Emergence and clonal expansion of in vitro artemisinin-resistant *Plasmodium falciparum* kelch13 R561H mutant parasites in Rwanda

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Artemisinin resistance (delayed *P. falciparum* clearance following artemisinin-based combination therapy), is widespread across Southeast Asia but to date has not been reported in Africa1-4. Here we genotyped the *P. falciparum* K13 (Pfkelch13) propeller domain, mutations in which can mediate artemisinin resistance5,6, in pretreatment samples collected from recent dihydroarteminisin-piperaquine and artemether-lumefantrine efficacy trials in Rwanda1. While cure rates were >95% in both treatment arms, the Pfkelch13 R561H mutation was identified in 19 of 257 (7.4%) patients at Masaka. Phylogenetic analysis revealed the expansion of an indigenous R561H lineage. Gene editing confirmed that this mutation can drive artemisinin resistance in vitro. This study provides evidence for the de novo emergence of Pfkelch13-mediated artemisinin resistance in Rwanda, potentially compromising the continued success of antimalarial chemotherapy in Africa.

Malaria represents a major public health issue in the tropics, with an estimated 228 million cases and 405,000 deaths in 2018 (refs. 5, 6). Of increasing concern is *P. falciparum* resistance to artemisinin (ART) derivatives, used worldwide as the core components of ART-based combination therapies (ACTs)5,6. ART resistance (ART-R), characterized by delayed *P. falciparum* clearance following treatment with artemisinin monotherapy or an ACT1,11, is now widespread in the Greater Mekong subregion (GMS), which consists of Cambodia, Thailand, Vietnam, Myanmar and Laos12,13. Resistance to the partner drugs piperaquine and mefloquine is also now common in the GMS, causing high rates of ACT treatment failure14,15.

The appearance of ART-R parasites in Africa would pose a major public health threat. Resistance to the first-line antimalarial chloroquine first arose in the GMS in the 1960s before spreading to Africa. Resistance to pyrimethamine (used in association with sulfadoxine) followed shortly thereafter16. The lost clinical efficacy of these compounds is suspected to have contributed to millions of additional malaria deaths in young African children in the 1980s17. In addition to the risk of imported resistance18, the likelihood of resistance emerging locally in Africa has increased in areas where control measures have reduced the disease transmission intensity. The resulting attenuation in naturally acquired human immunity can increase the frequency of symptomatic infections and the need for treatment, while decreasing parasite genetic diversity and reducing competition between sensitive and resistant parasites19. To date, the efficacy of ACTs has remained high outside Southeast Asia (SEA). Early detection of resistance provides the best chance of minimizing its lethal impact.

Mutations in the Pfkelch13 propeller domain (PF3D7_1343700) constitute the primary determinant of ART-R20-22. These mutations are suspected to reduce Pfkelch13 function, which is required for parasite-mediated endocytosis of host hemoglobin in the newly invaded intra-erythrocytic ring stages20,21. Pfkelch13 C580Y is the most widespread allele in SEA13,15 and has recently been detected in Guyana23 and Papua New Guinea24. In Africa, slow-clearing infections after ACT treatment have been observed at frequencies of <1%24. Previously we observed nonsynonymous Pfkelch13 mutations in <5% of African isolates, with >50% of the polymorphisms present in only a single *P. falciparum* infection. The most frequent Pfkelch13 mutation in Africa was A578S, which did not confer ART-R in vivo or in vitro25. Nonsynonymous Pfkelch13 mutations associated with delayed parasite clearance or day 3 positivity (day 3+) in the GMS (F446I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y, A675V) have only been rarely reported, if at all, in African parasites21,22,25.

Here we conducted an in-depth genetic analysis of *P. falciparum* samples collected from 2012 to 2015 at six Rwandan sites and performed gene-editing studies to evaluate the in vitro resistance phenotypes of parasites harboring the Pfkelch13 R561H or P574L mutations identified in these samples.
Results
Clinical drug efficacy trial design and outcomes. From September 2013 to December 2015, clinical drug efficacy studies to assess the efficacy of artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DP) for the treatment of uncomplicated P. falciparum malaria were conducted in patients enrolled at the Masaka and Ruhuha health facilities in Rwanda (Fig. 1; http://www.isrctn.com/ISRCTN63145981). The overall 42-day PCR-corrected efficacies of AL (95.2% (196 of 206); 95% CI, 91.3% to 97.7%) and DP (97.5% (212 of 217); 95% CI, 94.4% to 99.1%) were similar between both sites (P = 0.17, log-rank test). The day 3 positivity rate (day 3+), defined as the proportion of patients who were still parasitemic on day 3 after initiation of treatment, was low for both treatments: 1 of 263 (0.4%) for AL and 0 of 264 for DP (Table 1).

Pfkelch13 genotyping. Pfkelch13 propeller domain genotyping was performed on 534 pretreatment samples collected at Masaka and Ruhuha. Of the 507 successfully genotyped samples, 35 (6.9%) harbored 1 of 14 different Pfkelch13 nonsynonymous mutations. These included M460I, C469Y, R513L, V555A, R561H, P574L, R575I, A578S, G592E, E605K, A626E, V637I, E651K and P667R. One sample contained two clones, each with a different mutation (G592E and V637I). The Pfkelch13 561H variant, previously associated with delayed parasite clearance following ART monotherapy or ACT treatment in the GMS, was the most predominant mutant. This R561H mutation was observed exclusively in Masaka, where it was present in 7 of 58 samples in 2013–2014 and 12 of 199 samples in 2015 (19 of 257, 7.4%). We also detected two additional Pfkelch13 mutations (P574L and C469Y) previously associated with delayed parasite clearance. We did not observe significant changes in the proportion of Pfkelch13 nonsynonymous mutations over time (P = 0.3, chi-squared test, 1.92, d.f., 2) at either study site (Table 1).

Pfkelch13 genotyping was also carried out on 420 additional blood samples collected before AL treatment from patients enrolled in a study following the same clinical protocol that was conducted in 2012–2015 across four sites in Rwanda (ISRCTN63145981; Fig. 1). Among these, ten (2.4%) carried a Pfkelch13 nonsynonymous mutation (C469E, V487I, V555A, R561H, A578S, A578V or P667R). The Pfkelch13 R561H mutation was found in a sample from Rukara, which represented a modest but significant increase relative to the Dd2 WT line (P < 0.0001, Mann–Whitney U-test). The survival of the 561H line was comparable to that of Dd2 WT line (mean survival of 4.3%), which harbors the Pfkelch13 C580Y mutation (Fig. 2). These results demonstrate that the Pfkelch13 R561H mutation can yield ring-stage ART-R at a level that is comparable to the C580Y mutation that has swept across SEA.

Origins of the Rwandan Pfkelch13 561H haplotype and its relationship to other P. falciparum populations. To study the origin of the Pfkelch13 561H mutants found in Rwanda, we compared whole-genome sequences of 340 samples, comprising 25 Rwandan P. falciparum sequences generated for this study, and an earlier collection of 104 sequences from central, western and southern African locations, 164 from Bangladesh and SEA and 45 from South America, in addition to 2 reference genomes (3D7 from Africa and 7G8 from South America; Supplementary Table 2). Of the 25 Rwandan sequences, 16 were Pfkelch13 561H mutants and 9 were Pfkelch13 WT. The isolates from SEA (Myanmar and Thailand) included 17 561H mutants. All other parasite sequences either had distinct nonsynonymous Pfkelch13 mutations or were WT for Pfkelch13.

We also explored haplotype diversity across a 200-kb region surrounding the R561H mutation. This analysis used sequences from eight Rwandan Pfkelch13 mutant infections that seemed to be predominantly monoclonal (allelic depth of the WT allele < 0.05), as well as 17 sequences from Pfkelch13 561H mutants from SEA (Myanmar and Thailand). The presence of a single shared haplotype surrounding the 561H variant in the Rwandan samples was consistent with a single epidemiological origin for this mutation. These results confirmed that the Rwandan 561H mutants share no genetic relatedness to the 561H mutants previously detected in Myanmar and Thailand (Extended Data Fig. 1).

Next, we performed a principal coordinate analysis (PCoA) based on a pairwise genetic distance matrix (computed from a 200-kb window around the Pfkelch13 gene). This analysis confirmed that the African samples (including both the Rwandan Pfkelch13 561H mutants and WT parasites) clustered together and were distinct from Asian samples (Extended Data Fig. 2). In the eight Pfkelch13 561H mutants from Rwanda we identified an extended 494-kb region, encompassing the mutation that was identical across isolates (Extended Data Fig. 3). Although an ancient common ancestry cannot strictly be ruled out, our data provide compelling evidence that Rwandan Pfkelch13 561H is the product of a recent de novo local emergence.

Investigation of the genetic background of Rwandan Pfkelch13 561H mutants. To investigate further the genetic background of the Rwandan Pfkelch13 561H mutants, we searched for molecular
Fig. 1 | Genome-wide phylogenetic tree of 25 *P. falciparum* Rwandan isolates, together with 315 isolates collected worldwide (Africa, Asia and South America). Isolates were sourced from the MalariaGEN *P. falciparum* Community Project (https://www.malariagen.net/apps/pf/4.0). Locations of clinical drug efficacy study sites where Rwandan isolates were collected are indicated. Patients enrolled at Masaka and Ruhuha (black) were treated with AL or DP, whereas patients enrolled at Bugarama, Kibirizi, Nyarurema and Rukara (gray) were treated with AL. *Pfkelch13* nonsynonymous mutations identified in these regions and relative proportions of mutant alleles are detailed in Table 1. Each leaf represents one sample and is colored according to the country of collection. Rwandan parasites carrying the *Pfkelch13* R561H mutation or the *Pfkelch13* WT allele are identified by filled or unfilled red stars at the tip, respectively. Rwandan *Pfkelch13* R561H mutants are closely related to other African samples at a genomic level, demonstrating that they are the product of a local emergence event. Scale bar, 0.0001 nucleotide substitutions per character. Only branch confidence supports <95% are indicated.
signatures associated with resistance to other antimalarials, including the ACT partner drugs piperaquine and lumefantrine. We also screened for mutations that have been identified in founder populations common to SEA ART-R parasites (those that constitute a ‘genetic background’ for ART-R)\(^27\).

First, we investigated 233 isolates from Masaka and Ruhuha for amplification of the plasmepsin2 (pfpm2; PF3D7_1408000) and multidrug resistance-1 (pfmdr1; PF3D7_0523000) genes, considered markers of reduced susceptibility to piperaquine and lumefantrine/mefloquine, respectively\(^28–30\). Of these, we found 4 isolates

| Patients and samples | 2013 | 2014 | 2015 |
|----------------------|------|------|------|
| Patients (n)         | 32   | 102  | 400  |
| Site                 |      |      |      |
| Masaka               | 11   | 49   | 208  |
| Ruhuha               | 21   | 53   | 192  |

### Antimalarial treatment

|                  | 2013 | 2014 | 2015 |
|------------------|------|------|------|
| AL               |      |      |      |
| Masaka           | 15   | 49   | 202  |
| Ruhuha           | 5    | 23   | 103  |
| DP               |      |      |      |
| Masaka           | 10   | 26   | 99   |
| Ruhuha           | 17   | 53   | 198  |

### Parasitemia (per microliter)

|                  | 2013 | 2014 | 2015 |
|------------------|------|------|------|
| Geometric mean   | 8,922| 7,492| 8,730|
| Median (IQR)     | 9,478(3,360–19,656)| 7,580(2,320–19,600)| 9,600(3,200–23,920)|

### Day 3\(^*\) rate\(^*\)

|                  | 2013 | 2014 | 2015 |
|------------------|------|------|------|
| Excluded patients|      |      |      |
| AL               | 6    | 9    | 45   |
| DP               | 2    | 7    | 42   |

### Clinical outcome at day 42

|                  | 2013 | 2014 | 2015 |
|------------------|------|------|------|
| AL               | 9    | 37   | 150  |
| DP               | 15   | 45   | 152  |

### PfKelch13 genotyping

|                  | 2013 | 2014 | 2015 |
|------------------|------|------|------|
| WT               | 28   | 84   | 354  |
| Synonymous mutations\(^*\) | 1    | 5    |
| 460 (M > I)      | 1    |      |
| 469 (C > Y)      | 1    |      |
| 513 (R > L)      | 1    |      |
| 555 (V > A)      | 2    |      |
| 561 (R > H)      | 7    | 12   |
| 574 (P > L)      | 1    |      |
| 575 (R > I)      | 1    |      |
| 578 (A > S)      | 1    |      |
| 592 (G > E)/637 (V > I)\(^\ddagger\) | 1 |
| 605 (E > K)      | 1    |      |
| 626 (A > E)      | 1    |      |
| 651 (E > K)      | 1    |      |
| 667 (P > R)      | 2    | 1    |

\(^{*}\)Data were missing for seven patients. \(^{\dagger}\)Excluded patients were patients with new infections or those with undetermined or uncertain PCR genotyping data. \(^{\ddagger}\)Synonymous mutations were G544G (n = 1, detected in 2013), T478T (n = 2, detected in 2015) and V666V (n = 3, detected in 2015). \(^{\ddagger\ddagger}\)Polyclonal infection containing two clones with two different PfKelch13 nonsynonymous mutations. The R561H mutation shown in bold font is validated in our report as an ART-R conferring PfKelch13 mutation. The C469Y and P574L mutations shown in italic font have been previously associated with delayed clearance following artemisinin monotherapy or ACT treatment.
Letters

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P574L C580Y

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can confer resistance to chloroquine or piperaquine31,32. All 561H
pfcrt
resistance transporter gene (of the 20
Pfkelch13
561H mutants for mutations in the chloroquine
were WT for
(Supplementary Table 3). We also tested 14
Pfkelch13
(PF3D7_1362500) genes in either the
561H or WT iso-
and two (22.2%) in WT samples (Pfkelch13
PF3D7_1318100), two (15.4%) in
561H mutant samples
1606
Pfkelch13
PF3D7_0720700),
(pfmdr2
pfpib7
mul-
somal protein S10 precursor
No mutations were detected in the
P. falciparum
isolates with the D193Y mutation in the ferredoxin gene (pffd
observed between the two groups of isolates. We detected four
WT parasites and tested for mutations in the six markers defining
Rwandan
561H mutants and WT parasites. For this analysis, we used 14
Pfkelch13
561H mutants and 10 randomly selected
WT parasites and tested for mutations in the six markers defining
the SEA ART-R background. No significant differences were
observed between the two groups of isolates. We detected four
isolates with the D193Y mutation in the ferredoxin gene (pfdd
PF3D7_1318100), two (15.4%) in
Pfkelch13
561H mutant samples and two (22.2%) in WT samples (P = 0.69, Fisher’s exact test). No mutations were detected in the
P.falciparum apicoplast ribosomal protein S10 precursor
(pfrps10, PF3D7_1460900), multidrug resistance protein 2 (pfmdr2, PF3D7_1447900), pfpib7
(PF3D7_0720700), pfpbh
(PF3D7_1012700) or exonuclease
(PF3D7_1362500) genes in either the
Pfkelch13
561H or WT isolates (Supplementary Table 4).

**Discussion**

This study clearly shows early warning signs of ART-R in Rwanda. We provide evidence for the clonal expansion of an indigenous
Pfkelch13
561H lineage in two localities 100 km apart in Rwanda
(prevalence 7.4% in Masaka and 0.7% in Rukara). This expansion was not linked to delayed parasite clearance in vivo or clinical treatment failure following AL or DP treatments, likely due to the high efficacy of the partner drugs lumefantrine and piperaquine. Genetic analyses indicate that Rwandan
Pfkelch13
561H mutants are the product of recent de novo local emergence. These findings contrast with previous scenarios from the 1980s in which the emergence of chloroquine- and pyrimethamine-resistant parasites in Africa resulted from the westward spread of these parasites from SEA, and confirm that local emergence of ART-R is possible in Africa.

We used gene editing and the RSA 0–3h, a clinically validated in vitro phenotypic analysis to demonstrate that the
Pfkelch13
561H mutation is sufficient to confer ART-R in vitro. These experiments employed Dd2, which has been the most widely used
P. falciparum strain for
Pfkelch13 gene editing. Our results revealed that in Dd2 parasites, the R561H mutation confers survival at levels comparable to the C580Y mutation that predominates in SEA (with mean survival rates of 4.3% and 4.7%, respectively). Previous studies have shown that
Pfkelch13 mutations that afford resistance do so across all strains, with the parasite genetic background modulating resistance levels and with mutations conferring less resistance in Dd2 compared to contemporary SEA strains. While we did not test the impact of this mutation in Rwandan parasites due to a lack of availability of culture-adapted strains, we are confident that the resistance phenotype observed herein would be maintained across strains.

Fig. 2 | Survival rates of Dd2
\(^{\text{R561H}}\), Dd2
\(^{\text{P574L}}\), Dd2
\(^{\text{C580Y}}\) and Dd2
\(^{\text{WT}}\) lines in the ring-stage survival assay (RSA
\(^{\text{0–3h}}\)). Mean ± s.e.m. RSA
\(^{\text{0–3h}}\) survival rates (percentage of viable parasites) were as follows: Dd2
\(^{\text{R561H}}\) 4.3 ± 0.1% (n = 7 assays); Dd2
\(^{\text{P574L}}\) 2.1 ± 0.3% (n = 8 assays); Dd2
\(^{\text{C580Y}}\) 4.7 ± 0.4% (n = 9 assays); Dd2
\(^{\text{WT}}\) 0.6 ± 0.1% (n = 13 assays). All assays were performed in duplicate. Mann–Whitney U-tests (two-sided) were used to test for statistically significant differences between
Pfkelch13-edited clones and the Dd2
\(^{\text{WT}}\) comparator line. Survival rates of Dd2
\(^{\text{R561H}}\), Dd2
\(^{\text{P574L}}\) and Dd2
\(^{\text{C580Y}}\) all differed significantly from Dd2
\(^{\text{WT}}\) (**** P < 0.0001). The limit of detection of viable parasites was estimated at 0.1% parasitemia (lower limit of 50 parasitized red blood cells per total number of 50,000 counted for each line in each assay).

Table 2 | Parasitemia at day 3 and PCR-corrected clinical outcome at day 42 of the patients enrolled in clinical drug efficacy studies in Masaka and Ruhuha (2013–2015, AL or DP) and Bugarama, Kibirizi, Nyarurema and Rukara (2012–2015, AL), according to
Pfkelch13 genotypes detected in
P. falciparum isolates collected before ACT treatment

| Clinical data | WT/synonymous mutant | Nonsynonymous mutant |
|-------------|---------------------|---------------------|
| **Masaka and Ruhuha 2013–2015** | | |
| Parasitemia at day 3 | Positive | 0 |
| | Negative | 469 (AL = 237, DP = 232) |
| | | 33 (AL = 16, DP = 17) |
| Clinical outcome at day 42 | Cured | 373 (AL = 185, DP = 188) |
| | Recrudescent | 9 (AL = 5, DP = 4) |
| **Bugarama, Kibirizi, Nyarurema and Rukara 2012–2015** | | |
| Parasitemia at day 3 | Positive | 2 |
| | | 0 |
| Negative | 403 | 10 |
| Clinical outcome at day 42 | Cured | 312 |
| | Recrudescent | 14 |

For all mutations n = 1 unless otherwise indicated.

\(^{*}\) (1.7%) with two copies of pfmd2 and 12 isolates (5.1%) with two copies of pfmd1. All isolates carrying two copies of pfmd2 or pfmd1 were WT for
Pfkelch13 (Supplementary Table 3). We also tested 14 of the 20
Pfkelch13 561H mutants for mutations in the chloroquine resistance transporter gene (pfcrt; PF3D7_0709000), whose variants can confer resistance to chloroquine or piperaquine. All 561H mutants carried WT pfcrt (Supplementary Table 4).

Second, we tested whether the proportions of single-nucleotide polymorphisms (SNPs) associated with the emergence of ART-R in the SEA genetic background varied between Rwandan
Pfkelch13 561H mutants and WT parasites. For this analysis, we used 14 Rwandan
Pfkelch13 561H mutants and 10 randomly selected WT parasites and tested for mutations in the six markers defining the SEA ART-R background. No significant differences were observed between the two groups of isolates. We detected four isolates with the D193Y mutation in the ferredoxin gene (pfdd; PF3D7_1318100), two (15.4%) in
Pfkelch13 561H mutant samples and two (22.2%) in WT samples (P = 0.69, Fisher’s exact test). No mutations were detected in the
P.falciparum apicoplast ribosomal protein S10 precursor (pfrps10, PF3D7_1460900), multidrug resistance protein 2 (pfmdr2, PF3D7_1447900), pfpib7 (PF3D7_0720700), pfpbh (PF3D7_1012700) or exonuclease (PF3D7_1362500) genes in either the
Pfkelch13 561H or WT isolates (Supplementary Table 4).

1606
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At a genomic level, Rwandan Pfkelch13 561H mutants were phylogenetically closely related to other African samples and clustered unambiguously with Rwandan Pfkelch13 WT parasites. Haplotype analysis revealed that Rwandan Pfkelch13 561H mutants shared an identical haplotype surrounding the R561H mutation that differed from the haplotypes of SEA 561H mutants, strongly suggesting a single de novo epidemiological origin and recent spread of the mutation. No genetic relatedness was observed between Rwandan Pfkelch13 561H parasites and Pfkelch13 561H mutants previously detected in Myanmar and Thailand by PCoA.

The current rise and expansion of the in vitro ART-R Pfkelch13 R561H mutation in Rwanda is particularly notable in light of the observed absence of clinical outcomes typically associated with ART-R. We suspect that the absence of delayed parasite clearance in Rwandan patients harboring Pfkelch13 561H mutant parasites is due to high levels of naturally acquired immunity to P. falciparum in the study participants. Indeed, it has been shown that P. falciparum antibody titers are strongly associated with faster parasite clearance rates in patients living in high-transmission areas like Rwanda and that antibodies against P. falciparum blood stages enhance antimalarial efficacy. In our study, the ages of patients enrolled at both sites ranged from 1 to 14 years, with an estimated median age of 8 years (interquartile range (IQR): 5–11 years). Given that immunity is acquired gradually with age, a clinical drug efficacy trial limited to younger populations (≤5 years of age) might reveal a significant association between the presence of Pfkelch13 561H mutants in pre-treatment isolates and delayed parasite clearance. We hypothesize that early signs of clinical ART-R can lie undetected in populations with high levels of immunity, calling into question the relevance of the current clinical metrics used to detect ART-R in Africa. This hypothesis is supported by data from population-based mathematical modeling that showed that ART-R parasites might be able to circulate up to 10 years longer without detection in high-transmission areas than in low-transmission areas.

To date, the Pfkelch13 R561H mutation has been reported multiple times in SEA (Cambodia until 2006, Myanmar and Thailand) and in India and a few times in Africa (Democratic Republic of the Congo, Rwanda and Tanzania), but has only been associated with slow-clearing infections in SEA. Thus, the degree to which Pfkelch13 561H mutant parasites are able to withstand exposure to ART in vivo and how Pfkelch13 561H is successfully transmitted between patients in the absence of clinical recrudescence (Table 2) requires further elucidation. It is possible that the resistance advantage afforded by the Pfkelch13 561H mutation is slight and undetectable based on day 3* and recrudescence metrics, and thus would be evident only with ART monotherapy trials. Regarding transmission, we can offer several hypotheses. First, Pfkelch13 561H mutants could be less susceptible to ART due to an ability to enter into a dormant state and later produce transmissible gametocytes. Second, Pfkelch13 561H parasites may have a higher capacity to be transmitted due to an unknown genetic feature or Pfkelch13 561H gametocytes may be less susceptible to the gametocytocidal activity of artemisinin. However, it is most likely that the transmission of Pfkelch13 561H mutants in Rwanda is maintained by asymptomatic individuals or mildly symptomatic untreated patients with circulating Pfkelch13 561H mutants that have been selected by low levels of circulating drugs.

We did not detect the combination of background mutations earlier suspected to be linked to the ART-R phenotype in SEA in the Pfkelch13 561H Rwandan isolates. This suggests that the emergence of mutant Pfkelch13 that drives in vitro resistance is not dependent on the presence of secondary mutations within the parasite genome. So far, no gene-editing and in vitro phenotyping experiments have been performed to test the importance of these secondary mutations for resistance. Data from this study suggest that mutations in fδ, mdr2, arps10 and others represent the genetic architecture of regional ART-R in P. falciparum SEA parasite populations rather than secondary determinants of resistance.

The findings of this study have substantial implications for public health in confirming the de novo emergence and clonal expansion of an ART-R Pfkelch13 R561H lineage in Rwanda and in validating this mutation as a mediator of ART-R in vitro. In the absence of effective strategies to contain the spread of resistance across Rwanda and to neighboring countries, we may soon witness a rise of resistance to ACT partner drugs, which will in turn lead to high treatment failure rates, as has occurred in SEA. Recent studies have predicted that ACT treatment failures in Africa could be responsible for an additional 78 million cases and 116,000 deaths over a 5-year period.

Molecular surveillance of Pfkelch13-related ART-R currently implemented by the National Malaria Control Programme in Rwanda needs to be sustained and strengthened so that mutations can be identified before clinical phenotypes become apparent. Our findings argue for the need for more rapid collection of data, analysis and dissemination of information using new high-throughput field-based surveillance tools operable at a national level. Likewise, we have to reappraise the performances of the current clinical phenotypic metrics (delayed parasite clearance and day 3*) to detect the warning signs of ART-R in African populations with high immunity early on.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-020-1005-2.

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Methods
Clinical drug efficacy trial oversight and blood sample collection. The clinical drug efficacy trial (ISRCTN63145981, http://www.isrctn.com/ISRCTN63145981) was conducted by the Rwanda National Malaria Program between 2013 and 2013 at two health facilities in Rwanda (Masaka and Ruhuha, in the Kicukiro and Bugesera districts, respectively) to assess the efficacy of AL or DP for the treatment of uncomplicated *Plasmodium falciparum* malaria in children 1–14 years of age, presenting with suspected uncomplicated *P. falciparum* malaria. Patients at both sites were randomly assigned to receive a full course of AL (Co-artem, 20 mg artemether and 120 mg lumefantrine per tablet) or DP (Duo-cotecin, 40 mg dihydroartemisinin and 320 mg piperaquine per tablet) according to the manufacturer’s dosing schedule.

The primary outcome of the study was the PCR-adjusted clinical response to the designated treatment on day 42 (ref. 34). The secondary outcome was the day 3, defined as the proportion of patients who were still parasitemic on day 3 after initiation of treatment as assessed by thick blood smear (Supplementary Methods 1)35.

*Pfkelch13* genotyping and whole-genome sequencing. Genomic investigations were carried out on blood samples collected before ACT treatment (AL or DP) from patients enrolled at Masaka and Ruhuha. We also analyzed blood samples collected before AL treatment from patients enrolled in clinical drug efficacy studies conducted at four additional sites (Bugarama, Kibirizi, Nyarurema and Rukara) across Rwanda between 2012 and 2015.

Parasite DNA was extracted from dried blood samples (Fig. 1) using the QIAamp DNA Blood Mini Kit (Qiagen). Mutations in the propeller domain of *Pfkelch13* (PF3D7_1343700, codons 440–680, 720 bp) were identified by capillary sequencing of PCR products. Parasite whole-genome sequences were obtained by Illumina paired-end sequencing after capture-based enrichment of parasite DNA (Supplementary Methods 2).

Phylogenetic analysis. For each sequenced sample, read alignments against the chromosome sequences of *P. falciparum* 3D7 v45 were processed to infer consensus sequences. These consensus sequences were pooled and concatenated, leading to 17,313,072 aligned nucleotide characters that were used to infer a maximum-likelihood phylogenetic tree (Supplementary Methods 3).

Genotyping and haplotype analysis. The Genome Analysis Toolkit Haplotype Caller was used to identify SNPs in each isolate. We assessed the genetic identity of *Pfkelch13* 561H mutants from Rwanda and Asia by comparing alleles at loci within a 200-kb window around the mutation and recording the number of discrepancies between each sample and the mutant consensus sequence. PCoA was performed by computing pairwise Euclidean genetic distances between samples in an extended 494-kb window (Supplementary Methods 4).

Generation of gene-edited lines and in vitro susceptibility assays. Dd2Δ3011 and Dd2Δ297–2 gene-edited parasite lines, as well as Dd2WT and the Dd2C580Y lines used as controls, were generated by CRISPR-Cas9-mediated editing of the *Pfkelch13* locus using the pD2Cam-cospCas9-U6-hdhfr vector. In vitro ART susceptibility analyses of these lines were assessed using RSAk-a (Supplementary Methods 5–7).

Statistical analysis. Sample size calculations and clinical data management methods have been previously described. PCR-adjusted clinical efficacy rates at day 42 were calculated using Kaplan–Meier survival analysis. Survival curves were compared using the Mantel–Haenszel log-rank test (one-sided). Patients with new infections during the 42-day follow-up period and patients with undetermined or noninterpretable PCR genotyping data were excluded from the final analysis. Data were reported in Microsoft Excel (Office 2016) and analyzed with MedCalc v.12 (MedCalc Software) and Prism 8 (GraphPad Software). Mann–Whitney U-tests (two-sided) were used for nonparametric comparisons. For frequency data (expressed with percentages and 95% CIs), we used chi-squared or Fisher’s exact tests (one-sided). Relative risks were estimated using the Mantel–Haenszel test. All *P* values <0.05 were deemed significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support the findings of this study are available from the corresponding authors upon request. Parasite whole-genome sequences have been deposited in the repository https://www.ncbi.nlm.nih.gov/bioproject/PRJEB38946, and the sequence files are accessible under the accession numbers ERS4758427 to ERS4758451.

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Author contributions
A.U., JLM.N., A.M., D.A.F., D.M., E.L., P.R., N.U., D.N., T.M., JB.M., M.W., M.M., E.P. and K.M. contributed to study design. A.U., JLM.N., A.M., T.M. and JB.M. collected clinical samples and data. D.M. and E.L. prepared the DNA. D.M., E.L., F.A., P.C. and A.C. performed the sequencing and sequence analyses. B.H.S. and D.A.F. performed genome-editing and in vitro assays. A.U., JLM.N., A.M., D.M., P.R., P.C. and A.C. analyzed data. D.M., B.H.S. and D.A.F. wrote the report. All authors read and approved the final manuscript. P.R. is a staff member of the World Health Organization and is responsible for the views expressed in this publication, noting that they do not necessarily represent the decisions, policy or views of the World Health Organization.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | Comparison of mutant pseudo-haplotypes in a 200 kb window around the R561H mutation (100 kb on both sides of the mutation, on chromosome 13). Each cell represents a single SNP. The blocks of cells (grouped in columns) correspond to SNPs falling into the same 20 kb interval within the 200 kb window. The R561H mutation in Pfkelch13 (PF3D7_1343700) is flagged in the dark red cell at the top. Light blue cells correspond to the reference allele (that is the 3D7 genome), dark blue cells correspond to the alternate allele and grey cells to missing values. Each row corresponds to one isolate, with isolates color-coded according to the country of origin (red for Rwanda, cyan for Thailand and green for Myanmar). Mutant pseudo-haplotypes include eight *P. falciparum* monoclonal Rwandan samples and 18 Southeast Asian samples (from Myanmar and Thailand, sourced from the *Plasmodium falciparum* Community Project; https://www.malariagen.net/apps/pl/4.0). The presence of a single shared haplotype surrounding the R561H mutation in Rwandan *P. falciparum* isolates is consistent with a single epidemiological origin of the genetic background on which the mutation arose. This genetic background demonstrates no genetic relatedness to R561H mutants previously detected in Myanmar and Thailand.
Extended Data Fig. 2 | Principal Coordinate Analysis (PCoA) based on pairwise genetic distances in a 494 kb window around the PfKelch13 gene. Principal Coordinate Analysis including PfKelch13 wild type and 561H isolates including those sourced from a public database (small dots, the MalariaGEN Plasmodium falciparum Community Project, https://www.malariagen.net/apps/pf/4.0) and originating from different continents (Asia, Africa or South America). Isolates originating from populations where the PfKelch13 R561H mutation was found are emphasized (large dots). Empty large dots correspond to PfKelch13 wild-type isolates and filled large dots correspond to PfKelch13 561H mutants. While the mutants tend to cluster with individuals of similar origin, axis 1 clearly discriminates African (Rwanda) from Asian (Thailand and Myanmar) PfKelch13 561H mutants.
Extended Data Fig. 3 | Extent of the common core haplotype in the eight Rwandan Pfkelch13 R561H isolates (monoclonal isolates). a, Recombination breakpoints estimated based on the accumulation of discrepancies between the consensus core sequence of mutants and each haplotype on both sides of the Pfkelch13 R561H mutation. The analysis was performed on the eight isolates that appeared monoclonal. Genomic positions are indicated relative to the Pfkelch13 mutation (0 kb). b, Length of the corresponding core mutant haplotypes (obtained based on (A)). Dotted lines delineate a common core region of 494 kb within which all mutant haplotypes appear identical. Genomic positions are indicated relative to the Pfkelch13 mutation (relative position 0 kb). In the larger haplotypes, no clear recombination breakpoint was observed on chromosome 13, indicating a sequence identity along the whole chromosome. Each of the eight isolates are represented by a specific color, consistent between panel (a) and panel (b).
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

- Data collection: Microsoft Excel (Office 2016)
- Data analysis: MedCal (version 12), Prism (version 8), Whole-genome Data Manager (version 2.0), IQ-TREE v1.6.7.2 with evolutionary model GTR+FO +R10 and SH-aLRT branch supports (1,000 replicates), Genome Analysis Toolkit (GATK) Haplotype Caller (4.1.7.0).

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- A list of figures that have associated raw data
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The data that support the findings of this study are available from the corresponding authors upon reasonable request. Parasite whole-genome sequences have been deposited in repository https://www.ncbi.nlm.nih.gov/bioproject/PRJEB38946 and the sequence files are accessible under the accession numbers ERS4758427 – ERS4758451.
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
Sample size calculations were informed by results from the 2009 study on AL and DHP conducted in Rwanda (The Four Artemisinin-Based Combinations (4ABC) Study Group. A head-to-head comparison of four artemisinin-based combinations for treating uncomplicated malaria in African children: a randomized trial. PLoS Med 2011;8(11):e1001119). Using a two-sided type I error rate of 0.05 and an 80% power to detect a 5% difference between treatments, a sample of 268 patients per treatment arm was used. The total sample for each treatment arm was split evenly between the two study sites.

Data exclusions
In the clinical data analysis, a per-protocol analysis was conducted excluding patients with new infections during the follow-up period to calculate the proportion of the ACPR in the PCR-adjusted data set. Data were excluded from the PCR-adjusted analyses if the genotyping results were unclassifiable or identified a new infection. The exclusion criteria were pre-established.

Replication
All attempts at replication were successful (see data, Figure 2)

Randomization
A randomization list was computer generated for different age-strata (<2 years; 2-5 years; 5-10; 10-14 years) using MS-Excel. Sequentially numbered sealed envelopes containing the treatment group assignments were prepared from the randomization list for each age category. The study doctor assigned a study number to the participant and the study nurse administered treatment by opening the envelope corresponding to the treatment number.

Blinding
The randomization codes were secured in a locked cabinet accessible only by the study nurse. Only the study nurse and patients were aware of treatment assignments whereas the study doctor was blinded to the treatment assignments.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Antibodies            |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology         |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data         |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq              |
| ☒   | Flow cytometry        |
| ☒   | MRI-based neuroimaging |

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Plasmodium falciparum Dd2 line - MRA 156 - https://www.beiresources.org/Catalog/BEIParasiticProtozoa/MRA-156.aspx

Authentication
The authentication procedure of P. falciparum Dd2 line is described in the certificate of analysis for MRA-156 - https://www.beiresources.org/Catalog/BEIParasiticProtozoa/MRA-156.aspx#

Mycoplasma contamination
P. falciparum Dd2 cell line was tested negative for mycoplasma contamination

Commonly misidentified lines (See FLAC register)
No misidentified lines
Human research participants

Policy information about studies involving human research participants

Population characteristics
Children 1-14 years of age presenting with suspected uncomplicated Plasmodium falciparum malaria (temperature ≥37.5°C and/or a history of fever within the past 24h).

Recruitment
Children 1-14 years of age were enrolled if they were subsequently confirmed to have parasitemias ranging from 1,000 to 100,000 parasites per microliter and were able to attend follow-up visits until day 42 post initiation of treatment. Enrolled patients were randomly assigned to receive a full course of AL (Coartem®, 20 mg artemether and 120 mg lumefantrine per tablet) or DP (Duo-Cotecxin®, 40 mg dihydroartemisinin and 320 mg piperaquine per tablet) according to the manufacturer’s dosing schedule. A blood sample was collected prior to the initiation of treatment (day 0) and was spotted onto filter paper for genotyping. Additional blood samples were collected weekly (on days 7, 14, 21, 28, 35 and 42) during the 42-day follow-up period. Blood samples were also collected in cases of febrile recurrence to differentiate recrudescence (true treatment failure) from new infection.

Ethics oversight
We confirm that this clinical study was performed in accordance with relevant guidelines and regulations. Approval for conducting the study was obtained from the Rwandan National Ethics Committee (RNEC129/RNEC/2012).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

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Clinical trial registration
ISRCTN63145981 (http://www.isrctn.com/ISRCTN63145981)

Study protocol
The study protocol was approved by the Rwanda National Ethics Committee on 16 May 2012 (RNEC129/RNEC/2012). The full trial protocol is available from the corresponding authors upon request.

Data collection
Data were collected from clinical studies, coordinated by the Rwanda National Malaria Program and designed to assess the efficacy of artemether-lumefantrine (AL) or dihydroartemisinin-piperaquine (DP) for the treatment of uncomplicated falciparum malaria at Masaka and Ruhuha health facilities in 2013-2015 and at Bugarama, Kibirizi, Nyarurema and Rukara health facilities in 2012-2015.

Outcomes
The primary and secondary outcomes were pre-defined. The primary outcome of the study was the PCR-adjusted clinical response to the designated treatment on day 42. Patients were either classified as cured, or in the case of recurrence, as re-infected (new infection) or recrudescent (true treatment failure) according to the WHO 2009 protocol. The secondary outcome was the day 3 positivity rate (day 3+), defined as the proportion of patients who were still parasitemic on day 3 after initiation of treatment as assessed by microscopic examination of thick blood smears.