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1 *Ex vivo* susceptibility to antimalarial drugs and polymorphisms
2 in drug resistance genes of African *Plasmodium falciparum*,
3 2016-2023: a genotype-phenotype association study

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36

37 **Keywords:** Antimalarial drug resistance, *ex-vivo* susceptibility, IC50 assay, genotype

38 Abstract

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

41 **Methods:** We measured the susceptibility to six antimalarials using *ex vivo* growth inhibition
42 assays (IC₅₀) for a total of 805 *Plasmodium falciparum* isolates obtained from travelers returning
43 to France (2016-2023), mainly from West and Central Africa. Isolates were sequenced using
44 molecular inversion probes (MIPs) targeting fourteen drug resistance genes across the parasite
45 genome.

46 **Findings:** *Ex vivo* susceptibility to several drugs has significantly decreased in 2019-2023 versus
47 2016-2018 parasite samples: lumefantrine (median IC₅₀: 23·0 nM [IQR: 14·4-35·1] in 2019-2023
48 versus 13·9 nM [8·42-21·7] in 2016-2018, p<0·0001), monodesethylamodiaquine (35·4 [21·2-
49 51·1] versus 20·3 nM [15·4-33·1], p<0·0001), and marginally piperaquine (20·5 [16·5-26·2]
50 versus 18·0 [14·2-22·4] nM, p<0·0001). Only four isolates carried a validated *pfkelch13* mutation.
51 Multiple mutations in *pfert* and one in *pfmdr1* (N86Y) were significantly associated with altered
52 susceptibility to multiple drugs. The susceptibility to lumefantrine was altered by *pfert* and *pfmdr1*
53 mutations in an additive manner, with the wild-type haplotype (*pfert* K76-*pfmdr1* N86) exhibiting
54 the least susceptibility.

55 **Interpretation:** Our study on *P. falciparum* isolates from West and Central Africa indicates a low
56 prevalence of molecular markers of artemisinin resistance but a significant decrease in
57 susceptibility to the partner drugs that have been the most widely used since a decade -lumefantrine
58 and amodiaquine. These phenotypic changes likely mark parasite adaptation to sustained drug
59 pressure and call for intensifying the monitoring of antimalarial drug resistance in Africa.

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63 Institut de Recherche pour le Développement.

64 Research in context

65 Evidence before this study

66 Artemisinin-based combination therapies (ACTs) have been introduced since the 2000s as the
67 first-line curative treatment of malaria. ACTs combine an artemisinin derivative, which rapidly
68 reduces parasite load, with another antimalarial drug -known as partner drug- which eliminates the
69 remaining parasites thanks to its longer half-life. This approach reduces the likelihood of parasites
70 developing resistance to both drugs, thereby increasing treatment efficacy and delaying the
71 emergence of resistance. However, resistance to artemisinins and then to some partner drugs was
72 identified in Southeast Asia more than a decade ago and has spread throughout the region.
73 Artemisinin partial resistance is now emerging in the East and Horn of Africa. It manifests as
74 delayed parasite clearance from the bloodstream after treatment, increasing the parasite load in
75 contact with the partner drug only and the likelihood of selecting resistant parasites. It is, therefore,
76 important to monitor antimalarial drug susceptibility and drug resistance mutations in
77 contemporary African isolates, especially in the understudied West and Central African regions,
78 to anticipate the spread of multidrug-resistant parasites.

79 We searched for articles on antimalarial drug resistance published between January 1, 2000, and
80 July 1, 2024, using the PubMed search terms “antimalarial resistance”, “Africa”, and “*ex vivo*”.
81 Of the 69 published studies, only six encompassing a total of 827 isolates across five West and
82 Central African countries from 2016 to 2022 combined *ex vivo* drug assays with genotyping data.
83 Parasites with an increased rate of *ex vivo* survival to artemisinins were reported in one study from
84 Ghana (7/90 isolates in 2018) and another from The Gambia (4/41 isolates in 2017). Only the
85 Ghanaian study reported mutations in the non-propeller domain of *pfkelch13* gene, whereas the
86 Gambian study reported mutations associated with reduced susceptibility to lumefantrine (7%,
87 3/41). In Mali, Senegal and Burkina Faso, most isolates were susceptible to commonly used
88 antimalarial drugs (chloroquine, amodiaquine, piperaquine, mefloquine, lumefantrine and
89 dihydroartemisinin) using standard growth inhibition assays. In Ghana, reduced susceptibility to
90 artemisinin, mefloquine and amodiaquine was observed. The relative lack of recent data on
91 parasite susceptibility to antimalarial drugs in recent parasites from West and Central Africa
92 prompted us to conduct this study.

93

94 **Added value of this study**

95 *Ex vivo* susceptibility to six antimalarial drugs (dihydroartemisinin, lumefantrine, mefloquine,
96 chloroquine, monodesethylamodiaquine, and piperazine) and mutations in fourteen drug
97 resistance genes were evaluated in 805 isolates collected between January 2016 and February 2023
98 from 35 African countries, mainly from West and Central Africa.

99 Median IC₅₀ values were in the low nanomolar range, indicating good potency against *P.*
100 *falciparum*. However, worrying trends emerged from 2019 onwards, with median IC₅₀ values for
101 lumefantrine that increased from 13·9 nM in 2016-18 to 23 nM in 2019-23 and for amodiaquine
102 from 20·3 nM to 35·4 nM.

103 The high prevalence of resistance alleles in *pfdhfr*, *pfdhps*, *pfmdr1* and *pfcr1* genes underscores the
104 sustained pressure exerted by antimalarial drugs on parasite populations. Notably, although the
105 triple mutant *pfdhfr* N511-C59R-S108N was highly prevalent, the *dhfr-dhps* quintuple mutant
106 (with extra *pfdhps* A437G-K540E), which is responsible for sulfadoxine-pyrimethamine treatment
107 failure in adults and children, was rare. In addition, the analysis revealed some geographic and
108 temporal variations in mutation prevalence.

109 The genotype-phenotype association analysis performed in this study elucidates the relationship
110 between genetic variants and *ex vivo* drug susceptibility, providing valuable information for
111 understanding the molecular basis of resistance and informing future treatment strategies. For
112 example, mutations in the *pfcr1* and *pfmdr1* genes, mainly K76T and N86Y, were associated with
113 altered susceptibilities to most drugs. Haplotypic association analysis further indicated that the two
114 genes have cumulative effects on the susceptibility to lumefantrine, with the wild-type haplotype
115 (*pfcr1* K76-*pfmdr1* N86) exhibiting the least susceptibility.

116 **Implication of all the available evidence**

117 While the susceptibility to most antimalarials suggests continued efficacy, the observed decrease
118 in susceptibilities to lumefantrine and amodiaquine in parasites from West and Central Africa from
119 2019 onwards suggests an ongoing adaptation of parasites, possibly related to the increasing use
120 of ACT treatments in Sub-saharan Africa since a decade. These phenotypic changes over time
121 were accompanied by small changes in the prevalence of resistance alleles in *pfcr1* and *pfmdr1*
122 genes. Additional changes, potentially leading to larger decreases in drug susceptibilities, can be

123 expected over time. The large-scale analysis presented here provides invaluable, contemporary
124 insights into the current landscape of susceptibility to antimalarial drugs and molecular markers of
125 resistance in *P. falciparum* isolates from West and Central Africa. While the data suggests that
126 ACTs and sulfadoxine-pyrimethamine are likely to be effective in these regions, the phenotypic
127 changes we observed call for intensifying the monitoring of antimalarial drug resistance in Africa.

128 Introduction

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

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

150 To better understand the current situation in West and Central Africa, we analyzed the *ex vivo*
151 susceptibility to six drugs (DHA, LMF, mefloquine (MFQ), chloroquine (CQ), mono-
152 desethylamodiaquine (MDAQ), and PPQ; assessed by growth inhibition assays) of 805 *P.*
153 *falciparum* isolates collected between 2016 and 2023 from imported malaria cases in returned
154 travelers to mainly West and Central Africa. The isolates were also sequenced using high-
155 throughput molecular inversion probes (MIPs) targeting 815 overlapping targets across fourteen
156 drug resistance genes to determine the prevalence of drug resistance markers and to estimate
157 genetic correlates of drug susceptibility phenotypes.

158 Methods

159 Sample and data collection

160 The French National Malaria Reference Center (FNMRC) coordinates a nationwide network of
161 hospitals in mainland France to survey imported malaria. Blood samples from civilian or military
162 hospitals participating in the FNRMC network were sent to the FNRMC reference laboratory
163 (Bichat Hospital in Paris) (see Study Group). Inclusion criteria were: diagnosis of *P. falciparum*
164 malaria recorded between January 2016 and February 2023 and confirmed by microscopy and
165 PCR, available DNA sample, information on the country visited, and available IC₅₀ data against
166 CQ, MFQ, PPQ, MDAQ, LMF and DHA. A total of 813 isolates fulfilled the inclusion criteria.

167 *Ex vivo* assays

168 CQ, DHA, MDAQ, MFQ, LMF, and PPQ were purchased from Alsachim (Illkirch Graffenstaden,
169 France). The susceptibility of the isolates to the antimalarial drugs was assessed without culture
170 adaptation. 100 µL of parasitized erythrocytes (final parasitaemia of 0.5% and a final hematocrit
171 of 1.5%) were aliquoted into 96-well plates preloaded with a concentration gradient of antimalarial
172 drugs. The plates were incubated for 48 hours in a controlled atmosphere of 85% N₂, 10% O₂, 5%
173 CO₂ at 37°C. Drug susceptibilities were determined as half-maximal inhibitory concentrations
174 (IC₅₀s) using the standard 42-hour [³H]hypoxanthine uptake inhibition assay (**Supplementary**
175 **Methods**)²¹. Resistance thresholds for the six compounds were previously reported by Pradines,
176 Kaddouri and others.²²⁻²⁴

177 DNA extraction and MIP sequencing of drug resistance genes

178 Genomic DNA was extracted from 200 µL whole blood using a MagNA Pure automaton (Roche
179 Diagnostics, USA), eluted in 100 µL, and stored at -20°C. DNA extracts from the included samples
180 were sent to Brown University for MIP sequencing. All samples were then genotyped using
181 molecular inversion probes (MIPs, n=815) targeting known drug resistance SNP mutations in 14
182 genes across the *P. falciparum* genome, as previously described (**Table S1, Appendix 2**).^{12,25}
183 Sequencing was performed on an Illumina NextSeq 550 instrument (150 bp paired-end reads) at
184 Brown University (RI, USA).

185

186 **MIP data analysis and estimating drug resistance prevalence**

187 Sequencing data processing and variant calling were performed using MIPtools (v0.19.12.13;
188 <https://github.com/bailey-lab/MIPTools>). The prevalence for each drug resistance marker was
189 calculated as $p = x / n * 100$, where p is the prevalence, x is the number of infections with mutant
190 alleles, and n is the number of successfully genotyped infections, as previously described.¹² Mixed
191 genotypes, i.e. a sample with a reference allele and an alternative allele in a given sample, were
192 considered mutant regardless of the within-sample mutant allele frequency. Haplotypes were
193 reconstructed for all samples using the major allele in a given codon (**Supplementary Methods**).

194 **Phenotype–genotype association analysis**

195 Isolates with >30% missing genotype data were discarded. We then retained only biallelic non-
196 synonymous SNPs with minor allele frequency (MAF) >0.01 for phenotype-genotype association
197 analysis. Phenotype-genotype association was performed with the SNPassoc R package (v.2.1.0)
198 ²⁶ using the built-in WGassociation function under a dominant model. For each drug, a linear
199 regression model was fitted with continuous IC₅₀ values as the outcome and individual SNP
200 genotypes as independent variables. The model was adjusted for the complexity of infection (COI),
201 year, and the visited country of imported cases as covariates.

202 **Ethical aspects**

203 Informed consent was not required because the clinical and biological data were collected from
204 the FNMRC database by the common public health mission of all National Reference Centers in
205 France, in coordination with the Santé Publique France organization for malaria surveillance and
206 care. According to article L1221–1.1 of the Public Health Code in France, the study was considered
207 non-interventional research and only required the patient's non-opposition (according to article
208 L1211–2 of the Public Health Code). All data were anonymized before use. The FNMRC got the
209 authorization to use their databases according to the National Data Protection Commission (CNIL)
210 (number 1223103). Human DNA was not analyzed.

211 **Role of the funding source**

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

214 Results

215 Geographical origin and year of collection

216 From January 2016 to February 2023, 1,126 *P. falciparum* isolates from travelers were evaluated
217 for *ex vivo* antimalarial drug susceptibility at the FNMRC laboratory. Of these, 813 isolates were
218 included in this study, corresponding to samples with validated IC₅₀ for the following six drugs:
219 DHA, LMF, MFQ, MDAQ, CQ, and PPQ. According to reported travel history, infections
220 originated from thirty-five African countries, but most isolates were from travelers who had visited
221 West (59%, 471/805) and Central (38%, 306/805; **Figure 1A**) African countries. The most visited
222 countries were Côte d'Ivoire (27%, 217/805), Cameroon (21%, 169/805), Mali (9%, 69/805),
223 Guinea (7%, 58/805), and Republic of the Congo (6%, 45/805) (**Figure 1A** and **Table S2**). The
224 number of samples collected per year was higher in 2016 (165/805), 2017 (166/805) and 2018
225 (167/805) than in 2019 (100/805) and beyond (**Figure 1B**). No differences were seen in the
226 distribution of countries between 2016-2018 and 2019-2023 ($p > 0.05$, **Table S3**). Travelers were
227 predominantly male (62%), with a median age of 40 years and a median parasitemia of 1.7%
228 (**Table 1**).

229 Half-maximal inhibitory concentration (IC₅₀) for six antimalarial compounds

230 IC₅₀ values of *P. falciparum* 3D7 laboratory reference strain are indicated in **Table S4**. For the
231 isolates tested in this study, the median IC₅₀ estimated with the standard growth inhibition assay
232 was less than 30 nM for all assessed antimalarial compounds, consistent with potent activity. The
233 median IC₅₀ values for the six drugs were 1.1 nM [IQR: 0.8-1.7] for DHA, 16.7 nM [9.9-27.4]
234 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]
235 for CQ, and 18.5 nM [15.1-24.3] for PPQ. Using published resistance thresholds,²²⁻²⁴ 49% of the
236 isolates were classified as resistant to MFQ (IC₅₀ > 30 nM), 10% to CQ (IC₅₀ > 100 nM), and 3% to MDAQ
237 (IC₅₀ > 80 nM). Less than 1% of isolates had an IC₅₀ above the threshold for DHA (10 nM), LMF (150 nM)
238 and PPQ (135 nM) (**Figure S1**).

239 Remarkably, when compared to samples collected in 2016-2018, a significant decrease in
240 susceptibility was observed in 2019-2023 samples for LMF (median IC₅₀: 13.9 nM [IQR: 8.42-
241 21.7] in 2016-2018 versus 23.0 nM [14.4-35.1] in 2019-2023; $p < 0.001$), MDAQ (20.3 nM [15.4-
242 33.1] versus 35.4 nM [21.2-51.1]; $p < 0.001$), and modestly for PPQ (18.0 nM [14.2-22.4] versus
243 20.5 [16.5-26.2]; $p < 0.001$) (**Figure 2**, **Table S5**). In contrast, a modest improvement in

244 susceptibility was observed in 2019-2023 samples for CQ (27.7 nM [19.0-45.0] versus 25.1 nM
245 [17.3-38.9]; $p=0.05$), and there was no significant change for MFQ. Altered drug susceptibilities
246 between the two time periods were again detected after data stratification into West and Central
247 African countries, except for DHA, which was significantly altered only in samples from Central
248 Africa, and for CQ (**Table S6**), possibly because of less power for CQ. We note that these temporal
249 changes in drug susceptibility were not significant when using 1-year bins during the 2016-2023
250 study period (**Table S7**). The frequency of CQ-resistant isolates ($IC_{50} > 100$ nM) decreased nearly
251 by half in 2019-2023 compared to 2016-2018 (7% in 2019-2023 versus 12% in 2016-2018,
252 $p<0.05$) (**Table S8**).

253 There were several significant drug-drug IC_{50} correlations, most of which were positive (**Figure**
254 **S2**). The largest positive correlations involved the following drug pairs: LMF-MFQ, CQ-MDAQ,
255 DHA-LMF, and DHA-MFQ. The negative correlations were much less intense and involved CQ-
256 LMF and CQ-MFQ.

257 **Prevalence of validated antimalarial resistance mutations**

258 Sequence data generated with MIPs covered 43 exons from 14 genes reported to be associated
259 with drug resistance. 805 out of the 813 included isolates were successfully genotyped. The
260 average number of reads per sample was 120 (range: 48-258) (**Figure S3**). Nearly 60% of the
261 isolates (477/805) had a COI >1 . We first examined the prevalence of mutations in five robustly
262 validated drug resistance genes: *pfdhfr* and *pfdhps* (associated with resistance to pyrimethamine
263 and sulfadoxine, respectively), *pfert* and *pfmdr1* (associated with multidrug resistance), and
264 *pfkelch13* (associated with ART-R).

265 More than 85% of isolates carried the *pfdhfr* N51I, C59R, and S108N mutations (**Figure 3A**),
266 which were predominantly found as the IRN triple mutant haplotype and marginally as the ICN
267 and NRN double mutants (**Figure S4**), regardless of the country of infection (**Figure S5, Table**
268 **S9**). We found a low prevalence of the *pfdhps* I431V (8.5%), K540E (6%), A581G (8%) and
269 A613S (15%) mutations, and a large prevalence of S436A and A437G (53% and 78%,
270 respectively). At *pfdhps* codons 436-437-540-581 considered jointly (haplotypes, **Figure S4**),
271 single mutants AAKA and SGKA were the most common haplotypes (35% and 50% respectively).
272 The quintuple mutant *dhfr+dhps* haplotype IRN+A437G-K540E, which is responsible for SP
273 treatment failure in adults and children, was detected in 3% of samples (19/600).

274 In *pfmdr1*, the prevalence of the N86Y, Y184F, D1246Y mutations were 11%, 71%, and 1%,
275 respectively (**Fig 3A**), and the prevalence of the NYD, NFD and YFD haplotypes at codons 86-
276 184-1246 were 38·8%, 54·8% and 5·6%, respectively (**Figure S4**). Other minor haplotypes were
277 found in less than 1% of isolates.

278 The *pfprt* K76T mutation was found in 30% of isolates (**Figure 3A**) and was absent in the Central
279 African Republic (**Figure S5**). Regarding the classical eight *pfprt* codons 74-75-76-220-271-326-
280 356-371, three main haplotypes were detected (**Figure S4**; per country, see **Table S10** and **Figure**
281 **S6**): the wild-type MNKAQNIR (77%) and the mutants **IETSENTI** (also known as Cam783;
282 15%) and **IETSENI** (also known as GB4; 6%).

283 Four isolates carried a validated *pfkelch13* mutation in the propeller domain (**Figure 3B**): A675V
284 (2/734, from Rwanda in 2019 and Uganda in 2020), Y493H (1/705, from Mali in 2016), and I543T
285 (1/731, from Côte d'Ivoire in 2018). The two isolates carrying the A675V mutation showed a
286 susceptibility to DHA ($IC_{50} = 1\cdot78$ nM and $IC_{50} = 1\cdot67$ nM) larger than the upper quartile. The
287 A675V isolate from Rwanda also had elevated IC_{50} values for LMF ($IC_{50} = 41\cdot57$ nM) and MFQ
288 ($IC_{50} = 49\cdot12$ nM). Two other mutations were detected in the propeller domain: A578S (8 isolates)
289 that is known as a polymorphism and E612D (1 isolate). Most of the other non-synonymous
290 *pfkelch13* mutations occurred in the N-terminal-coding domain (**Figure 3B**, **Figure S7**), K189T
291 being very frequent (58·4%) and also known as polymorphism.

292 Finally, the prevalence of “background” mutations associated with ART-R in South East Asia ²⁷
293 was low: *pfdd* D193Y at 0·27%, *pfmdr2* T484I at 0%, *pfpi7* C1484F at 0·16%, and *pfpp* V1157L
294 at 0% (**Table S11**).

295 We then analysed mutation prevalence over time (**Figure 3C**, **Figures S8** and **S9**, and **Table S12**).
296 Remarkably, when compared to samples collected in 2016-2018, a significant decrease in
297 prevalence was observed in 2019-2023 samples for *pfprt* K76T (33% in 2016-2018 versus 25% in
298 2019-2023; $p < 0\cdot05$) and *pfmdr1* N86Y (14% versus 5%; $p < 0\cdot001$), and there was a nearly
299 significant decrease for *pfmdr1* Y184F (73% versus 66%; $p = 0\cdot07$) (**Figure 3C**). The prevalence
300 of the wild-type haplotype at *pfprt* 76 and *pfmdr1* 86 (K76-N86) was largely dominant across the
301 whole study period and slightly increased from 73% in 2016-2018 to 79% in 2019-2023 ($p = 0\cdot05$,
302 **Figure 3D**, **Figure S8B**). Analysis of SNP-SNP correlation in isolates from Côte d'Ivoire and

303 Cameroon indicated that *pfmdr1* N86Y and *pfprt* K76T were not in linkage disequilibrium with
304 each other (**Figure S10**).

305 **Association between SNPs and drug susceptibility**

306 Genotype-phenotype association analysis across 362 SNPs and susceptibilities to 6 drugs showed
307 several significant associations (**Figure 4**, **Tables S12** for the full list of associations, and **S13** and
308 **S14** after correction for multiple testing). Most strikingly, multiple well-known SNPs in *pfprt* were
309 strongly associated with susceptibilities to multiple drugs (CQ, MDAQ, DHA, LMF) and were
310 borderline significant after multiple testing correction for MFQ and PPQ. Mutant *pfprt* alleles were
311 associated with decreased susceptibility to CQ ($p = 10^{-38}$ – 10^{-50} for 7 amino acids) and MDAQ
312 ($p = 10^{-6}$ – 10^{-8} for 7 amino acids) whereas wild-type *pfprt* alleles were associated with decreased
313 susceptibility to DHA ($p = 10^{-6}$ for 6 amino acids) and LMF ($p = 10^{-5}$ for 3 amino acids). There
314 was also a strong association between the well-characterised mutation N86Y in *pfmdr1* and
315 susceptibility to MFQ ($p = 10^{-6}$), LMF ($p = 10^{-5}$), and DHA ($p = 0.0003$, although non-significant
316 after correction). Finally, the *pfubp1* N1704K and K1705N mutations were associated with
317 decreased susceptibility to CQ ($p = 2 \cdot 10^{-5}$ and 0.0001 respectively).

318 To control for the major *pfprt* effect, we added *pfprt* K76T as a covariate (**Table S13**). All the
319 associations involving another SNP in *pfprt* (except M74I for CQ) and *pfubp1* N1704K and
320 K1705N were lost, suggesting they were dependent on the *pfprt* K76T genotype. The associations
321 involving *pfmdr1* (with LMF and MFQ) were maintained and no new associations were detected.
322 A model with *pfmdr1* N86Y as a covariate abolished only the association of *pfubp1* K1705N for
323 CQ (but not the one of N1704K) (**Table S13**). These data indicate that, among the 362 tested SNPs,
324 *pfprt* and *pfmdr1* SNPs were the major drivers of susceptibility to most tested drugs and could
325 interact in a likely additive manner in the case of LMF.

326 **Effect of *pfprt* and *pfmdr1* haplotypes on IC₅₀s**

327 As compared to the wild-type *pfprt* (MNKAQNIR), mutant haplotypes **IETSENTI** and **IETSENII**
328 (that differ at I356T only) were significantly associated with decreased susceptibility to CQ and
329 MDAQ (Kruskal-Wallis test, p-value range: <0.0001 – 0.0003 ; **Figure S11**) and improved
330 susceptibility to DHA, LMF and PPQ (Kruskal-Wallis test, p-value range: <0.0001 – 0.05).
331 **IETSENTI**, but not **IETSENII**, was associated with improved susceptibility to MFQ (Kruskal-
332 Wallis test, $p = 0.0001$), suggesting that I356T has some role in MFQ transport activity.

333 Isolates carrying the *pfmdr1* NYD wild-type haplotype had decreased susceptibility to DHA, LMF,
334 MFQ and improved susceptibility to PPQ as compared to the mutant YFD (Kruskal-Wallis test,
335 p-value range: <0.001 - 0.01; **Figure S11**).

336 Finally, the analysis of *pfprt* K76T - *pfmdr1* N86Y haplotypes identified three main drug patterns
337 (**Figure 5**). The susceptibility to LMF, MFQ, and also marginally to DHA, improved with the
338 number of *pfprt-pfmdr1* mutations suggesting these mutations work in additive ways. The most
339 decreased susceptibility to LMF was associated with the wild-type haplotype (*pfprt* K76-*pfmdr1*
340 N86). The susceptibility to CQ and MDAQ decreased mostly with mutant *pfprt*, and the addition
341 of mutant *pfmdr1* had a lesser effect. The susceptibility to PPQ was more difficult to interpret with
342 little haplotypic effects.

343 Discussion

344 Whereas there are recent reports of emerging ART-R and decreased susceptibility to LMF in *P.*
345 *falciparum* parasites from East Africa,²⁸ few recent studies have investigated the resistance status
346 of contemporary West and Central African isolates at a large scale. Here, we report the temporal
347 trends of *ex vivo* susceptibilities to six antimalarials using standard growth inhibition assays and
348 their association with mutations in fourteen drug resistance genes in 805 *P. falciparum* samples
349 collected between 2016 and 2023 in returned travelers to West and Central African countries. We
350 found an overall excellent susceptibility of isolates to the six evaluated antimalarials, but the one
351 to ACT partner drugs LMF and MDAQ decreased in recent years. Concomitantly, the prevalence
352 of wild-type *pfprt* K76 and *pfmdr1* N86 alleles increased in recent years, consistent with a large
353 selective pressure exerted by the use of LMF and the discontinuation of CQ in these regions.
354 Finally, altered susceptibility to most drugs was strongly associated with *pfprt* and/or *pfmdr1*
355 genotypes.

356 Regarding molecular ART-R, only four isolates among 805 carried a WHO-validated *pfkelch13*
357 mutation. A675V was detected in two travelers returning from East Africa (Rwanda and Uganda),
358 consistent with the spread of this mutation in this region.^{10,29} The returned traveler from Uganda
359 showed delayed clearance under artesunate therapy,³⁰ whereas the A675V isolate from Rwanda,
360 had elevated IC₅₀ values for LMF (41.57 nM) and MFQ (49.12 nM), consistent with clinical and
361 *ex vivo* data reported by others for Ugandan A675V isolates.^{11,14,15} Two other validated mutations,

362 Y493H and I543T, were identified in one isolate from Mali in 2016 and one from Côte d'Ivoire in
363 2018, respectively. These isolates did not have unusual IC₅₀s against any of the drugs tested. As
364 these two mutations were not detected in more recent samples, these seem to be sporadic cases.
365 Altogether, *pfkelch13* ART-R mutations were detected but uncommon in Central and West African
366 isolates during the study period (2016-2023). Contrary to the current situation in East Africa,
367 *pfkelch13*-mediated ART-R is seemingly not emerging during 2016-2023 in West and Central
368 African regions. Continuous monitoring of *pfkelch13* mutations should however be reinforced as
369 they could either emerge locally or be introduced in these regions by intracontinental migration
370 from East Africa.

371 We found a significant reduction in susceptibility of *P. falciparum* to LMF and MDAQ for samples
372 collected in recent years. LMF and MDAQ are the two main partner drugs used in West and Central
373 Africa during the last decade. The use of ACTs may have selected parasites whose response to the
374 partner's drug is altered in these regions. A similar trend was previously reported for LMF in
375 northern Uganda,¹⁴ which possibly predated the emergence of ART-R in this region.¹⁰ The clinical
376 effects of the decreased susceptibilities to LMF and MDAQ observed in our study are unknown,
377 but the spread of ART-R may be facilitated in such phenotypic backgrounds.

378 Remarkably, wild-type *pfprt* and *pfmdr1* genes were individually associated with decreased
379 susceptibility to LMF, consistent with previous *in vitro* and clinical findings.^{31,32,33} Haplotypic
380 association analysis further suggests that the two genes have cumulative effects, with the wild-
381 type haplotype (*pfprt* K76-*pfmdr1* N86) exhibiting the largest decrease in susceptibility to LMF.
382 The increasing prevalence in recent years of parasites carrying the wild-type allele at either gene
383 or at both could, therefore, explain the LMF data. In contrast the double mutant haplotype (*pfprt*
384 76T-*pfmdr1* 86Y) exhibited the largest decrease in susceptibility to MDAQ. Taken together, these
385 findings support the rationale for implementing regimens with drug combinations that exert
386 opposing selective pressures.³⁴ Of note, susceptibility to MDAQ decreased in recent years,
387 whereas the prevalence of the *pfprt* K76T mutation decreased slightly. This is surprising as this
388 mutation is strongly associated with decreased susceptibility to MDAQ in our dataset and other
389 studies. We have no solid explanation for this result, but one possibility could be that other
390 mediators of altered susceptibility to MDAQ exist and that our targeted MIP sequencing and
391 association studies did not capture them.

392 Intriguingly, two novel *pfubp1* mutations, N1704K and K1705N, were associated with reduced
393 susceptibility to CQ in our data set. The effect of these *pfubp1* mutations seems to depend on the
394 *pfert* K76T genotype. However, as these two mutations have low prevalence (10.0 and 7.9%,
395 respectively) and likely a modest effect, we have low statistical power to conduct a haplotypic
396 analysis. Several studies reported an association of different *upb1* mutations with altered survival
397 or susceptibility to DHA in mouse malaria or *P. falciparum*,^{35,14} and recently to MFQ, LMF and
398 PPQ in the *Plasmodium yoelii* mouse malaria model.³⁶ However, as the two new mutations are
399 located in an N/K repeat protein region that is not part of the reported active site(s) of the enzyme,
400 gene editing experiments are needed to formally address their role in modulating drug response to
401 CQ.³⁷

402 The high prevalence of pyrimethamine resistance mutations in the study period indicates the high
403 level of SP pressure in West and Central Africa. Nevertheless, the low prevalence of *pfdhps* K540E
404 (<10%) and of the SP-resistant quintuple *dhfr-dhps* mutant (3%) is reassuring that SP is still
405 effective for preventive therapies. Indeed, the WHO recommends for countries to withdraw SP for
406 IPTp use when the prevalence of *Pfdhps* K540E>95% and A581G>10% and for IPTi use when
407 the prevalence of *Pfdhps* K540E >50%.³⁸

408 This study was not conducted without limitations. First, we have not evaluated *ex vivo* resistance
409 to artemisinin derivatives with the dedicated ring survival assay, the standard measure of in vitro
410 susceptibility to artemisinins. We, however, report very rare ART-R mutations in *pfkelch13*, and
411 at this time, these are the major determinants of ART-R in patients. Second, we have not evaluated
412 copy number variations in *pfmdr1* and *pfplasmepsins 1-2* that are associated with altered
413 susceptibility to MFQ and PPQ, respectively. Third, our geographical coverage of West and
414 Central Africa is biased, with two countries contributing to almost 50% of the total isolate sampling
415 (169 isolates from Cameroon in Central Africa and 217 isolates from Côte d'Ivoire in West Africa).
416 Therefore, trends at the country level could not be robustly drawn. Fourth, our analyses focused
417 on imported malaria cases to France and infections in travelers may differ in several aspects from
418 those in people living in endemic areas. Finally, we used a targeted sequencing approach to
419 genotype isolates and focused on validated and some candidate drug resistance genes. Detecting
420 emerging resistance mutations in novel genes would need a larger genomic coverage.

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

426 Author contributions

427 JR, AAF, SC, VS, RCo, JC, JAB, and SH designed the study. SC, VS, RZ, AB, LC, and LH did
428 the *ex-vivo* IC₅₀ assays, and archived data. LM, MT, BP, JC and SH provided administrative and
429 logistical support. JR, AAF, JC, RCr, and JAB analysed and did the genotyping. JR and AAF
430 verified and analysed the data and did the statistical analysis. All authors had full access to all the
431 data. All authors contributed to the writing of the manuscript.

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492 Conflict of interest

493 The authors declare no conflicts of interest.

494 Data availability

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

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Table 1. Epidemiological characteristics of travelers returning to France and description of isolates from 2016 - 2023

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

IQR: Interquartil range; RBC: Red blood cells. * Missing data for two isolates.

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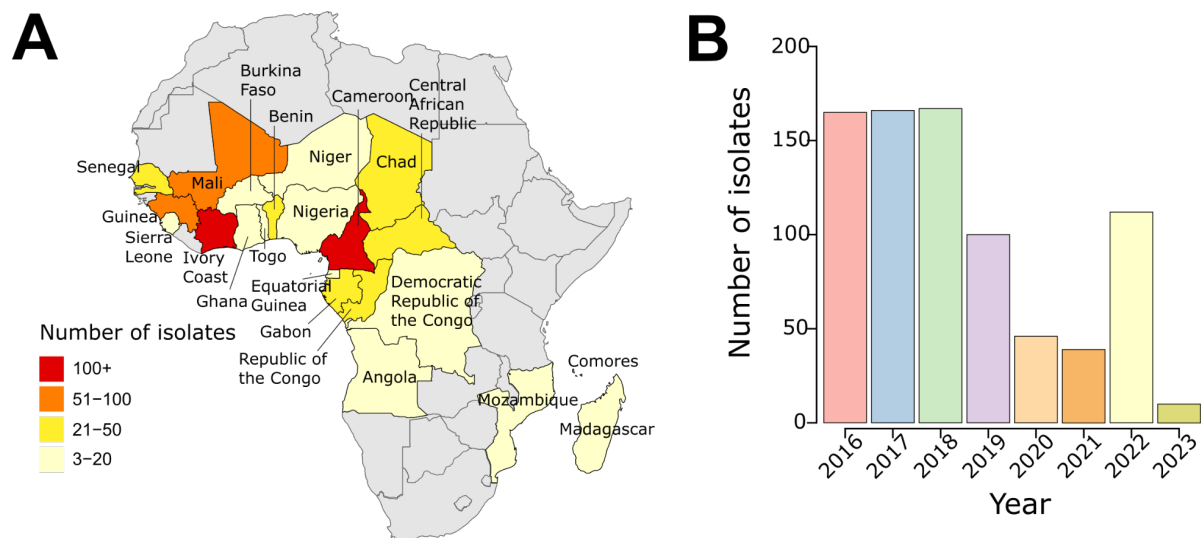
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655 **Figure 1. Geographical origin and collection year of isolates. A)** Map of Africa showing the origin of malaria
656 cases imported into France. Colors indicate sample size, and countries with fewer than three isolates are not shown.
657 **B)** Bar plot showing the temporal distribution of imported malaria cases included in this study.

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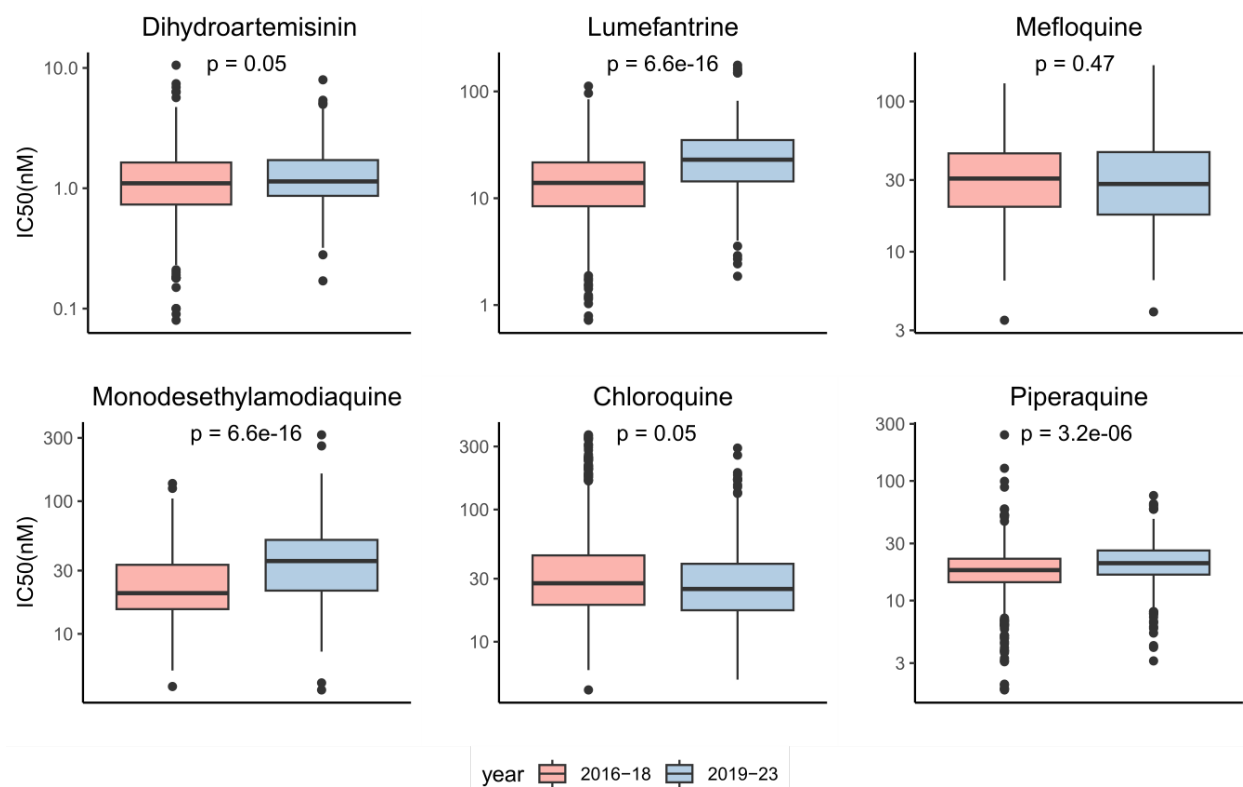
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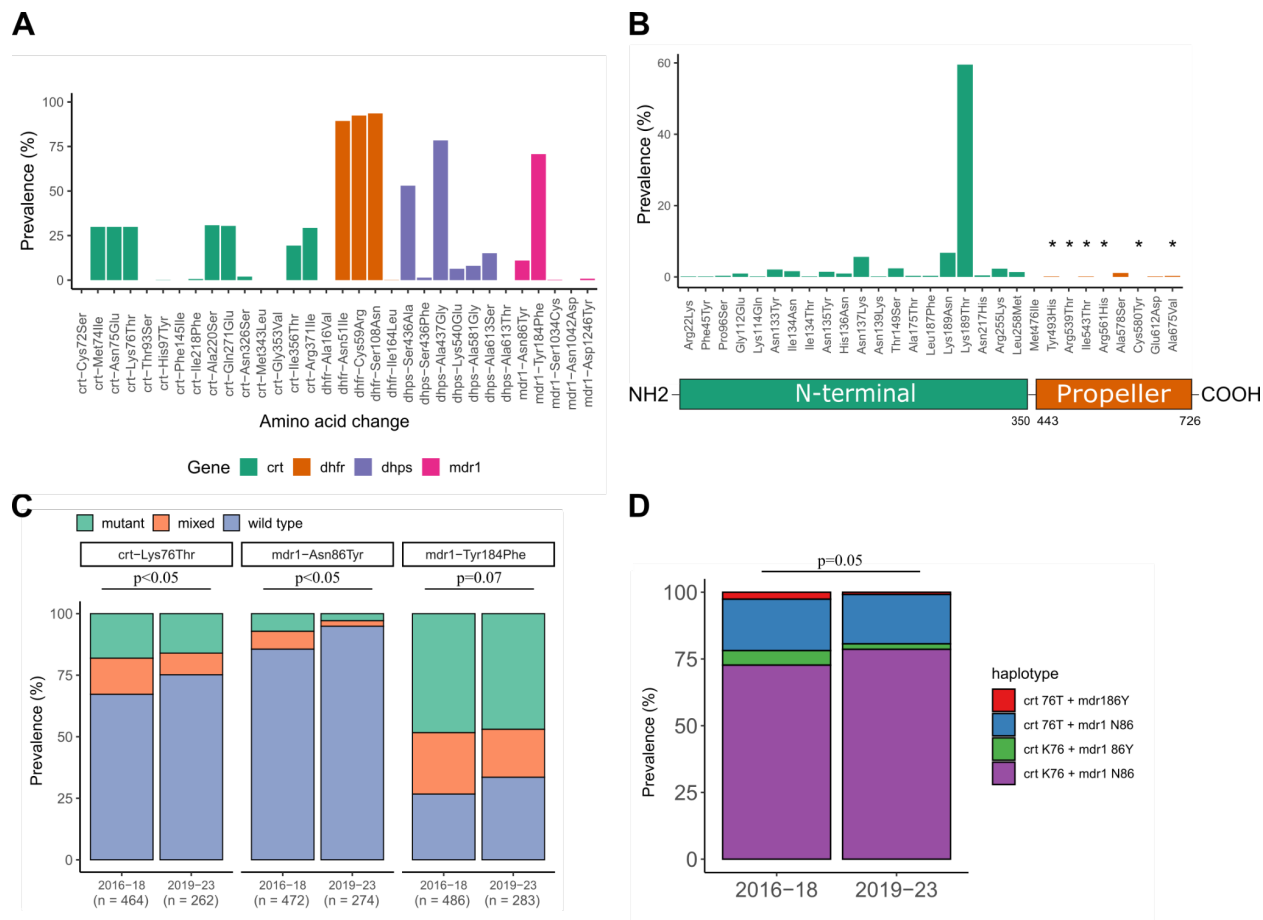
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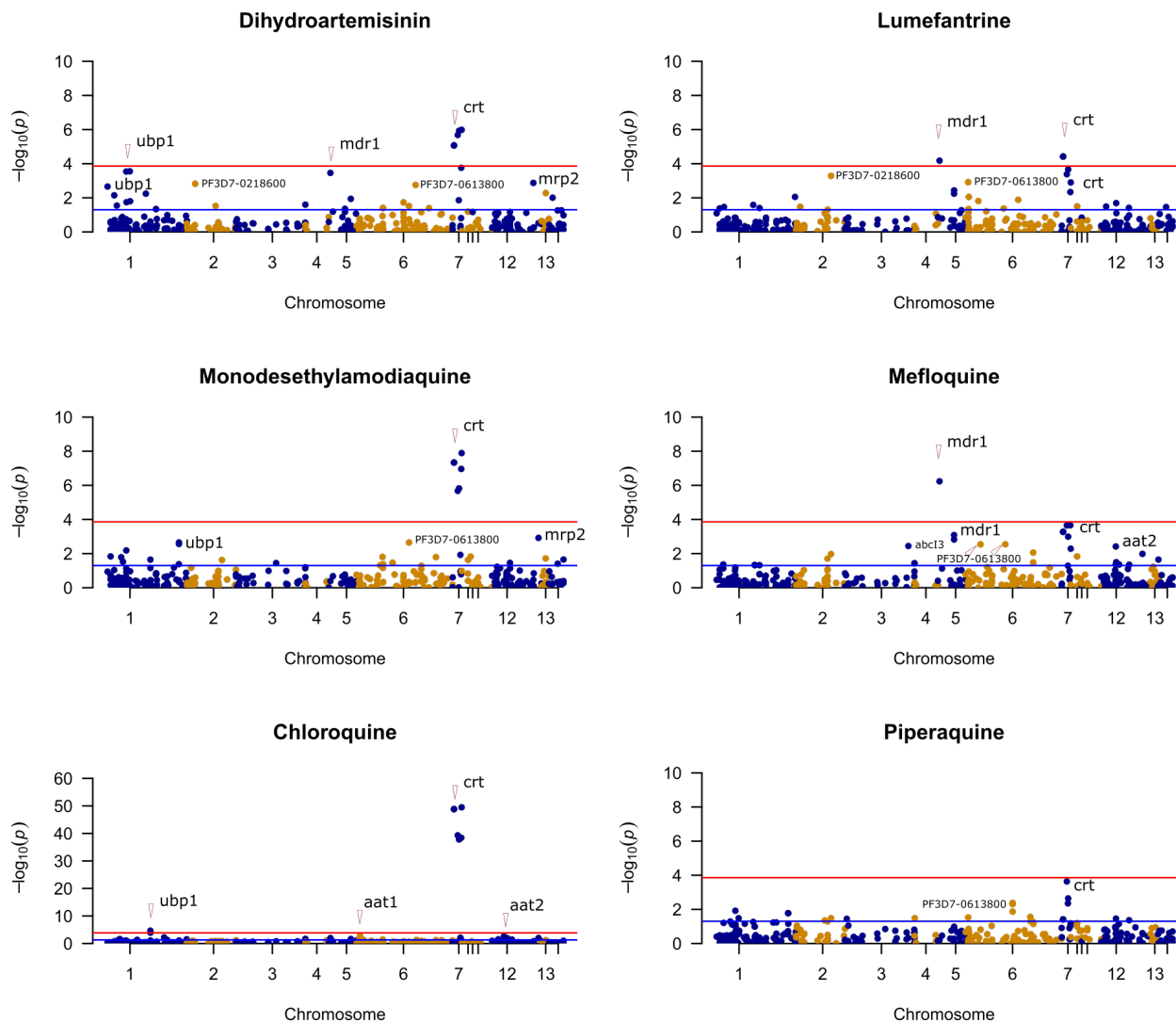
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667 **Figure 2. Half-maximal inhibitory concentration (IC₅₀) for six antimalarial drugs. A)** Distribution of IC₅₀ for the
668 six antimalarial drugs in 2016-18 and 2019-23. Box plots show the median IC₅₀ (in nM) and interquartile range.
669 Change in susceptibility over time was tested by Mann-Whitney test. P-values were adjusted using Benjamini-
670 Hochberg correction.

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 673 **Figure 3. Prevalence of key mutations associated with resistance to different drugs. A)** Prevalence of key
 674 mutations in *pfprt*, *pfdhfr*, *pfdhps* and *pfmdr1* genes detected in isolates from this study. **B)** Prevalence of mutations
 675 in the *pfkelch13* gene. Green bar plots indicate the prevalence of mutations in the N-terminal-coding domain, while
 676 orange bar plots indicate the prevalence of mutations in the propeller domain. SNPs marked with an asterisk (*) are
 677 validated or candidate SNPs by WHO. **C)** Prevalence of the main key mutations in *pfprt* and *pfmdr1* over time. **D)**
 678 Prevalence of *pfprt* 76-*pfmdr1* 86 haplotypes over time. Color code for the 4 haplotypes: purple, wild-type K76-
 679 N86; green, single mutant K76-86Y; blue, single mutant 76T-N86; red, double mutant 76T-86Y. P-values were
 680 calculated by Fisher's exact test.

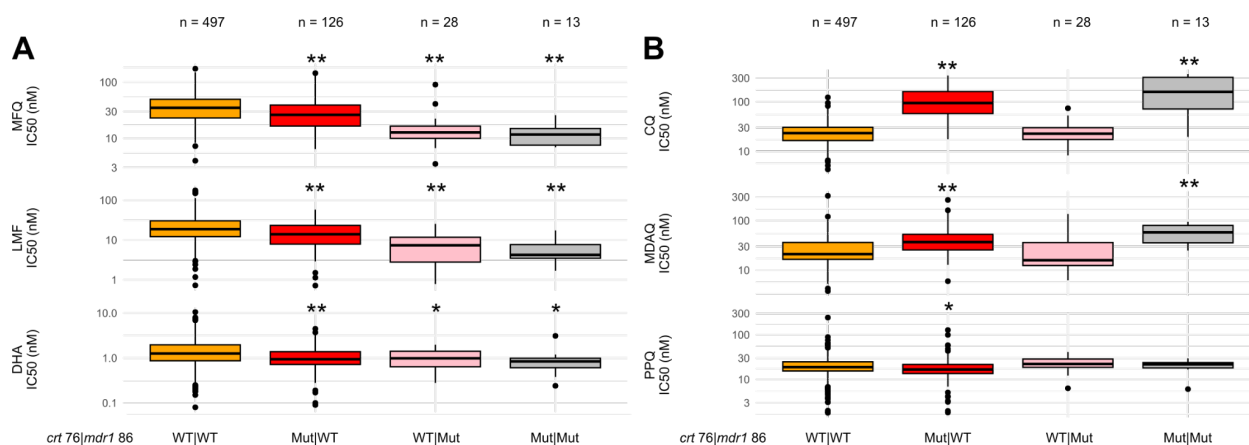
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683 **Figure 4. Manhattan plot showing the significance of SNPs associated with six antimalarial drugs.** Each dot
684 represents 1 of 362 SNPs with $MAF > 0.01$ colored by chromosome. The x-axis represents the chromosomal location
685 of the SNPs, and the y-axis represents the $-\log_{10}$ of the P-value obtained from the linear regression model analysis.
686 The blue line represents the nominal P-value ($P \leq 0.05$), and the red line represents the P-value after Bonferroni
687 correction ($P \leq 1 \times 10^{-4}$). The full list of SNPs associated with IC50 is shown in **Table S13**.

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690 **Figure 5. Effect of *pfprt* 76-*pfmdr1* 86 haplotypes on IC₅₀ for six drugs.** A) IC₅₀ for mefloquine (MFQ),
691 lumefantrine (LMF) and dihydroartemisinin (DHA) disaggregated by *pfprt*-*pfmdr1* haplotypes. B) IC₅₀ for
692 chloroquine (CQ), monodesethylamodiaquine (MDAQ) and piperazine (PPQ) disaggregated by *pfprt*-*pfmdr1*
693 haplotypes. Haplotypes were built with *pfprt* K76T and *pfmdr1* N86Y using the major allele per position. n: number
694 of isolates carrying the haplotype. Differences in the IC₅₀ of wild-type (WT|WT, crt-K76|mdr1-N86) versus single
695 mutants (Mut|WT, crt-76T|mdr1-N86), (WT|Mut, crt-K76|mdr1-86Y), and double mutant (Mut|Mut, crt-76T|mdr1-
696 86Y) were calculated with a Pairwise Wilcoxon tests with Benjamini-Hochberg correction. *: p<0.05; **: p<0.001.