malawi | ERR263677 | Vietnam | ERR337569 |
| Gambia | ERR1081231 | Mali | ERR666918 | Vietnam | ERR126377 |

**Table S10. Pairwise genetic differentiation (ϕST) among *P. falciparum* isolates depending on their origin.**

Genetic differentiation was estimated with an Analysis of Molecular Variance (AMOVA) based on Euclidean genetic distances.

| | Eritrea_WT | Eritrea_NA | Eritrea_622I | Congo_DR | Ethiopia | Kenya | Malawi | Tanzania | Uganda |
|---|---|---|---|---|---|---|---|---|---|
| Eritrea_WT | 0 | **0.008** | **0.120** | **0.085** | **0.038** | **0.115** | **0.083** | **0.076** | **0.111** |
| Eritrea_NA | **0.008** | 0 | **0.075** | **0.115** | **0.038** | **0.152** | **0.113** | **0.103** | **0.144** |
| Eritrea_622I | **0.120** | **0.075** | 0 | **0.290** | **0.133** | **0.331** | **0.295** | **0.288** | **0.322** |
| Congo_DR | **0.085** | **0.115** | **0.290** | 0 | **0.145** | 0.001 | 0.005 | 0.017 | 0.004 |
| Ethiopia | **0.038** | **0.038** | **0.133** | **0.145** | 0 | **0.194** | **0.141** | **0.136** | **0.180** |
| Kenya | **0.115** | **0.152** | **0.331** | 0.001 | **0.194** | 0 | 0.020 | 0.001 | 0.015 |
| Malawi | **0.083** | **0.113** | **0.295** | 0.005 | **0.141** | 0.020 | 0 | 0.002 | 0.014 |
| Tanzania | **0.076** | **0.103** | **0.288** | 0.017 | **0.136** | 0.001 | 0.002 | 0 | 0.019 |
| Uganda | **0.111** | **0.144** | **0.322** | 0.004 | **0.180** | 0.015 | 0.014 | 0.019 | 0 |

Values in bold correspond to $\phi_{ST}$ values that were significantly non-null ($p < 0.05$). *P*-values were calculated based on 999 permutations. Different types of Eritrean isolates are indicated: WT, *Pfkelch13* wild type; 622I, *Pfkelch13* 622I or NA, when information was not available.

**Table S11. Genetic backgrounds associated with antimalarial drug resistance and polymorphisms of *hrp2* and *hrp3* genes of Eritrean *Pfkelch13* wild-type and 622I variants isolates collected prior treatment, Eritrea, 2016-2019.**

| Genomic change | Gene | Gene ID | Polymorphism | % of day0 isolate per *Pfkelch13* genotypes (PF3D7_1343700) | |
|---|---|---|---|---|---|
| | | | | Wild-type | 622I |
| CNV | *Pfmdr-1* | PF3D7_0523000 | ≥2 | 0% (0/139) | 0% (0/29) |
| | *plasmepsin2* | PF3D7_1408000 | ≥2 | 32.4% (45/139) | 31.0% (9/29) |
| | | | | | |
| SNP | *Pfcrt* | PF3D7_0709000 | CVMNKTHFIMCGI | 32.5% (101/311) | 7.5% (5/67) |
| | | | CVIETTHFIMCGI | 66.9% (208/311) | 92.5% (62/67) |
| | | | CVIETTHFIMCGT | 0.6% (2/311) | 0% (0/67) |
| | *Pfmdr-1* | PF3D7_0523000 | NYSND | 10.0% (31/311) | 4.5% (3/67) |
| | | | NFSND | 85.9% (267/311) | 95.5% (64/67) |
| | | | YFSND | 4.2% (13/311) | 0% (0/67) |
| | *dhfr* | F3D7_0417200 | ACNCSI | 24.8% (77/311) | 1.5% (1/67) |
| | | | ACNCNI | 1.3% (4/311) | 0% (0/67) |
| | | | ACICNI | 49.5% (154/311) | 74.6% (50/67) |
| | | | ACIRNI | 24.4% (76/311) | 23.9% (16/67) |
| | *dhps* | PF3D7_0810800 | SGKAA | 58.5% (182/311) | 52.2% (35/67) |
| | | | AGKAA | 0.3% (1/311) | 0% (0/67) |
| | | | SAKAA | 10.0% (31/311) | 4.5% (3/67) |
| | | | SGEAA | 30.2% (94/311) | 40.3% (27/67) |
| | | | SGKAS | 0% (0/311) | 1.5% (1/67) |
| | | | AAKAA | 0.6% (2/311) | 0% (0/67) |
| | | | SAEAA | 0.3% (1/311) | 1.5% (1/67) |
| | | | | | |
| Deletion | No deletion | | Wild-type | 50.0% (140/280) | 13.8% (9/65) |
| | *hrp2* | PF3D7_0831800 | *hrp2* deletion | 5.7% (16/280) | 0% (0/65) |
| | *hrp3* | PF3D7_1372200 | *hrp3* deletion | 22.5% (63/280) | 69.2% (45/65) |
| | *hrp2* & *hrp3* | | *hrp2* & *hrp3* deletions | 21.8% (61/280) | 16.9% (11/65) |

Position of amino acid corresponds to codons 72, 73, 74, 76, 93, 97, 145, 218, 343, 350, 353 and 356 for *pfcrt*, to codons 86, 184, 1034, 1042 and 1246 for *Pfmdr-1*, to codons 16, 50, 51, 59, 108, 164 for *dhfr* and to codons 431, 436, 437, 540, 581, 613 for *dhps*. * Significance level (Chi-squared test).

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