Assessment of *ex vivo* antimalarial drug efficacy in African *Plasmodium falciparum* parasite isolates, 2016–2023: a genotype–phenotype association study

Jason Rosado,[a,n,*] Abebe A. Fola,[b,c,n] Sandrine Cojean,[d,e,f,n] Véronique Sarrasin,[d,n] Romain Coppée,[g] Justine Bailly,[a] Rizwana Zaffaroulah,[d] Azza Bouzayene,[d] Liliane Cicéron,[d] Ludivine Houzé,[d] Rebecca Crudale,[b,c] Lise Musset,[h] Marc Thellier,[i] Bruno Pradines,[j,k,l,m] Jérôme Clain,[a,o,**] Jeffrey A. Bailey,[b,c,o] and Sandrine Houzé,[a,d,o] Investigation Study Group[p]

[a]Université Paris Cité, IRD, Inserm, MERIT, Paris, F-75006, France
[b]Department of Pathology and Laboratory Medicine, Brown University, RI, 02906, USA
[c]Center for Computational Molecular Biology, Brown University, RI, 02906, USA
[d]Centre National de Référence du Paludisme, Assistance Publique-Hôpitaux de Paris, Hôpital Bichat-Claude-Bernard, Paris, France
[e]Université Paris-Saclay, Faculté de Pharmacie, Orsay, France
[f]UMR BIPAR, Anses, Laboratoire de Santé Animale, INRAE, Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France
[g]Université de Rouen Normandie, Laboratoire de Parasitologie-Mycologie, UR 7510 ESCAPE, Rouen, France
[h]Laboratoire de Parasitologie, World Health Organization Collaborating Center for Surveillance of Antimalarial Drug Resistance, Centre Nationale de Référence du Paludisme, Institut Pasteur de la Guyane, Cayenne, French Guiana
[i]Centre National de Référence du Paludisme, Sorbonne Université, Assistance Publique des Hôpitaux de Paris, Laboratoire de Mycologie et Parasitologie, Hôpital de la Pitié-Salpêtrière, Paris, France
[j]Unité Parasitologie et Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Marseille, France
[k]Aix Marseille Université, SSA, AP-HM, RITMES, Marseille, France
[l]Centre National de Référence du Paludisme, Marseille, France
[m]IHU-Méditerranée Infection, Marseille, France

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Summary

Background Given the altered responses to both artemisinins and lumefantrine in Eastern Africa, monitoring antimalarial drug resistance in all African countries is paramount.

Methods We measured the susceptibility to six antimalarials using *ex vivo* growth inhibition assays (IC_{50}) for a total of 805 *Plasmodium falciparum* isolates obtained from travellers returning to France (2016–2023), mainly from West and Central Africa. Isolates were sequenced using molecular inversion probes (MIPs) targeting forty-three genes across the parasite genome, of which nineteen are drug resistance genes.

Findings *Ex vivo* susceptibility of all assessed antimalarial compounds was consistent with their potent activity. The median IC_{50} values for the six drugs were 1.1 nM [IQR: 0.8–1.7] for DHA, 16.7 nM [9.9–27.4] for LMF, 29.5 nM [19.1–45.5] for MFQ, 23.4 nM [17.1–39.0] for MDAQ, 26.7 nM [18.0–41.2] for CQ, and 18.5 nM [15.1–24.3] for PPQ. Only four isolates carried a validated *pfkelch13* mutation. Multiple mutations in *pfcrt* and one in *pfmdr1* (Asn86Tyr) were significantly associated with altered susceptibility to multiple drugs, and their frequencies decreased over time. *Pfcrt* and *pfmdr1* mutations altered susceptibility to lumefantrine and mefloquine in an additive manner, with the wild-type haplotype (*pfcrt* K76-*pfmdr1* N86) exhibiting the lowest susceptibility.

Interpretation Our study on *P. falciparum* isolates from West and Central Africa indicates a low frequency of molecular markers associated with artemisinin resistance and a modest but significant decrease (1.6–2.3X) in the frequency of multidrug resistance markers. These genotypic changes likely mark parasite adaptation to sustained drug pressure and call for intensifying the monitoring of antimalarial drug resistance in Africa.

*Corresponding author.
**Corresponding author.
E-mail addresses: jjrosados@gmail.com (J. Rosado), jerome.clain@u-paris.fr (J. Clain).
[n]The first authors contributed equally to this work.
[o]Co-senior authors contributed equally to this work.
[p]The members of Investigation Study Group are listed in the Supplementary Materials section.

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Keywords: Antimalarial drug resistance; *Ex-vivo* susceptibility; Growth inhibition assay; Genotype

Research in context

Evidence before this study

We searched for articles on antimalarial drug resistance published between January 1, 2000 and July 1, 2024, using the PubMed search terms "antimalarial resistance", "Africa", and "*ex vivo*". Of the 69 published studies, only six encompassing a total of 827 isolates across five West and Central African countries from 2016 to 2022 combined *ex vivo* drug assays with genotyping data. Parasites with an increased rate of *ex vivo* survival to artemisinins were reported in one study from Ghana (7/90 isolates in 2018) and another from The Gambia (4/41 isolates in 2017). Only the Ghanaian study reported mutations in the non-propeller domain of *pfkelch13* gene, whereas the Gambian study reported mutations associated with reduced susceptibility to lumefantrine (7%, 3/41). In Mali, Senegal and Burkina Faso, most isolates were susceptible to commonly used antimalarial drugs (chloroquine, amodiaquine, piperaquine, mefloquine, lumefantrine, and dihydroartemisinin) using standard growth inhibition assays. In Ghana, reduced susceptibility to artemisinin, mefloquine and amodiaquine was observed. The relative lack of recent data on parasite susceptibility to antimalarial drugs in recent parasites from West and Central Africa prompted us to conduct this study.

Added value of this study

The large-scale analysis presented here provides invaluable, contemporary insights into the current landscape of susceptibility to antimalarial drugs and molecular markers of resistance in *Plasmodium falciparum* isolates from West and Central Africa between January 2016 and June 2023. We show a decreasing trend in the frequency of *pfcrt* Lys76Thr and *pfmdr1* Asn86Tyr mutations in recent years. Association analyses highlighted that *pfcrt* and *pfmdr1* polymorphisms were the major drivers of altered susceptibility to most tested drugs and could interact in a likely additive manner in the case of LMF.

Implications of all the available evidence

The observed decrease in the frequency of mutant alleles of *pfcrt* and *pfmdr1* genes in parasites from West and Central Africa suggests an ongoing adaptation of parasites, possibly related to the increasing use of ACT treatments in Sub-Saharan Africa over the past decade. While the data suggest that ACTs and sulfadoxine-pyrimethamine are likely to be effective in these regions, the genotypic changes we observed call for intensifying the monitoring of antimalarial drug resistance in Africa.

Introduction

Malaria continues to take a heavy toll on public health in Africa. Antimalarials remain a cornerstone in the fight against malaria, and the evolution of drug resistance poses a significant threat to the efficacy of treatment regimens.[1,2] Monitoring antimalarial drug resistance and treatment efficacy is paramount, as drug selection pressures are ongoing, and no alternatives to current first-line treatments will be available in the coming years.[3,4] Artemisinin-based combination therapies (ACTs) were introduced in the 2000s as first-line curative treatments. ACTs combine an artemisinin derivative, which rapidly reduces parasite load, with another antimalarial drug, known as a partner drug, which eliminates the remaining parasites due to its longer half-life.

However, the emergence of artemisinin partial resistance (ART-R) has raised concerns about the long-term efficacy of ACTs for effective malaria treatment.[5,6] After ART-R emerged, treatment failures with dihydroartemisinin (DHA)–piperaquine (PPQ) were reported in Southeast Asia as parasites acquired additional resistance to PPQ.[7,8] ART-R has recently emerged in East Africa and the Horn of Africa,[9–11] creating the potential for selection of parasites resistant to the partner drug(s) and threatening the long-term efficacy of ACTs in Africa.[12,13] Worryingly, decreased susceptibility of *P. falciparum* isolates to the partner drug lumefantrine (LMF) has also been reported in northern Uganda.[14,15] In West and Central Africa, treatment efficacy studies have confirmed the clinical efficacy of ACTs in several settings overall.[16,17] However, few studies have reported *ex vivo* antimalarial drug assays for West and Central African isolates, with little evidence of reduced susceptibility to partner drugs.[18,19] Molecular surveillance has also revealed the sporadic presence of validated ART-R *pfkelch13* mutations in these two regions.[20]

Here, we aimed to measure the frequencies of drug resistance genotypes, assess the level of *ex vivo* drug

susceptibilities, and investigate genotype–phenotype associations in isolates from West and Central Africa. Therefore, we analysed the *ex vivo* susceptibility to six drugs (DHA, LMF, mefloquine (MFQ), chloroquine (CQ), monodesethylamodiaquine (MDAQ), and PPQ; assessed by growth inhibition assays) of 805 *P. falciparum* isolates collected between 2016 and 2023 from imported malaria cases in returned travellers to mainly West and Central Africa. The isolates were also sequenced using high-throughput molecular inversion probes (MIPs) targeting 814 overlapping sequences across 43 genes, of which 19 are validated drug resistance genes.

Methods

Sample and data collection

The French National Malaria Reference Centre (FNMRC) coordinates a nationwide network of hospitals in mainland France to survey imported malaria. Blood samples from civilian or military hospitals participating in the FNRMC network were shipped at +4 °C to the FNRMC reference laboratory (Bichat Hospital in Paris) (see Study Group) and stored at +4 °C for a maximum of 48 h prior to *ex vivo* assays. Parasite viability was evaluated by microscopic observation of Giemsa-stained smears before testing. Parasite morphology was used as a surrogate for viability. Only isolates with a minimum of 0.2% viable parasites were further assessed. Sex data was self-reported by study participants. Inclusion criteria were: diagnosis of *P. falciparum* malaria recorded between January 2016 and June 2023 and confirmed by microscopy and PCR, available DNA sample, information on the country visited, and available IC_{50} data against CQ, MFQ, PPQ, MDAQ, LMF, and DHA. A total of 813 isolates fulfilled the inclusion criteria.

Ex vivo assays

CQ, DHA, MDAQ, MFQ, LMF, and PPQ were purchased from Alsachim (Illkirch Graffenstaden, France). The susceptibility of the isolates to the antimalarial drugs was assessed without culture adaptation. 100 µL of parasitised erythrocytes (final parasitaemia of 0.5% and a final haematocrit of 1.5%) were aliquoted into 96-well plates preloaded with a concentration gradient of antimalarial drugs. The plates were incubated for 48 h in a controlled atmosphere of 85% N_2, 10% O_2, 5% CO_2 at 37 °C. Drug susceptibilities were determined as half-maximal inhibitory concentrations (IC_{50}s) using the standard 42-h [^{3}H] hypoxanthine uptake inhibition assay.[21] We used the resistance thresholds for the six compounds previously reported by Pradines, Kaddouri and others.[22–24]

DNA extraction and MIP sequencing of drug resistance genes

Genomic DNA was extracted from 200 µL of whole blood using a MagNA Pure automaton (Roche Diagnostics, USA), eluted in 100 µL, and stored at −20 °C. DNA extracts from the included samples were sent to Brown University for MIP sequencing. All samples were then genotyped using molecular inversion probes (MIPs, n = 814) targeting 43 genes across the *P. falciparum* genome, of which 19 have known drug resistance SNP mutations, as previously described (Table S1, Appendix 2).[12,25,26] Sequencing was performed on an Illumina NextSeq 550 instrument (150 bp paired-end reads) at Brown University (RI, USA).

MIP data analysis and estimating drug resistance frequency

Sequencing data processing and variant calling were performed using MIPtools (v0.19.12.13; https://github.com/bailey-lab/MIPTools). Using MIPWrangler, raw reads from each captured MIP, identifiable by unique molecular identifiers (UMIs), were used to reconstruct sequences, and variant calling was performed on these samples using freebayes.[27] Biallelic variant SNP positions were retained for analysis. Variants were annotated using the 3D7 v3 reference genome. To reduce false positives due to PCR and sequencing errors, the alternative allele (SNP) must have been supported by more than one UMI within a sample with at least 5× coverage, and the allele must have been represented by at least 10 UMIs in the population.

We estimated the frequency of *pfkelch13* validated mutations as reported by the WHO. In addition, the frequency of reported "background mutations"[28] associated with ART-R, such as *ferredoxin* (*pffd*) Asp193Tyr, *multidrug resistance 2 transporter* (*pfmdr2*) Thr484Ile, *putative phosphoinositide binding protein* (*pfpib7*) Cys1484Phe, *protein phosphatase* (*pfpph*) Val1157Leu, and *pfcrt* Asn326Ser and Ile356Thr was also assessed. The frequency for each drug resistance marker was calculated as $p = (x/n) \times 100$, where p is the frequency, x is the number of infections with mutant alleles, and n is the number of successfully genotyped isolates, as previously described.[12] Mixed genotypes, i.e., a sample with a reference allele and an alternative allele in a given sample, were considered mutant regardless of the within-sample mutant allele frequency. Haplotypes were reconstructed for all samples using the major allele in a given codon, i.e., the allele with a within-sample allele frequency larger than 75%. Alleles with a frequency between 25% and 75% were considered unresolved and discarded from the analysis. Only samples with complete SNP data for the tested haplotypes were included in the analysis.

The CNV analysis utilised the *simple_copy_caller* function from the *cnv_caller* module in MIPTools. To ensure reliable copy number calls, we limited the analysis to samples with a minimum of 25 unique molecular identifiers (UMIs) per probe for the target gene. Then, UMI counts for each sample were normalised by dividing each value by the mean UMI count

of the corresponding sample, allowing for comparability across samples. Probes exhibiting high variability across samples were excluded from the analysis to enhance accuracy. Subsequently, the sample-wise normalised values for each probe were further normalised by dividing them by the probe's mean value. This dual normalisation approach ensured that the expected normalised value for each probe was approximately 1 for genes with a "normal" copy number, while the average normalised value for each sample provided an estimate of the gene's copy number. Copy numbers were estimated using the depth of sequence coverage from 31 unique probes for *pfmdr1* and 31 probes for the *plasmepsin 1–3* locus.

Ethics

The FNRCM surveillance system was approved by the National Data Protection Commission (CNIL) in the declaration number 1223103. According to article L1121–1.1 of the Public Health Code in France, the study was considered non-interventional research. According to article L1211–2 of the Public Health Code in France, biomedical databases and research resources containing in-depth health information can be used several times for scientific purposes as long as informed consent is obtained from patients and in cases involving children, parents, or a legal representative had to report their opposition to the hospital. Physicians informed participants that their samples might be used for non-interventional research and that they could object to this if they wished. No participants' objections were reported to the FNMRC. All data were pseudonymised before use. Human DNA was not analysed. According to French legislation, no institutional review board approval was required regarding samples from the FNRCM.

Statistics

No sample size calculation was performed; instead, all available participant data were included. Inclusion criteria are described in the section on sample and data collection. Normality was visually assessed using histogram frequency distributions and the Shapiro test. Monotonic temporal trends in the IC_{50}s data were tested using the Mann–Kendall trend test of the Kendall R package (version 2.2.1), as the IC_{50} data did not have a normal distribution. Changes in the frequency of mutant genotypes over time were evaluated using a Cochran–Armitage test, as implemented in the DescTools R package (version 0.99.6), which is designed to detect linear trends and directional changes. Temporal changes in the frequency of the *crt-mdr1* haplotypes were examined using logistic regression models, with haplotype presence as the outcome and year as the predictor. Correlations between drug log10 transformed IC_{50}s were assessed using a two-sided Pearson correlation test implemented via the rstatix R package (version 0.7.2).

For phenotype–genotype association analysis, isolates with more than 30% missing genotype data were discarded. We then retained only biallelic non-synonymous SNPs with minor allele frequency (MAF) > 0.01 for phenotype–genotype association analysis. Phenotype-genotype association was performed with the SNPassoc R package (v.2.1.0)[29] using the built-in WGassociation function under a dominant model. As the IC_{50} data were right-skewed, they were log10 transformed to satisfy the normality of residuals and homoscedasticity assumptions. For each drug, a linear regression model was fitted with continuous log10 IC_{50} values as the outcome and individual SNP genotypes as independent variables. The model was adjusted for the complexity of infection (COI), year, and the visited country of imported cases as covariates. We applied the Bonferroni correction to define a significance threshold of $p \leq 10^{-4}$ for all linear regression analyses. Association analysis results were visualised with a Manhattan plot using the qqman R package (v.0.1.9). COI was estimated using the R package RealMcCoil (v.1.3.1)[30] using bi-allelic SNPs. As a secondary analysis, conditional regression using *pfcrt* or *pfmdr1* SNPs was performed to remove any effect of *pfcrt*/*pfmdr1* loci and to detect novel variants associated with drug susceptibility.

We then selected significant SNPs to proceed to a haplotype analysis. We evaluated the effect of haplotypes on the IC_{50}s of the six drugs using a Pairwise Wilcoxon test with Benjamini-Hochberg multiple testing correction. A multiple linear regression analysis was performed to investigate whether the increment of log10 IC_{50} of the six drugs was dependent on haplotype frequency over time.

All analyses were performed in R (version 4.3.1). The geographic distribution of traveller isolates was plotted using the R packages 'rnaturalearth' (v.1.0.1) and 'rnaturalearthdata' (v.1.0.0). Other graphical outcomes were created in ggplot (version 3.4.4) within R version 4.3.1.

Role of funders

The funders of the study had no role in the design, data collection, data analysis, data interpretation, or writing of the report.

Results

Geographical origin and year of collection

From January 2016 to June 2023, 9966 *P. falciparum* isolates from travellers were initially included in the study. Only 4788 had the information on the country of infection. Of these, 1125 isolates met the criteria for *ex vivo* antimalarial drug susceptibility testing at the

FNMRC laboratory. Of these, 813 isolates (samples) were included in this study, corresponding to samples with information on the country of infection, DNA available for genomic studies and validated IC_{50} for the following six drugs: DHA, LMF, MFQ, MDAQ, CQ, and PPQ. 805 out of the 813 included isolates were successfully genotyped. According to reported travel history, infections originated from thirty-five African countries, but most isolates were from travellers who had visited West (59%, 471/805) and Central (38%, 306/805; Fig. 1a) African countries. The geographical representation of this subset is consistent with the geographic distribution of the initial isolates included in the study (Fig. S1). The most visited countries were Côte d'Ivoire (27%, 217/805), Cameroon (21%, 169/805), Mali (9%, 69/805), Guinea (7%, 58/805), and the Republic of the Congo (6%, 45/805) (Fig. 1a and Table S2). The number of samples collected per year was higher in 2016 (165/805), 2017 (166/805), and 2018 (167/805) than in 2019 (100/805) and beyond (Fig. 1b). The small number of imported malaria cases from 2019 to 2023 compared to 2016 to 2018 reflects the effect of COVID-19-related travel restrictions in France in 2020, and having limited data on the growth inhibitory assay against six drugs (DHA, LMF, MFQ, CQ, MDAQ, and PPQ) as main inclusion criteria. Travellers were predominantly male (62%), with a median age of 40 years and a median parasitaemia of 1.7% (Table 1).

Half-maximal inhibitory concentration (IC_{50}) for six antimalarial compounds

IC_{50} values of *P falciparum* 3D7 laboratory reference strain are indicated in Table S3. For the isolates tested in this study, the median IC_{50} estimated with the standard growth inhibition assay was less than 30 nM for all assessed antimalarial compounds, consistent with potent activity. The median IC_{50} values for the six drugs were 1.1 nM [IQR: 0.8–1.7] for DHA, 16.7 nM [9.9–27.4] for LMF, 29.5 nM [19.1–45.5] for MFQ, 23.4 nM [17.1–39.0] for MDAQ, 26.7 nM [18.0–41.2] for CQ, and 18.5 nM [15.1–24.3] for PPQ. Using published resistance thresholds,[22–24] 49% of the isolates were classified as resistant to MFQ (IC_{50} > 30 nM), 10% to CQ (IC_{50} > 100 nM), and 3% to MDAQ (IC_{50} > 80 nM). Less than 1% of isolates had an IC_{50} above the threshold for DHA (10 nM), LMF (150 nM), and PPQ (135 nM) (Fig. S2, Table S4). West and Central African countries showed similar median IC_{50}s for most drugs except for PPQ, which was slightly increased in the latter (18.1 nM [14.3–22.8] vs 19.6 nM [16.0–26.1], p = 0.0016, Table S5). We used a Mann–Kendall test to assess the temporal trend of IC_{50}s with year-on-year data, and we did not see monotonic trends (Fig. 2, Table S6).

There were several significant drug-drug IC_{50} correlations, most of which were positive (Fig. S3). The largest positive correlations involved the following drug pairs: LMF-MFQ, CQ-MDAQ, DHA-LMF, and DHA-MFQ. The negative correlations were much less intense and involved CQ-LMF.

Frequency of validated antimalarial resistance mutations

Sequence data generated with MIPs covered exons from 43 genes that included 19 genes reported to be associated with drug resistance. The average number of reads per sample was 120 (range: 48–258) (Fig. S4). Nearly 60% of the isolates (477/805) had a COI >1. We initially

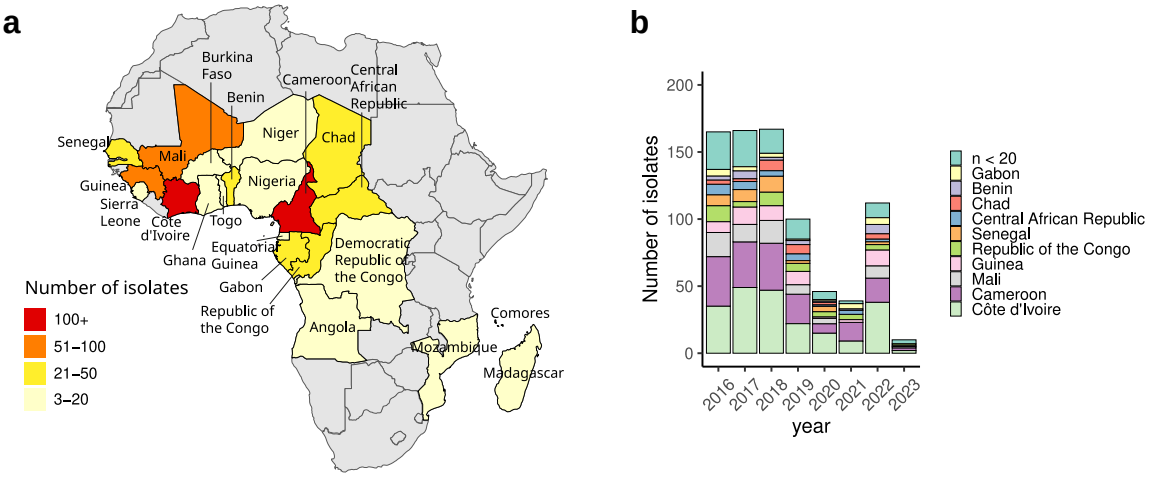

***Fig. 1:* Geographical origin and collection year of isolates. a)** Map of Africa showing the origin of malaria cases imported into France. Colours indicate sample size, and countries with fewer than three isolates are not shown. **b)** Bar plot showing the temporal distribution of imported malaria cases included in this study (2016, n = 165; 2017, n = 166; 2018, n = 167; 2019, n = 100; 2020, n = 46; 2021, n = 39; 2022, n = 112; 2023, n = 10). The top ten countries (n > 21 samples) are displayed. Countries with fewer than 20 samples are represented in the group "n < 20".

	n = 805
Participant characteristics	
Age, median [IQR]	39.2 [27.1, 51.40]
Sex, n (%)	
Female	306 (38.0%)
Male	499 (62.0%)
Isolates characteristics	
Parasitaemia[a] % (Parasites/100 RBC), median [IQR]	1.70 [0.81, 3.46]

IQR: Interquartile range; RBC: Red blood cells. [a]Missing data for two isolates.

Table 1: **Epidemiological characteristics of travellers returning to France and description of isolates from 2016 to 2023.**

identified 2398 SNPs. After filtering data for missingness (retaining only SNPs genotyped in >70% of samples) and MAF at ≥1%, we got 362 bi-allelic SNPs corresponding to 36 genes. We first examined the frequency of mutations in five robustly validated drug resistance genes: *pfdhfr* and *pfdhps* (associated with resistance to pyrimethamine and sulfadoxine, respectively), *pfcrt* and *pfmdr1* (associated with multidrug resistance), and *pfkelch13* (associated with ART-R).

More than 85% of isolates carried the *pfdhfr* Asn51Ile (646/723), Cys59Arg (668/723), and Ser108Asn (669/715) mutations (Fig. 3a), which were predominantly found as the IRN (85.4%, 561/657) triple mutant haplotype and marginally as the ICN (1%, 10/657) and NRN (5.8%, 38/657) double mutants (Fig. S5), regardless of the country of infection (Table S7). We found a low frequency of the *pfdhps* Ile431Val (8.5%, 59/692), Lys540Glu (6%, 46/720), Ala581Gly (8%, 60/748), and Ala613Ser (15%, 111/735) mutations, and a large frequency of Ser436Ala and Ala437Gly (53% [352/664] and 78% [425/542], respectively). At *pfdhps* codons 436-437-540-581 considered jointly (haplotypes, Fig. S5), single mutants AAKA and SGKA were the most common haplotypes (35% [209/657] and 50% [298/657], respectively). The quintuple mutant haplotype IRN-GE (*pfdhfr* IRN and *pfdhps* 437Gly and 540Glu), which is responsible for treatment failure with SP in adults and children, was detected in 3% of samples (19/600).

In *pfmdr1*, the frequency of the Asn86Tyr, Tyr184Phe, and Asp1246Tyr mutations were 11% (82/746), 71% (544/769), and 1% (6/711), respectively (Fig. 3a), and the frequency of the NYD, NFD, and YFD haplotypes at codons 86-184-1246 were 38.8% (241/621), 54.8% (340/621), and 5.6% (35/621), respectively (Fig. S5). Other minor haplotypes were found in less than 1% of the isolates.

The *pfcrt* Lys76Thr mutation was found in 30% (217/726) of isolates (Fig. 3a) and was absent in the Central African Republic. No isolates carried the Cys72Ser mutation. Regarding the classical eight *pfcrt*

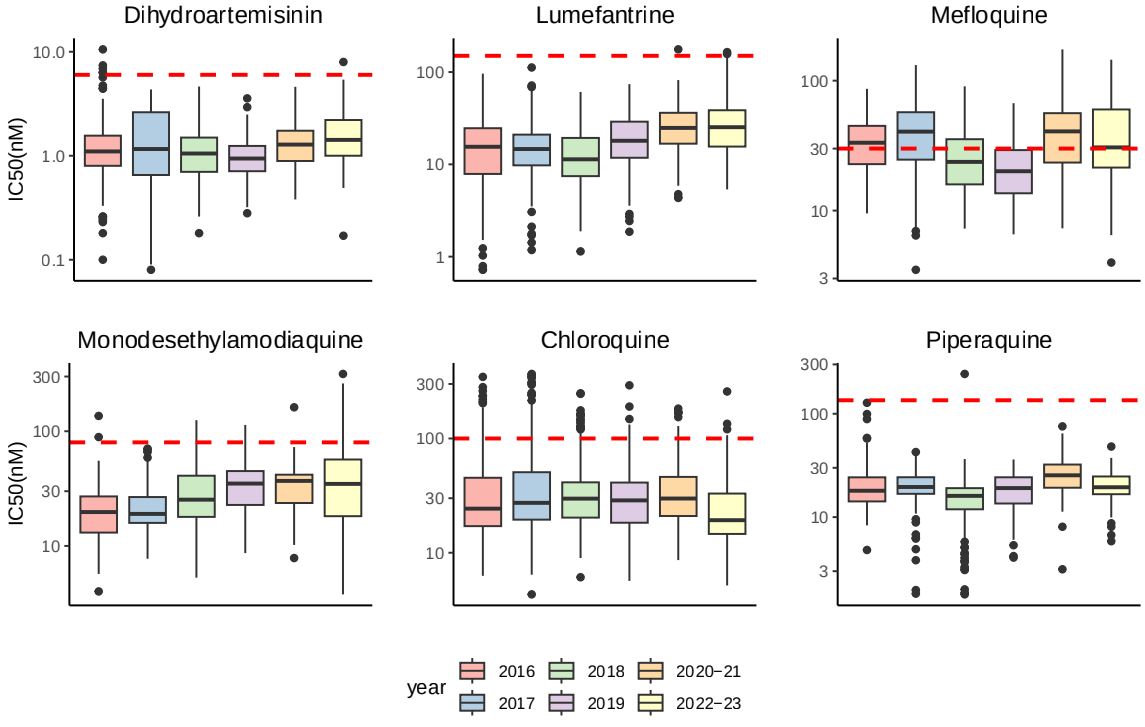

Fig. 2: **Half-maximal inhibitory concentration (IC_{50}) for six antimalarial drugs.** Distribution of IC_{50} for the six antimalarial drugs from 2016 to 2023 (2016, n = 165; 2017, n = 166; 2018, n = 167; 2019, n = 100; 2020–21, n = 85; 2022–23, n = 122). Box plots show the median IC_{50} (in nM) and interquartile range. Dashed red lines indicated the IC_{50}s cut-offs for drug resistance as defined in some studies (see Table S4).

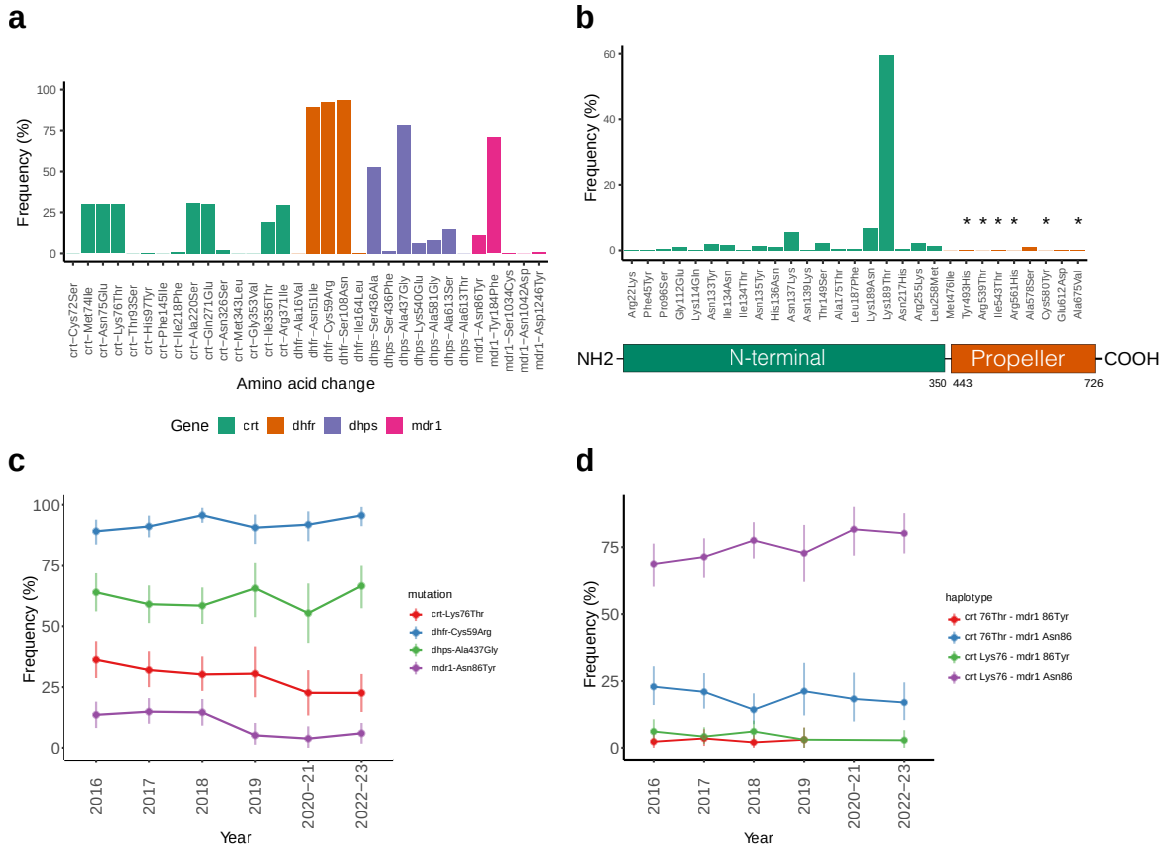

Fig. 3: **Frequency of key mutations associated with resistance to different drugs. a)** Frequency of key mutations in *pfcrt*, *pfdhfr*, *pfdhps*, and *pfmdr1* genes detected in isolates from this study (n = 427–785). **b)** Frequency of mutations in the *pfkelch13* gene (n = 611–761). Green bar plots indicate the frequency of mutations in the N-terminal-coding domain, while orange bar plots indicate the frequency of mutations in the propeller domain. SNPs marked with an asterisk (*) are validated or candidate SNPs by WHO. **c)** Frequency of the main key mutations in *pfcrt*, *pfdhfr*, *pfdhps*, and *pfmdr1* by year (n = 796). **d)** Frequency of *pfcrt* Lys76Thr-*pfmdr1* Asn86Tyr haplotypes over time (n = 664). Colour code for the 4 haplotypes: purple, wild-type Lys76-Asn86; green, single mutant Lys76-86Tyr; blue, single mutant 76Thr-Asn86; red, double mutant 76Thr-86Tyr. a–c: mixed genotypes, i.e., a sample with a reference allele and a mutant allele in a given sample, were considered mutant regardless of the within-sample mutant allele frequency. c & d: 95% confidence intervals are represented by error bars. In panel d, haplotypes were reconstructed for all samples using the major allele in a given codon (i.e., the allele with a within-sample allele frequency larger than 75%); alleles with a frequency between 25% and 75% were considered unresolved and discarded from the analysis.

codons 74-75-76-220-271-326-356-371, three main haplotypes were detected (Fig. S5; per country, see Fig. S6): the wild-type MNKAQNIR (77%, 374/487) and the mutants IETSENTI (also known as Cam783; 15% [75/487]) and IETSENII (also known as GB4; 6% [30/487]).

Four isolates carried a validated *pfkelch13* mutation in the propeller domain (Fig. 3b): Ala675Val (2/734, from Rwanda in 2019 and Uganda in 2020), Tyr493His (1/705, from Mali in 2016), and Ile543Thr (1/731, from Côte d'Ivoire in 2018). The two isolates carrying the Ala675Val mutation showed an IC_{50} to DHA (IC_{50} = 1.78 nM and IC_{50} = 1.67 nM) larger than the upper quartile. The Ala675Val isolate from Rwanda also had elevated IC_{50} values for LMF (IC_{50} = 41.57 nM) and MFQ (IC_{50} = 49.12 nM). Two other mutations were detected in the propeller domain: Ala578Ser (8/714), known as polymorphism, and Glu612Asp (1/664). Most other non-synonymous *pfkelch13* mutations occurred in the N-terminal coding domain (Fig. 3b), with Lys189Thr particularly frequent (58.4%, 378/635) and known as a polymorphism.

Copy number estimates of genes associated with decreased susceptibility to MFQ (*pfmdr1*) and PPQ (*plasmepsin 1*, *2*, and *3*) were based on UMI depth. The dataset of 805 isolates was filtered for high coverage, leaving 377 isolates for copy number estimation. Duplication of *pfmdr1* was detected in one isolate (0.26%, 1/369). Mean copy estimates were 1.01 (range 0.72–1.99; n = 369) for *pfmdr1*, 1.01 (0.66–1.44; n = 318) for *plasmepsin 1*, 1.01 (0.69–1.29; n = 341) for *plasmepsin 2*, and 1.01 (0.69–1.61; n = 332) for *plasmepsin 3* (Fig. S7).

The frequency of "background" mutations associated with ART-R in South East Asia[28] was low: *pffd* Asp193Tyr at 0.27% (2/737), *pfmdr2* Thr484Ile at

0% (0/628), *pfpib7* Cys1484Phe at 0.16% (1/642), and *pfpph* Val1157Leu at 0% (0/677) (Table S8).

We then analysed mutation frequency over time using a Cochran–Armitage trend test (Fig. 3c and Table S9). The *pfcrt* Lys76Thr (Cochran–Armitage test, Z = –2.87, p = 0.0040; frequency of 36% [53/146] in 2016 and 22.6% [26/115] in 2022–23) and *pfmdr1* Asn86Tyr mutations (Z = –3.27, p = 0.0011; frequency of 14% [20/147] in 2016 and 6% [7/117] in 2022–23) exhibited a significant decreasing trend over time. In contrast, mutations at other loci (*pfdhps* Ala437Gly and *pfdhfr* Cys59Arg) displayed no significant temporal trends (all p > 0.05) (Fig. 3c). The frequency of the wild-type haplotype at *pfcrt* 76 and *pfmdr1* 86 (K76-N86) was largely dominant across the whole study period and showed a slight but not significant increase from 68.7% (90/131) in 2016 to 80% (85/106) in 2022–2023 (Z = 0, p > 0.05, Fig. 3d).

Association between SNPs and IC_{50}s

Genotype-phenotype association analysis across 362 SNPs and IC_{50}s to 6 drugs showed several significant associations (Fig. 4, Tables S10 for the complete list of associations, and S11 and S12 after correction for multiple testing). Multiple well-known SNPs in *pfcrt* were strongly associated with IC_{50}s for the six drugs. Mutant *pfcrt* alleles were associated with increased IC_{50} to CQ ($p = 10^{-43}$–10^{-65} for 7 amino acids) and MDAQ ($p = 10^{-9}$–10^{-13} for 7 amino acids) whereas wild-type *pfcrt* alleles were associated with increased IC_{50} to DHA ($p = 10^{-5}$–10^{-6} for 7 amino acids), LMF ($p = 10^{-7}$–10^{-13} for 3 amino acids), MFQ ($p = 10^{-5}$–10^{-10} for 6 amino acids), and PPQ ($p = 10^{-4}$ for 1 amino acid). There was also a strong association between *pfmdr1* mutations and IC_{50} to MFQ ($p = 10^{-6}$–10^{-7} for 3 amino acids: Asn86Tyr, Asp650Asn, and Asn652Asp), LMF ($p = 10^{-7}$–10^{-13} for

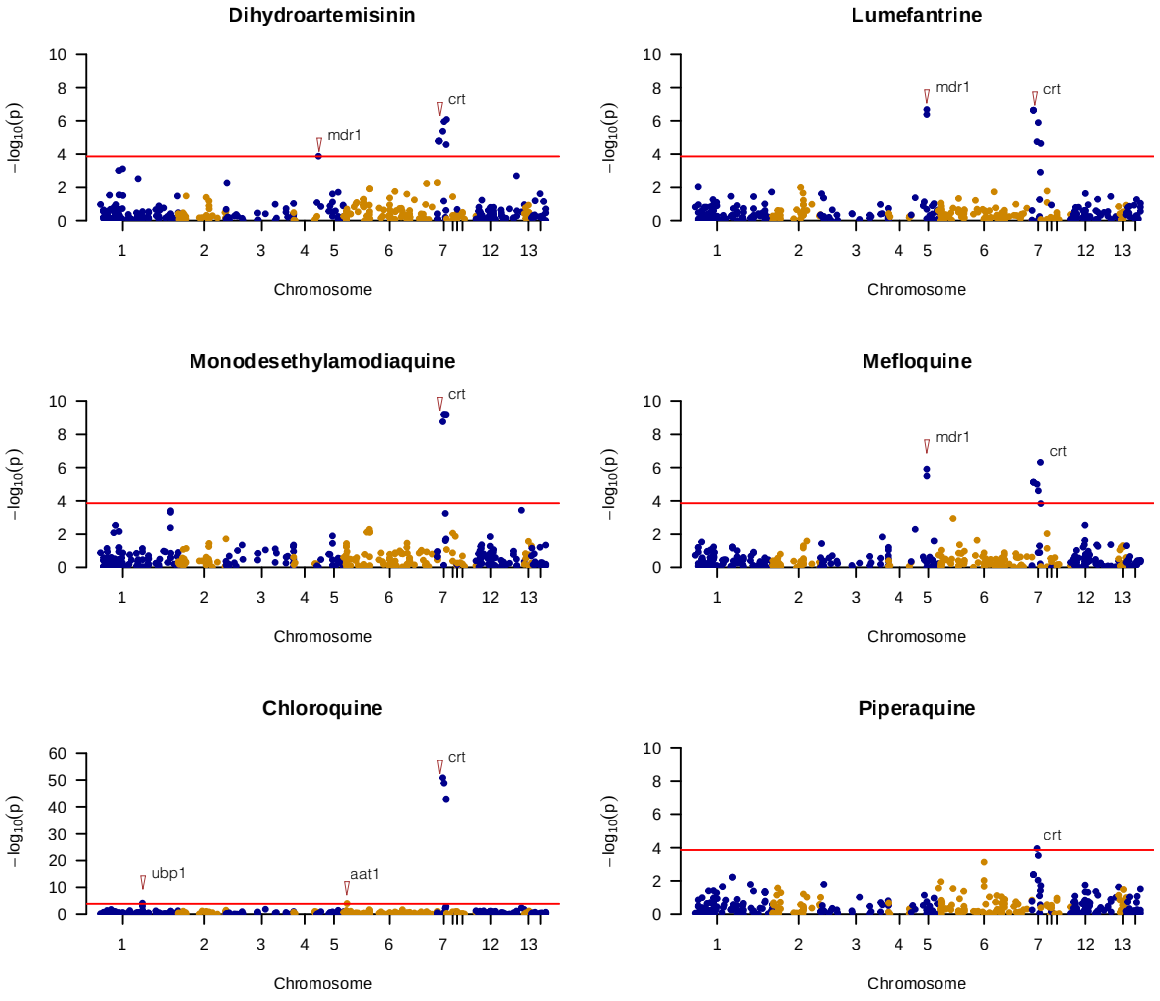

***Fig. 4:* Manhattan plot showing the significance of SNPs associated with six antimalarial drugs.** Each dot represents 1 of 362 SNPs with MAF > 0.01 coloured by chromosome. The x-axis represents the chromosomal location of the SNPs, and the y-axis represents the –log10 of the p-value obtained from the linear regression model analysis. The red line represents the p-value threshold after Bonferroni correction ($p \leq 10^{-4}$). The full list of SNPs associated with IC_{50} is shown in Table S10.

3 amino acids: Asn86Tyr, Asp650Asn, and Asn652Asp) and DHA ($p = 10^{-4}$ for Asn86Tyr). Finally, the *pfubp1* Asn1704Lys and *pfaat1* Ser258Leu mutations were associated with increased IC_{50} to CQ ($p < 10^{-4}$ for both).

To control for the major *pfcrt* effect, we added *pfcrt* Lys76Thr as a covariate (Table S11). All the associations involving another SNP in *pfcrt* (except Met74Ile and Asn75Glu for CQ), *pfubp1* Asn1704Lys, and *pfaat1* Ser258Leu were lost, suggesting they were dependent on the *pfcrt* Lys76Thr genotype. The associations involving *pfmdr1* (with LMF and MFQ) were maintained, and no new associations were detected. Similarly, a model with *pfmdr1* Asn86Tyr as a covariate abolished only the association of *pfmdr1* Asp650Asn and Asn652Asp for LMF and MFQ (Table S11). These data indicate that, among the 362 tested SNPs, *pfcrt* and *pfmdr1* SNPs were the major drivers of susceptibility to most tested drugs and could interact in a likely additive manner in the case of LMF.

Effect of *pfcrt* and *pfmdr1* haplotypes on IC_{50}s

Since *pfcrt* and *pfmdr1* SNPs were associated with IC_{50}s, we further analysed the effect of haplotypes on the evaluated drugs. As compared to the wild-type *pfcrt* (MNKAQNIR), mutant haplotypes IETSENTI and IETSENII (that differ at Ile356Thr only) were significantly associated with increased IC_{50} to CQ and MDAQ (Pairwise Wilcoxon test, p-value range: <0.0001–0.0003; Fig. S8) and decreased IC_{50} to DHA, LMF, and PPQ (Pairwise Wilcoxon test, p-value range: <0.0001–0.05). IETSENTI, but not IETSENII, was associated with decreased IC_{50} to MFQ (Pairwise Wilcoxon test, $p = 0.0001$), suggesting that Ile356Thr has some role in MFQ transport activity.

Isolates carrying the *pfmdr1* NYD wild-type haplotype had increased IC_{50} to DHA, LMF, MFQ and decreased IC_{50} to PPQ as compared to the mutant YFD (Pairwise Wilcoxon test, p-value range: <0.001–0.01; Fig. S8).

Finally, the analysis of *pfcrt* K76T-*pfmdr1* N86Y haplotypes identified three main drug patterns (Fig. 5). The IC_{50} to LMF, MFQ, and also marginally to DHA, decreased with the number of *pfcrt-pfmdr1* mutations, suggesting these mutations work in additive ways and make parasites even more drug susceptible. The highest IC_{50} to LMF was associated with the wild-type haplotype (*pfcrt* K76-*pfmdr1* N86). The IC_{50} to CQ and MDAQ increased mainly with mutant *pfcrt*, and the addition of mutant *pfmdr1* had a much lower effect. The IC_{50} to PPQ was more difficult to interpret, with little haplotypic effect. A multiple linear regression model for LMF, DHA, CQ, MDAQ, and PPQ IC_{50}s suggested that both year and the *pfcrt* K76T-*pfmdr1* N86Y haplotypes impacted the variance of IC_{50} of these drugs, with the exception of MFQ, which was only associated with the haplotypes (Table S13). For instance, each one-year increase was associated with a 9.8% increase in LMF IC_{50} ($p < 10^{-10}$) and 10.3% increase in MDAQ IC_{50} ($p < 10^{-15}$). The *pfcrt* 76T-*pfmdr1* N86 single-mutant haplotype reduced the levels of LMF IC_{50} by 28.3% ($p < 10^{-5}$) and increased the MDAQ IC_{50} by 60% ($p < 10^{-15}$), while *pfcrt* K76-*pfmdr1* 86Y and *pfcrt* 76T-*pfmdr1* 86Y haplotypes had the stronger effects for LMF (decrease by 66.6%, $p < 10^{-13}$ and 70%, $p < 10^{-8}$, respectively), and only *pfcrt* 76T-*pfmdr1* 86Y haplotype for MDAQ (increase by 155%, $p < 10^{-8}$).

Discussion

Whereas there are recent reports of emerging ART-R and decreased susceptibility to LMF in *P. falciparum* parasites from East Africa,[31] few recent studies have investigated the resistance status of contemporary West and Central African isolates at a large scale. Here, we report the temporal trends of *ex vivo* susceptibilities to six antimalarials using standard growth inhibition assays and their association with mutations in fourteen drug resistance genes in 805 *P. falciparum* samples collected between 2016 and 2023 in returned travellers to West and Central African countries. We found an overall excellent susceptibility of isolates to the six evaluated antimalarials, but the frequency of mutant *pfcrt* Lys76Thr and *pfmdr1* Asn86Tyr alleles decreased by approximately half in recent years. This pattern could be explained by the large use of LMF that selects, although moderately, these two wild-type alleles and the fitness cost of the mutant alleles following the discontinuation of CQ in these regions. Most samples carried one copy of *pfmdr1*, *plasmepsin-1*, *-2*, and *-3* genes, ruling out their role in altering the drugs' IC_{50} in our study. Finally, altered susceptibility to most drugs was strongly associated with *pfcrt* and/or *pfmdr1* genotypes.

Regarding molecular ART-R, only four isolates among 805 carried a WHO-validated *pfkelch13* mutation. Ala675Val was detected in two travellers returning from East Africa (Rwanda and Uganda), consistent with the spread of this mutation in this region.[10,32] The returned traveller from Uganda showed delayed clearance under artesunate therapy,[33] whereas the Ala675Val isolate from Rwanda had elevated IC_{50} values for LMF (41.57 nM) and MFQ (49.12 nM), consistent with clinical and *ex vivo* data reported by others for Ugandan Ala675Val isolates.[11,14,15] Two other validated mutations, Tyr493His and Ile543Thr, were identified in one isolate from Mali in 2016 and one from Côte d'Ivoire in 2018, respectively. These isolates did not exhibit unusual IC_{50}s against any of the drugs tested. As these two mutations were not detected in more recent samples, these seem to be sporadic cases. Altogether, *pfkelch13* ART-R mutations were detected but were uncommon in Central and West African isolates during the study period (2016–2023). Contrary to the current situation in East Africa, *pfkelch13*-mediated ART-R is seemingly not emerging during 2016–2023 in West and Central

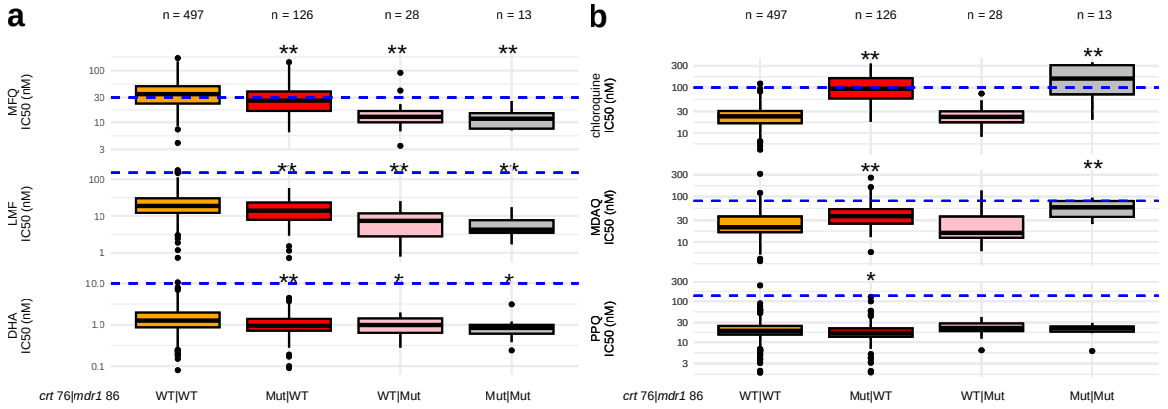

Fig. 5: **Effect of *pfcrt* Lys76Thr-*pfmdr1* Asn86Tyr haplotypes on IC_{50} for six drugs. a)** IC_{50} for mefloquine (MFQ), lumefantrine (LMF), and dihydroartemisinin (DHA) disaggregated by *pfcrt-pfmdr1* haplotypes. **b)** IC_{50} for chloroquine (CQ), monodesethylamodiaquine (MDAQ), and piperaquine (PPQ) disaggregated by *pfcrt-pfmdr1* haplotypes. Haplotypes were built with *pfcrt* Lys76Thr and *pfmdr1* Asn86Tyr using the major allele per position. Dashed lines indicated the IC_{50}s cut-offs. n: number of isolates carrying the haplotype. Differences in the IC_{50} of wild-type (WT|WT, crt-K76|mdr1-N86, n = 497) versus single mutant (Mut|WT, crt-76T|mdr1-N86, n = 126), wild-type vs single mutant (WT|Mut, crt-K76|mdr1-86Y, n = 28), and wild-type vs double mutant (Mut|Mut, crt-76T|mdr1-86Y, n = 13) were calculated with Pairwise Wilcoxon tests with Benjamini–Hochberg correction. *: $p < 0.05$; **: $p < 0.001$.

African regions. Continuous monitoring of *pfkelch13* mutations should, however, be reinforced as they could either emerge locally or be introduced in these regions by intracontinental migration from East Africa.

Mutations in *pfcrt* and *pfmdr1* genes were individually associated with decreased IC_{50} to LMF, consistent with previous *in vitro* and clinical findings.[34–36] Remarkably, two unreported *pfmdr1* mutations, Asp650Asn and Asn652Asp, were also associated with decreased IC_{50} to LMF (and MFQ). These new associations implicating Asp650Asn and Asn652Asp depend on the *pfmdr1* Asn86Tyr genotype and could, therefore, be a hitchhiking effect. The two new mutations are located in a *Plasmodium*-specific Asn/Asp repeat protein region that is disordered, situated at an extremity of the cytoplasmic nucleotide-binding domain 1. They are not part of the modelled MFQ binding sites nor at the known regulatory active site(s) of the transporter.[37] Gene editing experiments are essential to formally investigate their role in modulating drug response to LMF and MFQ.

Haplotypic association analysis further suggests that the two main polymorphisms in *pfcrt* and *pfmdr1* have cumulative effects, with the wild-type haplotype (*pfcrt* K76-*pfmdr1* N86) exhibiting the largest IC_{50} to LMF. The increasing frequency in recent years of parasites carrying the wild-type allele at either gene or at both could, therefore, be due at least partly to the drug pressure exerted by LMF on parasite populations in recent years.[38,39] In contrast, the double mutant haplotype (*pfcrt* 76T-*pfmdr1* 86Y) exhibited the largest IC_{50} to MDAQ, suggesting an additive effect of both mutations against this drug. Taken together, these findings support the rationale for implementing regimens with drug combinations that exert opposing selective pressures.[40] We found a slight yearly increase (~10%) in LMF and MDAQ IC_{50} levels using linear regression analysis. Double mutant haplotypes were significantly linked to the lower and largest IC_{50} values, respectively. However, trend tests revealed no clear temporal patterns, meaning the yearly effect was too small to create a consistent trend. The haplotype effects remained stable but impactful without directional selection over time.

Intriguingly, one novel *pfubp1* mutation, Lys1705Asn, was associated with increased IC_{50} to CQ in our dataset. The effect of this *pfubp1* mutation seems to depend on the *pfcrt* Lys76Thr genotype. Several studies have reported an association between different *upb1* mutations and altered survival or susceptibility to DHA in mouse malaria or *P. falciparum*,[14,41] and recently to MFQ, LMF and PPQ in the *Plasmodium yoelii* mouse malaria model.[42] However, as the new mutation is located in an Asn/Lys repeat protein region that is not part of the reported active site(s) of the enzyme, gene editing experiments are needed to formally address their role in modulating drug response to CQ.[43]

The high frequency of pyrimethamine resistance mutations in the study period indicates the high level of SP pressure in West and Central Africa. Nevertheless, the low frequency of *pfdhps* Lys540Glu (<10%) and of the SP-resistant quintuple *pfdhfr-pfdhps* mutant (3%) is reassuring that SP is still effective for preventive therapies. Indeed, the WHO recommends that countries withdraw SP for IPTp use when the frequency of *pfdhps* Lys540Glu > 95% and Ala581Gly > 10% and for IPTi use when the frequency of *pfdhps* Lys540Glu > 50%.[44]

This study was not conducted without limitations. First, we have not evaluated *ex vivo* resistance to

artemisinin derivatives with the dedicated ring survival assay, the standard measure of *in vitro* susceptibility to artemisinins. We, however, report very rare ART-R mutations in *pfkelch13*, and at this time, these are the major determinants of ART-R in patients. Second, our geographical coverage of West and Central Africa is biased, with two countries contributing to almost 50% of the total isolate sampling (169 isolates from Cameroon in Central Africa and 217 isolates from Côte d'Ivoire in West Africa). Therefore, trends at the country level could not be robustly drawn. Third, our analyses focused on imported malaria cases to France and infections in travellers may differ in several aspects from those in people living in endemic areas. Finally, we used a targeted sequencing approach to genotype isolates and focused on validated and some candidate drug resistance genes. Detecting emerging resistance mutations in novel genes would require a larger genomic coverage.

In conclusion, our study provides much-needed information on molecular and phenotypic drug resistance in contemporary isolates from West and Central Africa. It shows how current and past control efforts have been affecting malaria parasite populations in those regions and calls for intensifying the monitoring to inform as quickly as possible on future changes regarding antimalarial drug susceptibilities and resistance.

Contributors

The Imported Malaria Study Group of the FNMRC collected the blood samples and clinical data. JR, AAF, SC, VS, RCo, JC, JAB, and SH designed the study. SC, VS, JB, RZ, AB, LC, and LH did the *ex-vivo* IC_{50} assays and archived data. LM, MT, BP, JC, and SH provided administrative and logistical support. JR, AAF, JC, RCr, and JAB analysed and did the genotyping. JR and AAF verified and analysed the data and did the statistical analysis. All authors had full access to all the data. All authors contributed to the writing of the manuscript. All authors read and approved the final version.

Data sharing statement

All sequencing data are available under accession no. SAMN42141774 to SAMN42142576 at the Sequence Read Archive (SRA), and the associated BioProject ID is PRJNA1129163. De-identified datasets generated during the current study and used to make all figures are available as supplementary files or tables. The data and R scripts developed in this study were deposited in the GitHub repository (https://github.com/jrosados/CNR_IC50_MIPs). Additional software packages and tools that are useful when working with MIP data are available at https://github.com/bailey-lab/MIPTools and https://github.com/Mrc-ide/mipanalyzer.

Declaration of interests

The authors declare no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105835.

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