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1 **Modulation of *in vitro* antimalarial responses by polymorphisms in *Plasmodium***  
2 ***falciparum* ABC transporters (*pfmdr1* and *pfmdr5*)**

3

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30 **Abstract**

31 The emergence of resistance to artemisinin-based combination therapies (ACT) was described  
32 in Southeast Asia. In this context, the identification of molecular markers of ACT resistance  
33 partner drugs is urgently needed for monitoring the emergence and spread of resistance.  
34 Polymorphisms in transporter genes, especially of the ATP-binding cassette (ABC)  
35 superfamily, have been involved in anti-malarial drug resistance. In this study, the association  
36 between the mutations in the *P. falciparum* multidrug resistance 1 gene (*pfmdr1*, N86Y,  
37 Y184F, S1034C, N1042D and D1246Y) or repetitive amino acid motifs in *pfmdr5* and the *ex*  
38  *vivo* susceptibility to anti-malarial drugs was evaluated. Susceptibility to chloroquine, quinine,  
39 monodesethylamodiaquine, lumefantrine, piperazine, pyronaridine, mefloquine and  
40 dihydroartemisinin was assessed in 67 Senegalese isolates. The shorter DNNN motif ranged  
41 from 2 to 11 copy repeats, and the longer DHHNDHNNDNNN motif ranged from 0 to 2 in  
42 *pfmdr5*. The present study showed the association between repetitive amino acid motifs  
43 (DNNN-DHHNDHNNDNNN) in *pfmdr5* and *in vitro* susceptibility to 4-aminoquinoline-  
44 based antimalarial drugs. The parasites with 8 and more copy repeats of DNNN in *pfmdr5*  
45 were significantly more susceptible to piperazine. There was a significant association  
46 between parasites whose DHHNDHNNDNNN motif was absent and replaced by  
47 DHHNDNNN, DHHNDHNNDHNNDNNN or DHHNDHNNDHNNDHNNDNNN and  
48 increased susceptibility to chloroquine, monodesethylamodiaquine and pyronaridine. A  
49 significant association between both the wild-type allele N86 in *pfmdr1* and the N86-184F  
50 haplotype and reduced susceptibility to lumefantrine was confirmed. Further studies with a  
51 large number of samples are required to validate the association between these *pfmdr5* alleles  
52 and the modulation of 4-aminoquinoline-based antimalarial drug susceptibility.

53 **Keywords** Malaria, *Plasmodium falciparum*, Antimalarial drug, *in vitro*, Resistance,  
54 Molecular marker, ABC transporter, *pfmdr5*, *pfmdr1*

## 56 **1. Introduction**

57 The spread of antimalarial drug resistance from Southeast Asia to Africa has previously  
58 occurred with chloroquine and sulfadoxine-pyrimethamine (Wootton et al., 2002; Mita et al.,  
59 2011). The emergence of resistance to artemisinin, as manifested by delayed parasite  
60 clearance after artemisinin-based combination therapies (ACT), was described in Southeast  
61 Asia (Dondorp et al., 2009; Asley et al., 2014; Ménard et al., 2016). Drug resistance is  
62 currently emerging also to dihydroartemisinin-piperaquine, the most recently commercialized  
63 ACT, in Cambodia (Leang et al., 2015; Spring et al., 2015; Amaratunga et al., 2016) and in  
64 Vietnam (Phuc et al., 2017; Thanh et al., 2017). In areas where artemisinin resistance is  
65 emerging, combination efficacy is based now on partner drugs. In this context, it is essential  
66 to find novel markers of resistance to monitor the emergence and spread of resistance to these  
67 partner drugs.

68 Gene polymorphisms in the transporter ATP-binding cassette (ABC) superfamily are known  
69 to be involved in antimalarial drug resistance (Pradines et al., 2009). Studies of the *P.*  
70 *falciparum* multidrug resistance 1 gene (*pfmdr1*) have identified two single-nucleotide  
71 polymorphisms (SNPs) which are more prevalent in African and Asian parasites (N86Y and  
72 Y184F) and three SNPs which are more common in South American isolates (S1034C,  
73 N1042D and D1246Y). The N86Y mutation is involved in parasite susceptibility to most of  
74 the partner drugs of ACT, including artemisinin, lumefantrine, amodiaquine, mefloquine and  
75 piperaquine (Wurtz et al., 2014; Veiga et al., 2016). The role of these mutations was proved  
76 by transfection studies that demonstrated the susceptibility modulation of *P. falciparum*  
77 parasites to anti-malarial drugs (Sidhu et al., 2005; Veiga et al., 2016). Additionally, clinical  
78 assays in East Africa have shown the selection of the N86 allele in recurrent infections after  
79 treatment with artemether plus lumefantrine (Sisowath et al., 2005; Dokomajilar et al., 2006)

80 or artesunate plus mefloquine (Price et al., 2004). In Uganda, the use of dihydroartemisinin-  
81 piperazine selected for the 86Y mutation (Nankabirwa et al., 2016).  
82 However, SNPs and overexpression of *pfmdr1* do not entirely explain responses observed in  
83 resistant *P. falciparum* parasites, suggesting that other polymorphisms may be involved in  
84 antimalarial drug susceptibility. The *P. falciparum* genome is rich in sequences encoding low-  
85 complexity regions which comprise repetitions of short motifs. Existence of these repeat  
86 polymorphisms has been identified in ABC transporters (Okombo et al., 2013; Veiga et al.,  
87 2014). But very few studies explored association between these repeat polymorphisms and  
88 parasite susceptibility to anti-malarial drugs. Interestingly, the presence of 7 and 9 Asn repeats  
89 in *pfmdr6* (PF3D7\_1352100) were associated with modulation of *in vitro* lumefantrine and  
90 dihydroartemisinin drug responses in parasites from the China-Myanmar border area (Wang  
91 et al., 2012). Parasites with 6 Asn repeats were significantly less susceptible to lumefantrine,  
92 whereas those with 8 Asn repeats were less susceptible to piperazine in parasites collected in  
93 Kenya (Okombo et al., 2013). The presence of 7 or 9 Asn repeats was also associated with  
94 reduced *in vitro* susceptibility to quinine in 77 Senegalese *P. falciparum* isolates (Gendrot et  
95 al., 2017).

96 Repetitive amino acid motifs in *pfmdr5* (PF3D7\_1339900), which codes a protein localized  
97 at the plasma membrane of the parasite (Kavishe et al., 2009), were associated with reduced  
98 *in vitro* susceptibility to lumefantrine (Okombo et al., 2013). Additionally, deletion of *pfmdr5*  
99 in *P. falciparum* parasites resulted in a minor decrease in the inhibiting concentration 50%  
100 (IC<sub>50</sub>) of artemisinin (van der Velden et al., 2015).

101 The objectives of this study were to explore the existence of repeat polymorphisms in  
102 *pfmdr5* in Senegalese isolates and to evaluate the influence of these repetitive amino acid  
103 motifs on the *ex vivo* susceptibilities to ACT partners, including monodesethylamodiaquine  
104 (DQ), mefloquine (MQ), lumefantrine (LMF), piperazine (PPQ), pyronaridine (PND) and

105 dihydroartemisinin (DHA), and standard antimalarial drugs, such as chloroquine (CQ) and  
106 quinine (QN). The re-affirmation of the established relationships between in vitro properties  
107 and *pfmdr1* will allow to valid the relationships obtained between phenotypes and *pfmdr5*.

108

## 109 **2. Materials and methods**

### 110 *2.1. Patients and sample collection*

111 Sixty-seven *P. falciparum* isolates from patients with uncomplicated or severe falciparum  
112 malaria, who were recruited at the Hôpital Principal de Dakar after the rainy seasons from  
113 2013 to 2015, were successfully evaluated (13 from 2013, 22 from 2014 and 32 from 2015).  
114 Among the studied patients, 55 (82%) were recruited from the emergency department and 6  
115 patients (9%) had travelled within Senegal in the previous month. Antimalarial treatment prior  
116 to admission was not recorded. The patients were treated with quinine until 2014 and then  
117 with artesunate, artemether-lumefantrine or artesunate-amodiaquine.

118 Peripheral venous blood samples were collected in Vacutainer<sup>®</sup> ACD tubes (Becton  
119 Dickinson, Rutherford, NJ) prior to patient treatment. The parasitemia, ranged from 0.006 to  
120 14.1%, was estimated on thin blood smears that were stained using a RAL<sup>®</sup> kit (Réactifs  
121 RAL, Paris, France).

122

### 123 *2.2. Drugs*

124 CQ, QN and DHA were obtained from Sigma (Saint Louis, MO, USA), DQ from the World  
125 Health Organization (Geneva, Switzerland), MQ from Roche (Paris, France), LMF from  
126 Novartis Pharma (Basel, Switzerland), and PPQ and PND from Shin Poong Pharm Co (Seoul,  
127 Korea). QN, DQ, MQ, DHA and PPQ were first dissolved in absolute methanol and then  
128 diluted in water to final concentrations that ranged from 6 to 3,149 nM for QN (stock solution  
129 31,490 nM), 1.9 to 1,988 nM for DQ (stock solution 19,880 nM), 1.5 to 392 nM for MQ

130 (stock solution 40,000 nM), 0.1 to 107 nM for DHA (stock solution 26,750 nM) and 1.9 to  
131 998 nM for PPQ (stock solution 10,000 nM). CQ and PND were resuspended and diluted in  
132 water to final concentrations ranging from 6 to 3,231 nM and 0.4 to 199 nM, respectively.  
133 LMF was resuspended and diluted in absolute ethanol to obtain final concentrations ranging  
134 from 0.6 to 310 nM (stock solution 3,000 nM).  
135 Each batch of plates were validated on the CQ-resistant W2 strain (isolated in Indochina; ref  
136 MRA-157 obtained from MR4, VA) in four independent experiments using the same  
137 conditions described below. The mean IC<sub>50</sub> values for the chloroquine-resistant W2 strain for  
138 the different batches used during the three years were 292 nM for CQ, 275 nM for QN, 72 nM  
139 for DQ, 13.7 nM for LMF, 15.4 nM for MQ, 32.5 nM for PPQ, 26.4 nM for PND and 1.27  
140 nM for DHA. A comparison of W2 susceptibility data for the nine anti-malarial drugs  
141 indicated that there was no significant difference in the responses to antimalarial drugs over  
142 the three years ( $0.39 < p < 0.95$ ).

143 The polymorphic genetic markers *msp1* and *msp2* and microsatellite markers specific to *P.*  
144 *falciparum* were genotyped at least once a month to verify W2 clonality (Bogreau et al., 2006;  
145 Henry et al., 2006).

146

### 147 2.3. *Ex vivo* assay

148 The susceptibility of the 67 isolates to the eight antimalarial drugs was once assessed by  
149 ELISA sandwich based assay targeting the *P. falciparum* histidin-rich protein 2 antigen  
150 without culture adaptation as previously described (Fall et al., 2015; Gendrot et al., 2016). For  
151 the *ex vivo* assay, 100 µl of parasitized red blood cells (final parasitaemia, 0.5%; final  
152 haematocrit, 1.5%) was aliquoted into the 96-well plates pre-dosed with antimalarial drugs.  
153 The cut-off values for the reduced *in vitro* susceptibility or resistance were as follows: 77 nM

154 (CQ), 611 nM (QN), 61 nM (DQ), 30 nM (MQ), 115 nM (LMF), 135 nM (PPQ), 60 nM  
155 (PND) and 12 nM (DHA) (Fall et al., 2011; Pascual et al., 2015).

156

#### 157 *2.4. Nucleic acid extraction*

158 The total genomic DNA of each isolate was isolated using the QIAamp DNA Blood minikit  
159 according to the manufacturer's recommendations (Qiagen, Hilden, Germany).

160

#### 161 *2.5. Gene sequence polymorphism analysis*

162 Two genes, *pfmdr5* (PF3D7\_1339900), and *pfmdr1* (PF3D7\_0523000) were amplified by  
163 polymerase chain reaction using the oligonucleotide primer pairs described in Table 1. The  
164 primers used amplified a region of *pfmdr5* gene of about 900 bp. Two primer pairs were used  
165 to amplify the *pfmdr1* fragments carrying the five mutations in two regions of 610 pb and 890  
166 pb. The reaction mixture consisted of 200 ng of genomic DNA, 0.32  $\mu$ M of each primer, 1X  
167 final of reaction buffer (750 mM Tris-HCl, 200 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 % (v/v) Tween 20 and  
168 stabilizer, pH 8.8), 200  $\mu$ M of deoxynucleoside triphosphate mixture (dGTP, dATP, dTTP  
169 and dCTP) (Euromedex, Souffelweyersheim, France), variable concentrations of  $\text{MgCl}_2$   
170 (Table 1) and 0.2 U of Red Diamond Taq® polymerase (Eurogentec, Liège, Belgium) in a  
171 final volume of 25  $\mu$ L. The thermal cycler (T3 Biometra, Biometra GmbH, Göttingen,  
172 Germany) was programmed as follows: 94°C for 10 min, then 40 cycles alternating among  
173 94°C for 30 sec, hybridization temperature (Table 1) for 45 sec, and 72°C for extension at  
174 1 min per 1,000 bp, followed by a 10-min final extension step at 72°C. The amplified  
175 products were sequenced using the PCR primers on an ABI Prism 3100 analyzer (Applied  
176 Biosystems) according to the manufacturer's instructions. The Vector NTI advance (TM)  
177 software (version 11, Invitrogen, Cergy Pontoise, France) was used to perform data analysis.

178 The whole amplicons obtained were analyzed to identify any other possible single nucleotide  
179 polymorphism (SNP).

180 Poor-quality sequences were either resequenced or discarded and repeat polymorphisms  
181 were retained for analysis if clean individual peaks were observed in the electropherogram.  
182 Mixed genotypes were excluded from further analysis.

183

## 184 2.6. Statistical analysis

185 In this study, the association between the mutations in *pfmdr1* (N86Y, Y184F, S1034C,  
186 N1042D and D1246Y) or repetitive amino acid motifs in *pfmdr5* and the ex vivo  
187 susceptibility to antimalarial drugs was evaluated. The data were analyzed using the R  
188 software (version 3.0.2). Differences in the IC<sub>50</sub>s of the isolates and the *pfmdr5* and *pfmdr1*  
189 polymorphisms were compared using the Kruskal-Wallis test and the Welch two-sample *t*  
190 test. The differences between susceptible and resistant *P. falciparum* parasites and  
191 polymorphisms were analyzed by logistic regression. The significance level was calculated  
192 using the correction of Bonferroni. The significance threshold retained for *pfmdr5* analysis  
193 was 0.007.

194

## 195 3. Results

196 The distribution and the average parameters of the IC<sub>50</sub> values for the eight antimalarial drugs  
197 are presented in Table 2 and Fig. 1.

198

### 199 3.1. *Pfmdr5*

200 Fourteen different allelic groups were identified in the 67 isolates that were genotyped at  
201 *pfmdr5* (Table 3). The shorter DNNN motif ranged from 2 to 11.

202 The longer DHHNDHNNDNNN motif existed in single- or double-copy repeat. This motif  
203 was not found in 5 isolates (7.5%) and was replaced by DHHNDNNN (4.5%),  
204 DHHNDHNNDHNNDNNN (1.5%) and DHHNDHNNDHNNDHNNDNNN (1.5%) motifs.  
205 No new SNP was identified the Senegalese *P. falciparum* parasites.

206 The proportion of isolates with the dominant allele was 25.5% (allele 5-1), 23.9% (allele 6-  
207 1), 19.4% (allele 7-1), 9.0% (allele 8-1) and 4.5% (allele 3-0).

208 The 8 DNNN -1 DHHNDHNNDNNN (8-1) allele showed a significant association with  
209 increased susceptibility to PPQ (30.6 versus 54.3 nM;  $p=0.042$ ; Welch two-sample *t* test)  
210 (Fig. 2A). There was a significant association between parasites with 8 and more than 8  
211 DNNN motifs and increased susceptibility to PPQ (14.0 versus 40.6 nM;  $p=0.003$ ) or PND  
212 (2.7 versus 10.5 nM;  $p=0.04$ ) (Fig. 2B). After Bonferroni correction, only parasites with 8 and  
213 more than 8 motif repeats were significantly more susceptible to PPQ. There was a significant  
214 association between the allele 3-0 and increased susceptibility to CQ (30.8 versus 117.0 nM;  
215  $p<0.001$ ), DQ (10.7 versus 39.3 nM;  $p<0.001$ ), PND (5.1 versus 15.2 nM;  $p<0.001$ ) and MQ  
216 (18.5 versus 37.0 nM;  $p=0.021$ ). After Bonferroni correction, allele 3-0 was associated only  
217 with increased susceptibility to CQ, DQ and PND. This study also showed a significant  
218 association between the alleles n-0 (alleles 3-0 + 7-0 + 8-0) and increased susceptibility to CQ  
219 (28.2 versus 120.0 nM;  $p<0.001$ ), DQ (8.3 versus 40.4 nM;  $p<0.001$ ), PND (6.0 versus 15.5  
220 nM;  $p<0.001$ ) and LMF (2.4 versus 7.7 nM;  $p=0.037$ ). After Bonferroni correction, significant  
221 association was retained only for CQ, DQ and PND.

222

### 223 3.2. *Pfmdr1*

224 The 1034C, 1042D and 1246Y *pfmdr1* mutations were not found. No new SNP was identified  
225 the Senegalese *P. falciparum* parasites. The 86Y mutation in *pfmdr1*, identified in 5.5% of the  
226 samples, was significantly associated with increased susceptibility to LMF (2.1 versus 9.9

227 nM;  $p=0.002$ ; Welch two-sample  $t$  test) (Table 4) (Fig. 3). However, this result should be  
228 taken with caution due to the low frequency of the 86Y SNP. There was no significant  
229 difference in  $IC_{50}$  between the isolates with Y184-184F haplotype ( $p$  values from 0.062 to  
230 0.956; Welch two-sample  $t$  test). Nevertheless, there was a significant association between the  
231 N86-184F haplotype, identified in 53% of the samples, and decreased susceptibility to LMF  
232 (12.3 versus 6.3 nM;  $p=0.049$ ) (Fig. 4).

233

### 234 3.3. *Pfmdr5/pfmdr1*

235 There was no significant association between the alleles n-0 (alleles 3-0 + 7-0 + 8-0), 8-1 or  
236  $\geq 8-x$  and the N86Y-Y184F haplotypes in *pfmdr1* ( $p=0.982$ ; Pearson's Chi-squared test).

237

## 238 4. Discussion

239 The emergence of resistance to ACT in Southeast Asia (Dondorp et al., 2009; Asley et al.,  
240 2014; Ménard et al., 2016), even to the most recently commercialized DHA-PPQ in Southeast  
241 Asia (Amaratunga et al., 2016; Phuc et al., 2017), needs the identification of molecular  
242 markers of resistance to ACT partner drugs for monitoring emergence and spread of  
243 resistance (Parobek et al., 2017).

244 ABC transporters have been involved in antimalarial drug resistance. PfMDR5 is a  
245 transporter that belongs to the ABC B family, like PfMDR1, PfMDR2 or PfMDR6. However,  
246 PfMDR5 is a half transporter and contains only one membrane domain (MD) with 6 putative  
247  $\alpha$ -helical transmembrane segments (TMS) and only one nucleotide-binding domain (NBD)  
248 containing the "Walker A" and the "Walker B" motifs and a short but highly conserve  
249 sequence which is typical for the members of the ABC family (Kavishe et al., 2009; Pradines  
250 et al., 2005; Pradines et al., 2009). The repetitive amino acid motifs analyzed in this study,  
251 DNNN and DHHNDDHNNNDNNN, which occurred within a 180 bp region of the open

252 reading frame of *pfmdr5*, are located in the NBD (repeat position above 520 amino acids).

253 Polymorphisms on gene region encoding the NBD of ABC B transporters have already been

254 shown to be involved in antimalarial drug resistance. The 1246Y mutation in *pfmdr1* gene,

255 which is located in the NBD2 is selected in association with a mutation at codon 86 (86Y) and

256 an absence of mutation in codon 184 (Y184) in resistance to dihydroartemisinin-piperaquine

257 treatment (Pillai et al., 2012; Taylor et al., 2016). Polymorphisms in NBD region might be

258 involved in resistance to antimalarial drugs. This study showed that the repetitive amino acid

259 motifs were associated with an increased susceptibility to antimalarial drugs. The shorter

260 DNNN motif ranged from 2 to 11 copy repeats, and the longer DHHNDHNNNDNNN motif,

261 commonly present in single- or double-copy repeats, was replaced by other motifs in 5

262 isolates (7.5%). This change in longer motif was not found in isolates that were collected in

263 Kilifi, Kenya (Okombo et al., 2013). Among the 14 different allelic groups identified in this

264 study, 7 alleles have never been identified (alleles 2-2, 3-0, 3-1, 3-2, 7-0, 8-0 and 11-1).

265 Except for allele 3-0 (4.5%), the alleles which were mostly identified in Senegalese *P.*

266 *falciparum* isolates, were the same alleles as previously observed in Kenyan parasites: allele

267 5-1 (25.5% versus 32.8% in Kenyan isolates), allele 6-1 (23.9% versus 10.3%), allele 7-1

268 (19.4% versus 25.9%) and allele 8-1 (9.0% versus 17.2%) (Okombo et al., 2013). However,

269 the proportions for the most frequent alleles were different between Dakar, Senegal and Kilifi,

270 Kenya. Additionally, the most frequent alleles observed in *P. falciparum* referent strains from

271 Africa, Asia or South America were 5-1, 7-1 or 8-1 (Okombo et al., 2013). More generally,

272 parasites with 8 and more than 8 short motifs were significantly more susceptible to PPQ than

273 isolates with less short motifs. None of the parasites bearing 8 and more than 8 short motifs

274 showed reduced susceptibility to PPQ ( $IC_{50} > 135$  nM). Okombo and colleagues found that

275 allele 5-1 was significantly associated with an increased LMF  $IC_{50}$  on Kenyan isolates, but

276 without correction of the threshold of significance in multiple comparisons (Okombo et al.,

277 2013). In our study on Senegalese isolates, no significant associations were found between the  
278 allele 5-1 and antimalarial drug susceptibility. Parasites with the DHHNDHNNDNNN motif  
279 replaced by DHHNDNNN (3-0 allele), DHHNDHNNDHNNDNNN (8-0 allele) and  
280 DHHNDHNNDHNNDHNNDNNN (7-0 allele) were significantly more susceptible to CQ,  
281 QN and PND, antimalarial drugs that contain a 4-aminoquinoline based chemical core.  
282 However, these results came from a limited number of samples. Further studies with a larger  
283 number of samples are required to validate the association between these *pfmdr5* alleles and  
284 the modulation of antimalarial drug susceptibility. *Pfmdr5* seems to be not linked with *pfmdr1*  
285 SNP. However, biochemical studies will be required to eliminate the hypothesis that *pfmdr5*  
286 may be linked to another gene which has a genuine effect on anti-malarial responses and may  
287 be not involved in resistance.

288 The *pfmdr1* N86 was significantly associated with decreased susceptibility to LMF (9.9  
289 versus 2.1 nM). However, this result should be taken with caution due to the low frequency of  
290 the 86Y SNP. But this result is in accordance with previous *in vitro* studies in Asia (Munghin  
291 et al., 2010), Kenya (Mwai et al., 2009), Benin (Dahlström et al., 2014) and Senegal (Wurtz et  
292 al., 2014). The role of N86Y mutation in LMF susceptibility modulation was proved by  
293 transfection study (Veiga et al., 2016). Additionally, clinical assays in Africa have shown the  
294 selection of the N86 allele in recurrent infections after treatment with artemether-lumefantrine  
295 (Baraka et al., 2015; Sondo et al., 2016). The presence of the N86 mutation was a risk factor  
296 for recrudescence in patients who were treated with artemether-lumefantrine (Venkatesan et  
297 al., 2014).

298 Furthermore, there was a significant association between the N86-184F haplotype and  
299 decreased susceptibility to LMF (12.3 for the N86-184F haplotype versus 6.3 nM for the N86-  
300 Y184). This result is consistent with an *in vitro* study previously conducted in Senegal (Wurtz  
301 et al., 2014). Additionally, no mutations for the 1246 codon (1246Y) were found. This result

302 supports the findings of *in vivo* studies that showed that the N86-184F-D1246 haplotype was  
303 present in artemether-lumefantrine reinfections in Senegal and East Africa (Mbaye et al.,  
304 2016; Otiennoburu et al., 2016).

305 In this study, no association was highlighted between N86 and 86Y alleles with *in vitro*  
306 susceptibility modulation to DQ. However, this result should be taken with caution due to the  
307 low frequency of the 86Y SNP. The data on the 86Y mutation and its involvement in DQ  
308 susceptibility are contradictory. Previous works showed no association between the  
309 haplotypes, N86 and 86Y, and DQ IC<sub>50</sub> (Dahltröm et al., 2014), whereas other studies showed  
310 associations between 86Y mutation and increased (Wurtz et al., 2014) or decreased  
311 susceptibility to DQ (Folarin et al., 2011). The replacement by transfection of the 86Y  
312 mutation by the wild-type N86 resulted in increased susceptibility to DQ (Veiga et al., 2016).  
313 Nevertheless, the 86Y mutation was found to be associated with treatment failures after  
314 amodiaquine monotherapy (Holmgren et al., 2006; Danquah et al., 2010) or artesunate-  
315 amodiaquine (Venkatesan et al., 2014; Sondo et al., 2016; Yeka et al., 2016).

316

## 317 **5. Conclusion**

318 In summary, the present study showed the association between repetitive amino acid motifs  
319 (DNNN-DHHNDDHNNDNNN) in *pfmdr5* and *in vitro* susceptibility to 4-aminoquinoline  
320 based antimalarial drugs. In particular, parasites bearing 8 and more than 8 short motifs  
321 (DNNN) were more susceptible to piperazine than isolates with less short motifs, and  
322 isolates with atypical long motif repeat (DHHNDDHNNDNNN) were more susceptible to  
323 pyronaridine, chloroquine and monodesethylamodiaquine. However, these results should be  
324 taken with caution due to the low frequency of the different alleles. The present results  
325 validated the association between the wild-type alleles N86 in *pfmdr1* and N86-184F  
326 haplotype with reduced susceptibility to lumefantrine. It will be important in future to analyze

327 the potential association between piperaquine susceptibility modulation and *pfmdr5* and other  
328 genes which are involved in piperaquine resistance in Asia but not yet validated in African  
329 parasites, including *plasmepsin 2-3*, *exonuclease* or *pfcr1* genes (H97Y, F145I, M343L or  
330 G353V) (Amato et al., 2016; Witkowski et al., 2016; Agrawal et al., 2017; Ross et al., 2018).  
331 The multiple copies of the *plasmepsin 2* gene, one of the most promising candidates, does not  
332 explain *ex vivo* or *in vitro* reduced susceptibility to piperaquine or clinical failure with  
333 dihydroartemisinin-piperaquine in Africa (Rasmussen et al., 2017; Inoue et al., 2018; Malvy et  
334 al., 2018; Robert et al., 2018; Robert et al., 2019; Russo et al., 2018). Further studies with a  
335 larger number of samples are required to validate the association between these *pfmdr5* alleles  
336 and modulation of antimalarial drug susceptibility.

337

#### 338 **Ethics approval and consent to participate**

339 Verbal consent was obtained from all the patients or their parents/guardians before blood  
340 collection. Bio-banking of human clinical samples used for malaria diagnostics and secondary  
341 uses for scientific purposes is possible as long as the corresponding patients are informed and  
342 have not indicated any objections. This requirement was fulfilled here by giving verbal  
343 information to the patients, and no immediate or delayed patient opposition was reported to  
344 the hospital clinicians. The ethical committee of the Hôpital Principal de Dakar approved the  
345 study.

346

#### 347 **Conflicts of interest**

348 The authors declare that they have no competing interests.

349

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359

### 360 **References**

- 361 Agrawal, S., Moser, K.A., Morton, L., Cummings, M.P., Parihar, A., Dwivedi, A., Shetty,  
362 A.C., Drabek, E.F., Jacob, C.G., Henrich, P.P., Parotek, C.M., Jongsakul, K., Huy, R.,  
363 Spring, M.D., Lanteri, C.A., Chaorattanakawee, S., Lon, C., Fukuda, M.M., Saunders,  
364 D.L., Fidoch, D.A., Lin, J.T., Juliano, J.J., Plowe, C.V., Silva, J.C., Takala-Harrison, S.,  
365 2017. Association of a novel mutation in the *Plasmodium falciparum* chloroquine  
366 transporter with decreased piperazine sensitivity. *J. Infect. Dis.* 216, 468-476.
- 367 Amaratunga, C., Lim, P., Suon, S., Sreng, S., Mao, S., Sopha, C., Sam, B., Dek, D., Try, V.,  
368 Amato, R., Blessborn, D., Song, L., Tullo, G.S., Fay, M.P., Anderson, J.M., Tarning, J.,  
369 Fairhurst, R.M., 2016. Dihydroartemisinin-piperazine resistance in *Plasmodium*  
370 *falciparum* malaria in Cambodia: a multisite prospective cohort study. *Lancet Infect. Dis.*  
371 16, 357-365.
- 372 Amato, R., Lim, P., Miotto, O., Amaratunga, C., Dek, D., Pearson, R.D., Almagro-Garcia, J.,  
373 Neal, A.T., Sreng, S., Suon, S., Drury, E., Jyothi, D., Stalker, J., Kwiatkowski, D.P.,  
374 Fairhurst, R.M., 2016. Genetic markers associated with dihydroartemisinin-piperazine

375 failure in *Plasmodium falciparum* malaria in Cambodia: a genotype–phenotype association  
376 study. *Lancet Infect. Dis.* 17, 164-173.

377 Ashley, E.A., Dhorda, M., Fairhurst, R.M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson,  
378 J.M., Mao, S., Sam, B., Sopha, C., Chuor, C.M., Nguon, C., Sovannaroeth, S., Pukrittayakamee, S.,  
379 Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T.T.,  
380 Thuy-Nhien, N.T., Thanh, N.V., Phu, N.H., Htut, Y., Han, K.T., Aye, K.H., Mokuolu, O.A.,  
381 Olaosebikan, R.R., Folaranmi, O.O., Mayxay, M., Khanthavong, M., Hongvanthong, B., Newton,  
382 P.N., Onyamboko, M.A., Fanello, C.I., Tshefu, A.K., Mishra, N., Valecha, N., Phyo, A.P., Nosten,  
383 F., Yi, P., Tripura, R., Borrmann, S., Bashraheil, M., Peshu, J., Faiz, M.A., Ghose, A., Hossain,  
384 M.A., Samad, R., Rahman, M.R., Hasan, M.M., Islam, A., Miotto, O., Amato, R., MacInnis, B.,  
385 Stalker, J., Kwiatkowski, D.P., Bozdech, Z., Jeeyapant, A., Cheah, P.Y., Sakulthaew, T., Chalk, J.,  
386 Intharabut, B., Silamut, K., Lee, S.J., Vihokhern, B., Kunasol, C., Imwong, M., Tarning, J., Taylor,  
387 W.J., Yeung, S., Woodrow, C.J., Flegg, J.A., Das, D., Smith, J., Venkatesan, M., Plowe, C.V.,  
388 Stepniewska, K., Guerin, P.J., Dondorp, A.M., Day, N.P., White, N.J., 2014. Spread of artemisinin  
389 resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med.* 371, 411-423.

390 Baraka, V., Tinto, H., Valea, I., Fitzhenry, R., Delgado-Ratto, C., Mbonye, M.K., van  
391 Overmeir, C., Rosanas-Urgell, A., van geertruyden, J.P., D’Alessandro, U., Erhart, A.,  
392 2015. In vivo selection of *Plasmodium falciparum* *Pfcr* and *Pfmdr1* variants  
393 by artemether-lumefantrine and dihydroartemisinin-piperaquine in Burkina Faso.  
394 *Antimicrob. Agents Chemother.* 59, 734-737.

395 Bogreau, H., Renaud, F., Bouchiba, H., Durand, P., Assi, S.B., Henry, M.C., Garnotel, H.,  
396 Pradines, B., Fusai, T., Wade, B., Adehossi, E., Parola, P., Kamil, M.A., Puijalon, O.,  
397 Rogier, C., 2006. Genetic diversity and structure of African *Plasmodium falciparum*  
398 populations in urban and rural areas. *Am. J. Trop. Med. Hyg.* 74, 953-959.

399 Dahlström, S., Aubouy, A., Maïga-Ascofaré, O., Faucher, J.F., Wakpo, A., Ezinmègnon, S.,  
400 Massougbodji, A., Houzé, P., Kendjo, E., Deloron, P., Le Bras, J., Houzé, S., 2014.

401 *Plasmodium falciparum* polymorphisms associated with ex vivo drug susceptibility and  
402 clinical effectiveness of artemisinin-based combination therapies in Benin. *Antimicrob.*  
403 *Agents Chemother.* 58, 1-10.

404 Danquah, I., Coulibaly, B., Meissner, P., Petruschke, I., Müller, O., Mockenhaupt, F.P., 2010.  
405 Selection of *pfmdr1* and *pfcr1* alleles in amodiaquine treatment failure in north-western  
406 Burkina Faso. *Acta Trop.* 114, 63-66.

407 Dokomajilar, C., Nsobya, S.L., Greenhouse, B., Rosenthal, P.J., Dorsey, G., 2006. Selection  
408 of *Plasmodium falciparum pfmdr1* alleles following therapy with artemether-lumefantrine  
409 in an area of Uganda where malaria is highly endemic. *Antimicrob. Agents Chemother.* 50,  
410 1893-1895.

411 Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyto, A.P., Tarning, J., Lwin, K.M., Ariey, F.,  
412 Hanpithakpong, W., Lee, S.J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K.,  
413 Lim, P., Herdman, T., An, S.S., Yeung, S., Singhasivanon, P., Day, N.P.J., Lindegardh, N.,  
414 Socheat, D., White, N.J., 2009. Artemisinin resistance in *Plasmodium falciparum* malaria.  
415 *N. Engl. J. Med.* 361, 455-467.

416 Fall, B., Camara, C., Fall, M., Nakoulima, A., Dionne, P., Diatta, B., Diémé, Y., Wade, B.,  
417 Pradines, B., 2015. *Plasmodium falciparum* susceptibility to standard and potential anti-  
418 malarial drugs in Dakar, Senegal, during the 2013-2014 malaria season. *Malar. J.* 14, 60.

419 Fall, B., Diawara, S., Sow, K., Baret, E., Diatta, B., Fall, K.B., Mbaye, P.S., Fall, F., Diémé,  
420 Y., Rogier, C., Wade, B., Bercion, R., Pradines, B., 2011. Ex vivo susceptibility of  
421 *Plasmodium falciparum* isolates from Dakar, Senegal, to seven standard anti-malarial  
422 drugs. *Malar. J.* 10, 310.

423 Folarin, O.A., Bustamante, C., Gbotosho, G.O., Sowunmi, A., Zalis, M.G., Oduola,  
424 A.M., Happi, C.T., 2011. In vitro amodiaquine resistance and its association with

425 mutations in *pfprt* and *pfmdr1* genes of *Plasmodium falciparum* isolates from Nigeria. *Acta*  
426 *Trop.* 120, 224-230.

427 Gendrot, M., Diawara, S., Madamet, M., Kounta, M.B., Briolant, S., Wade, K.A., Fall, M.,  
428 Benoit, N., Nakoulima, A., Amalvict, R., Diémé, Y., Fall, B., Wade, B., Diatta, B.,  
429 Pradines, B., 2017. Association between polymorphisms in the *Pfmdr6* gene and *ex vivo*  
430 susceptibility to quinine in *Plasmodium falciparum* parasites from Dakar, Senegal.  
431 *Antimicrob. Agents Chemother.* 61, e01183-16.

432 Gendrot, M., Fall, B., Madamet, M., Fall, M., Wade, K.A., Amalvict, R., Nakoulima, A.,  
433 Benoit, N., Diawara, S., Diémé, Y., Diatta, B., Wade, B., Pradines, B., 2016. Absence of  
434 association between polymorphisms in the RING E3 ubiquitin protein ligase gene and *ex*  
435 *vivo* susceptibility to conventional antimalarial drugs in *Plasmodium falciparum* isolates  
436 from Dakar, Senegal. *Antimicrob. Agents Chemother.* 60, 5010-5013.

437 Henry, M., Diallo, I., Bordes, J., Ka, S., Pradines, B., Diatta, B., M'Baye, P.S., Sane, M.,  
438 Thiam M., Gueye, P.M., Wade, B., Touze, J.E., Debonne, J.M., Rogier, C., Fusai, T., 2006.  
439 Urban malaria in Dakar, Senegal: chemosusceptibility and genetic diversity of *Plasmodium*  
440 *falciparum* isolates. *Am. J. Trop. Med. Hyg.* 75, 146-151.

441 Holmgren, G., Gil, J.P., Ferreira, P.M., Veiga, M.I., Obonyo, C.O., Björkman, A., 2006.  
442 Amodiaquine resistant *Plasmodium falciparum* malaria in vivo is associated with selection  
443 of *pfprt* 76T and *pfmdr1* 86Y. *Infect. Genet. Evol.* 6, 309-314.

444 Inoue, J., Silva, M., Fofana, B., Sanogo, K., Martensson, A., Sagara, I., Björkman, A., Veiga,  
445 M.I., Ferreira, P.E., Djimde, A., Gil, J.P., 2018. *Plasmodium falciparum* *plasmepsin 2*  
446 duplications, West Africa. *Emerg. Infect. Dis.* 24, 1591-1593.

447 Kavishe, R.A., van den Heuvel, J.M.W., van de Vegte-Bolmer, M., Luty, A.J.F., Russel,  
448 F.G.M., Koenderink, J.B., 2009. Localization of the ATP-binding cassette (ABC) transport

449 proteins PfMRP1, PfMRP2, and PfMDR5 at the *Plasmodium falciparum* plasma  
450 membrane. *Malar. J.* 8, 205.

451 Leang, R., Taylor, W.R.J., Bouth, D.M., Song, L., Tarning, J., Char, M.C., Kim, S.,  
452 Witkowski, B., Duru, V., Domergue, A., Khim, N., Ringwald, P., Menard, D., 2015.  
453 Evidence of *Plasmodium falciparum* malaria multidrug resistance to artemisinin and  
454 piperazine in Western Cambodia: Dihydroartemisinin-piperazine open-label multicenter  
455 clinical assessment. *Antimicrob. Agents Chemother.* 59, 4719-4726.

456 Malvy, D., Torrentino-Madamet, M., L'Ollivier, C., Receveur, M.C., Jeddi, F., Delhaes, L.,  
457 Piarroux, R., Millet, P., Pradines, B., 2018. *Plasmodium falciparum* recrudescence two  
458 years after treatment of an uncomplicated infection without return to an area where malaria  
459 is endemic. *Antimicrob. Agents Chemother.* 62, e01892-17.

460 Mbaye, A., Dieye, B., Ndiaye, Y.D., Bei, A.K., Muna, A., Deme, A.B., Yade, M.S., Diongue,  
461 K., Gaye, A., Ndiaye, I.M., Ndiaye, T., Sy, M., Diallo, M.A., Badiane, A.S., Ndiaye, M.,  
462 Seck, M.C., Sy, N., Koita, O., Krogstad, D.J., Nwakanma, D., Ndiaye, D., 2016. Selection  
463 of N86F184D1246 haplotype of *Pfmr1* gene by artemether-lumefantrine drug pressure on  
464 *Plasmodium falciparum* populations in Senegal. *Malar. J.* 15, 433.

465 Ménard, D., Khim, N., Beghain, J., Adegnika, A.A., Alam, M.S., Amodu, O., Awab, G.R.,  
466 Barnadas, C., Berry, A., Boum, Y., Bustos, M.D., Cao, J., Chen, J.H., Collet, L., Cui, L.,  
467 Das Thakur, G., Dieye, A., Djalle, D., Dorkenoo, M.A., Eboumbou Moukoko, C.E.,  
468 Espino, E., Fandeur, T., Ferreira-Da-Cruz, M.F., Fola, A.A., Fuehrer, H.P., Hassan, A.M.,  
469 Herrera, S., Hongvanthong, B., Houze, S., Karim, M.J., Jiang, L., Kano, S., Khan, W.A.,  
470 Khanthavong, M., Kremsner, P.G., Lacerda, M., Leang, R., Leelawong, M., Li, M., Lin,  
471 K., Laminou, I.M., Mazarati, J.B., Menard, S., Morlais, I., Muhindo Mavoko, H., Musset,  
472 L., Na-Bangchang, K., Nambozi, M., Niare, K., Noedl, H., Ouedraogo, J.B., Pillai, D.R.,  
473 Pradines, B., Quang Phuc, B., Ramharter, M., Randrianarivelosoa, M., Sattabongkot, J.,

474 Sheikh Omar, R., Silue, K.D., Sirima, S.B., Sutherland, C., Syafruddin, D., Tahar, R.,  
475 Tang, L.H., Toure, O.A., Tshibangu, P., Vigan-Womas, I., Warsame, M., Wini, L., Zakeri,  
476 S., Kim, S., Eam, R., Berne, L., Khean, C., Chy, S., Ken, M., Loch, K., Canier, L., Duru,  
477 V., Legrand, E., Barale, J.C., Stokes, B., Straimer, J., Witkowski, B., Fidock, D.A., Rogier,  
478 C., Ringwald, P., Ariey, F., Mercereau-Puijalon, O. ; for the K13 Artemisinin Resistance  
479 Multicenter Assessment consortium (KARMA), 2016. A worldwide map of *Plasmodium*  
480 *falciparum* K13-propeller polymorphisms. N. Engl J. Med. 374, 2453-2464.

481 Mita, T., Venkatesan, M., Ohashi, J., Culleton, R., Takahashi, N., Tsukahara, T., Ndounga,  
482 M., Dysoley, L., Endo, H., Hombhanje, F., Ferreira, M.U., Plowe, C.V., Tanabe, K., 2011.  
483 Limited geographical origin and global spread of sulfadoxine-resistant *dhps* alleles in  
484 *Plasmodium falciparum* populations. J. Infect. Dis. 204, 1980-1988.

485 Mungthin, M., Khositnithikul, R., Sitthichot, N., Suwandittakul, N., Wattanaveeradej, V.,  
486 Ward, S.A., Na-Bangchang, K., 2010. Association between the *pfmdr1* gene and in  
487 vitro artemether and lumefantrine sensitivity in Thai isolates of *Plasmodium falciparum*.  
488 Am. J. Trop. Med. Hyg. 83, 1005-1009.

489 Mwai, L., Kiara, S.M., Abdirahman, A., Pole, L., Rippert, A., Diriye, A., Bull, P., Marsh, K.,  
490 Borrmann, S., Nzila, A., 2009. In vitro activities of piperazine, lumefantrine, and  
491 dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in *pfprt*  
492 and *pfmdr1*. Antimicrob. Agents Chemother. 53, 5069-5073.

493 Nankabirwa, J.I., Conrad, M.D., Legac, J., Tukwasibwe, S., Tumwebaze, P., Wandera, B.,  
494 Brooker, S. J., Staedke, S.G., Kanya, M.R., Nsoya, S.L., Dorsey, G., Rosenthal, P.J.,  
495 2016. Intermittent preventive treatment with dihydroartemisinin-piperazine in Ugandan  
496 schoolchildren selects for *Plasmodium falciparum* transporter polymorphisms that modify  
497 drug sensitivity. Antimicrob. Agents Chemother. 60, 5649-5654.

498 Okombo, J., Abdi, A.I., Kiara, S.M., Mwai, L., Pole, L., Sutherland, C.J., Nzila, A., Ochola-  
499 Oyier, L.I., 2013. Repeat polymorphisms in the low-complexity regions of *Plasmodium*  
500 *falciparum* ABC transporters and associations with *in vitro* antimalarial responses.  
501 Antimicrob. Agents Chemother. 57, 6196-6204.

502 Otienoburu, S.D., Maïga-Ascofaré, O., Schramm, B., Jullien, V., Jones, J.J., Zolia, Y.M.,  
503 Houzé, P., Ashley, E.A., Kiechel, J.R., Guérin, P.J., Le Bras, J., Houzé, S., 2016. Selection  
504 of *Plasmodium falciparum* *pfprt* and *pfmdr1* polymorphisms after treatment with  
505 artesunate-amodiaquine fixed dose combination or artemether-lumefantrine in Liberia.  
506 Malar. J. 15, 452.

507 Parobek, C. M., Parr, J.B., Brazeau, N.F., Lon, C., Chaorattanakawee, S., Gosi, P., Barnett,  
508 E.J., Norris, L.D., Meshnick, S.R., Spring, M.D., Lanteri, C.A., Bailey, J.A., Saunders,  
509 D.L., Lin, J.T., Juliano, J.J., 2017. Partner-drug resistance and population substructuring of  
510 artemisinin-resistant *Plasmodium falciparum* in Cambodia. Genome Biol. Evol. 9, 1673-  
511 1686.

512 Pascual, A., Madamet, M., Briolant, S., Gaillard, T., Amalvict, R., Benoit, N., Travers, D.,  
513 Pradines, B., 2015. Multinormal *in vitro* distribution of *Plasmodium falciparum*  
514 susceptibility to piperazine and pyronaridine. Malar. J. 14, 49.

515 Phuc, B.Q., Rasmussen, C., Duong, T.T., Dong, L.T., Loi, M.A., Ménard, D., Tarning, J.,  
516 Bustos, D., Ringwald, P., Galappaththy, G.L., Thieu, N.Q., 2017. Treatment failure of  
517 dihydroartemisinin/piperazine for *Plasmodium falciparum* malaria, Vietnam. Emerg.  
518 Infect. Dis. 23, 715-717.

519 Pillai, D.R., Lau, R., Khairnar, K., Lepore, R., Via, A., Staines, H.M., Krishna, S., 2012.  
520 Artemether resistance *in vitro* is linked to mutations in PfATP6 that also interact with  
521 mutations in PfMDR1 in travellers returning with *Plasmodium falciparum* infections.  
522 Malar. J. 11, 131.

523 Pradines, B., Pagès, J.M., Barbe, J., 2005. Chemosensitizers in drug transport mechanisms  
524 involved in protozoan resistance. *Curr. Drug Targets Infect. Disord.* 5, 411-431.

525 Pradines, B., Parquet, V., Orlandi-Pradines, E., 2009. ABC transporters in *Plasmodium* and  
526 their involvement in resistance to antimalarial drugs, in: Ponte-Sucre, A. (ed), ABC  
527 Transporters in Microorganisms. Caister Academic Press, Wymondham, UK, pp 113-128.

528 Price, R.N., Uhlemann, A.C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel,  
529 R., Looareesuwan, S., White, N.J., Nosten, F., Krishna, S., 2004. Mefloquine resistance in  
530 *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet* 364, 438-447.

531 Rasmussen, S.A., Ceja, F.G., Conrad, M.D., Tumwebaze, P.K., Byaruhanga, O., Katairo, T.,  
532 Nsohya, S.L., Rosenthal, P.J., Cooper, R.A., 2017. Changing antimalarial drug sensitivities  
533 in Uganda. *Antimicrob. Agents Chemother.* 61, e01516-17.

534 Robert, M.G., Foguim Tsombeng, F., Gendrot, M., Diawara, S., Madamet, M., Kounta, M.B.,  
535 Wade, K.A., Fall, M., Gueye, M.W., Benoit, N., Nakoulima, A., Bercion, R., Amalvict, R.,  
536 Fall, B., Wade, B., Diatta, B., Pradines, B., 2019. Baseline *ex vivo* and molecular responses  
537 of *Plasmodium falciparum* isolates to piperazine before implementation of  
538 dihydroartemisinin-piperazine in Senegal. *Antimicrob. Agents Chemother.* 63, e02448-  
539 18.

540 Robert, M.G., Foguim Tsombeng, F., Gendrot, M., Mosnier, J., Amalvict, R., Benoit, N.,  
541 Torrentino-Madamet, M., Pradines, B., the French National Reference Centre for imported  
542 Malaria Study Group., 2018. Absence of high level of duplication of the *plasmepsin 2* gene  
543 in Africa. *Antimicrob. Agents Chemother.* 62, e00374-18.

544 Ross, L.S., Dhingra, S.K., Mok, S., Yeo, T., Wicht, K.J., Kumpornsin, K., Takala-Harrison,  
545 S., Witkowski, B., Fairhurst, R.M., Ariey, F., Ménard, D., Fidock, D.A., 2018. Emerging  
546 Southeast Asian *Pfcr* mutations confer *Plasmodium falciparum* resistance to the first-line  
547 antimalarial piperazine. *Nat. Commun.* 9, 3314.

548 Russo G, L'Episcopia M, Menegon M, Souza SS, Dongho BGD, Vullo V, Lucchi NW,  
549 Severini C. 2018. Dihydroartemisinin-piperaquine treatment failure in uncomplicated  
550 *Plasmodium falciparum* malaria case imported from Ethiopia. Infection 2018 Jul 6. doi:  
551 10.1007/s15010-018-1174-9

552 Sidhu, A.B., Valderramos, S.G., Fidock, D.A., 2005. *Pfmdr1* mutations contribute to quinine  
553 resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*.  
554 Mol. Microbiol. 57, 913-926.

555 Sisowath, C., Stromberg, J., Martensson, A., Msellem, M., Obondo, C., Björkman, A., Gil,  
556 J.P., 2005. In vivo selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by  
557 artemether-lumefantrine (Coartem). J. Infect. Dis. 191, 1014-1017.

558 Sondo, P., Derra, K., Diallo Nakanabo, S., Tarnagda, Z., Kazienga, A., Zampa, O., Valéa, I.,  
559 Sorgho, H., Owusu-Dabo, E., Ouédraogo, J.B., Guiguemdé, T.R., Tinto, H., 2016.  
560 Artesunate-amodiaquine and artemether-lumefantrine therapies and selection of *Pfprt*  
561 and *Pfmdr1* alleles in Nanoro, Burkina Faso. PLoS One 11, e0151565.

562 Spring, M.D., Lin, J.T., Manning, J.E., Vanachayangkul, P., Somethy, S., Bun, R., Se, Y.,  
563 Chann, S., Ittiverakul, M., Sia-Ngam, P., Kuntawunginn, W., Arsanok, M., Buathong, N.,  
564 Chaorattanakawee, S., Gosi, P., Ta-Aksorn, W., Chanarat, N., Sundrakes, S., Kong, N.,  
565 Heng, T.K., Nou, S., Teja-Isavadharm, P., Pichyangkul, S., Phann, S.T., Balasubramanian,  
566 S., Juliano, J.J., Meshnick, S.R., Chour, C.M., Prom, S., Lanteri, C.A., Lon, C., Saunders,  
567 D.L., 2015. Dihydroartemisinin-piperaquine failure associated with a triple mutant  
568 including kelch13 C580Y in Cambodia: an observational cohort study. Lancet Infect. Dis.  
569 15, 683-691.

570 Taylor, A.R., Flegg, J.A., Holmes, C.C., Guérin, P.J., Sibley, C.H., Conrad, M.D., Dorsey, G.,  
571 Rosenthal, P.J., 2016. Artemether-lumefantrine and dihydroartemisinin-piperaquine exert

572 inverse selective pressure on *Plasmodium falciparum* drug sensitivity-associated  
573 haplotypes in Uganda. Open Forum Infect. Dis. 4, ofw229.

574 Thanh, N.V., Thuy-Nhien, N., Tuyen, N.T., Tong, N.T., Nha-Ca, N.T., Dong, L.T., Quang,  
575 H.H., Farrar, J., Thwaites, G., White, N.J., Wolbers, M., Hien, T.T., 2017. Rapid decline in  
576 the susceptibility of *Plasmodium falciparum* to dihydroartemisinin-piperaquine in the  
577 south of Vietnam. Malar. J. 16, 27.

578 Van der Velden, M., Rijpma, S.R., Russel, F.G.M., Sauerwein, R.W., Koenderink, J.B., 2015.  
579 PfMDR2 and PfMDR5 are dispensable for *Plasmodium falciparum* asexual parasite  
580 multiplication but change *in vitro* susceptibility to anti-malarial drugs. Malar. J. 14, 76.

581 Veiga, M.I., Dhingra, S.K., Henrich, P.P., Straimer, J., Gnägig, N., Uhlemann, A.C., Martin,  
582 R.E., Lehane, A.M., Fidock, D.A., 2016. Globally prevalent PfMDR1 mutations modulate  
583 *Plasmodium falciparum* susceptibility to artemisinin-based combination therapies. Nature  
584 Commun. 7, 11553.

585 Veiga, M.I., Osorio, N.S., Ferreira, P.E., Franzen, O., Dahlstrom, S., Lum, J.K., Nosten, F.,  
586 Gil, J.P., 2014. Complex polymorphisms in the *Plasmodium falciparum* multidrug  
587 resistance protein 2 gene and its contribution to antimalarial response. Antimicrob. Agents  
588 Chemother. 58, 7390-7397.

589 Venkatesan, M., Gadalla, N.B., Stepniewska, K., Dahal, P., Nsanzabana, C., Moriera, C.,  
590 Price, R.N., Martensson, A., Rosenthal, P.J., Dorsey, G., Sutherland, C.J., Guérin, P.,  
591 Davis, T.M.E., Ménard, D., Adam, I., Ademowo, G., Arze, C., Baliraine, F.N., Berens-  
592 Riha, N., Björkman, A., Borrmann, S., Checchi, F., Desai, M., Dhorba, M., Djimdé, A.A.,  
593 El-Sayed, B.B., Eshetu, T., Eyase, F., Falade, C., Faucher, J.F., Fröberg, G., Grivoyannis,  
594 A., Hamour, S., Houzé, S., Jonson, J., Kamugisha, E., Kariuki, S., Kiechel, J.R., Kironde,  
595 F., Kofoed, P.E., Le Bras, J., Malmberg, M., Mwai, L., Ngasala, B., Nosten, F., Nsoya,  
596 S.L., Nzila, A., Oguike, M., Dahlström Otiennoburu, S., Ogutu, B., Ouédraogo, J.B., Piola,

597 P., Rombo, L., Schramm, B., Somé, A.F., Thwing, J., Ursing, J., Wong, R.P.M., Zeynudin,  
598 A., Zongo, I., Plowe, C.V., Hopkins Sibley, C., 2014. Polymorphisms in *Plasmodium*  
599 *falciparum* chloroquine resistance transporter and multidrug resistance 1 genes: parasite  
600 risk factors that affect treatment outcomes for *P. falciparum* malaria after artemether-  
601 lumefantrine and artesunate-amodiaquine. *Am. J. Trop. Med. Hyg.* 91, 833-843.

602 Wang, Z., Parker, D., Meng, H., Wu, L., Li, J., Zhao, Z., Zhang, R., Fan, Q., Wang, H., Cui,  
603 L., Yang, Z., 2012. In vitro sensitivity of *Plasmodium falciparum* from China-Myanmar  
604 border area to major ACT drugs and polymorphisms in potential target genes. *PLoS One* 7,  
605 30927.

606 Witkowski, B., Duru, V., Khim, N., Ross, L.S., Saintpierre, B., Beghain, J., Chy, S., Kim, S.,  
607 Ke, S., Kloeung, N., Eam, R., Khean, C., Ken, M., Loch, K., Bouillon, A., Domergue, A.,  
608 Ma, L., Bouchier, C., Leang, R., Huy, R., Nuel, G., Barale, J.C., Legrand, E., Ringwald, P.,  
609 Fidock, D.A., Mercereau-Puijalon, O., Ariey, F., Ménard, D., 2016. A surrogate marker of  
610 piperazine-resistant *Plasmodium falciparum* malaria: a phenotype–genotype association  
611 study. *Lancet Infect. Dis.* 17, 174-183.

612 Wootton, J.C., Feng, X., Ferdig, M.T., Cooper, R.A., Mu, J., Baruch, D.I., Magill, A.J., Su,  
613 X.Z., 2002. Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*.  
614 *Nature* 418, 320-323.

615 Wurtz, N., Fall, B., Pascual, A., Fall, M., Baret, E., Camara, C., Nakoulima, A., Diatta, B.,  
616 Fall, K.B., Mbaye, P.S., Diémé, Y., Bercion, R., Wade, B., Pradines, B., 2014. Role of  
617 *Pfmdr1* in *in vitro* susceptibility to chloroquine, quinine, monodesethylamodiaquine,  
618 mefloquine, lumefantrine and dihydroartemisinin. *Antimicrob. Agents Chemother.* 58,  
619 7032-7040.

620 Yeka, A., Kigozi, R., Conrad, M.D., Lugemwa, M., Okui, P., Katureebe, C., Belay, K.,  
621 Kapella, B.K., Chang, M.A., Kanya, M.R., Staedke, S.G., Dorsey, G., Rosenthal, P.J.,

622 2016. Artesunate/amodiaquine versus artemether/lumefantrine for the treatment of  
623 uncomplicated malaria in Uganda: A randomized trial. *J. Infect. Dis.* 213:1134-1142.

624

625

626 **Table 1**627 Forward and reverse primers, hybridization temperature (T<sub>m</sub>) and MgCl<sub>2</sub> concentration used

628 for PCR

629

Gene	Forward and Reverse primers	T <sub>m</sub>	[MgCl <sub>2</sub> ]
<i>pfmdr5</i> (PF3D7_1339900)	Pfmdr5-F	52°C	2.5 mM
	5'-CAAAAAGTGAAACGCAAACG-3'		
	Pfmdr5-R		
	5'-GAGGATCTTCATCTTGTGTTTCG-3'		
<i>pfmdr1</i> (PF3D7_0523000)	MDR1-1F	52°C	2.5 mM
	5'-AGA GAA AAA AGA TGG TAA CCT CAG-3'		
	MDR1-1R		
	5'-ACC ACA AAC ATA AAT TAA CGG-3'	56°C	2.5 mM
	MDR1-2F		
	5'-CAG GAA GCA TTT TAT AAT ATG CAT-3'		
MDR1-2R	56°C	2.5 mM	
5'-CGT TTA ACA TCT TCC AAT GTT GCA-3'			

630

631

632 **Table 2**

633 Distribution of the average parameters of *P. falciparum* susceptibility to chloroquine (CQ),  
 634 quinine (QN), monodesethylamodiaquine (DQ), lumefantrine (LMF), mefloquine (MQ),  
 635 piperazine (PPQ), pyronaridine (PND) and dihydroartemisinin (DHA)

636

Drugs	Quartile 1	Median	Quartile 3	Mean	Standard error	Cut off <sup>1</sup>	Reduced susceptibility (%) <sup>2</sup>
CQ	26.2	85.7	154.8	112.9	14.6	77	46.3
QN	54.6	138.3	286.0	221.2	29.0	611	9.2
DQ	8.1	21.0	50.3	38.6	5.1	61	22.4
LMF	1.7	4.7	12.5	9.8	1.5	115	0
MQ	13.8	29.3	46.1	35.4	3.2	30	49.2
PPQ	23.6	48.4	69.3	54.4	5.2	135	6.6
PND	5.6	11.3	17.3	14.1	1.9	60	3.5
DHA	0.7	1.8	4.3	2.9	0.4	12	1.7

637 <sup>1</sup>The threshold values for the reduced *in vitro* susceptibility or resistance were previously  
 638 estimated (Fall et al., 2011; Pascual et al., 2015).

639 <sup>2</sup>This column showed the proportion of isolates with IC<sub>50</sub> above the threshold value.

640

641 **Table 3**

642 Association between the *pfmdr5* genotypes analyzed among the 67 clinical isolates from  
 643 Dakar, Senegal and *ex vivo* susceptibility to chloroquine (CQ), quinine (QN),  
 644 monodesethylamodiaquine (DQ), lumefantrine (LMF), mefloquine (MQ), piperazine (PPQ),  
 645 pyronaridine (PND) and dihydroartemisinin (DHA)  
 646

Allele <sup>a</sup>	No <sup>b</sup>	Frequency (%)	IC <sub>50</sub> mean in nM for parasites bearing these genotypes versus IC <sub>50</sub> mean in nM of those that do not ( <b>p is displayed only when p&lt;0.05</b> ) <sup>c</sup>							
			CQ	QN	DQ	LMF	MQ	PPQ	PND	DHA
5-1	17	25.5	106.6/115.4	350.0/196.2	31.5/40.3	5.1/8.0	39.1/34.9	57.1/51.0	16.7/13.9	3.3/2.8
6-1	16	23.9	122.8/110.1	187.2/251.3	37.6/38.2	10.5/6.2	36.5/36.0	58.5/51.0	23.6/12.0	3.0/2.5
7-1	13	19.1	180.6/96.9	229/238	54.7/34.0	7.7/7.2	44.4/34.4	55.0/52.2	11.5/15.3	1.9/2.8
8-1	6	9.0	97.0/114.7	209.7/239.0	42.7/37.6	9.5/7.1	33.9/36.2	<b>30.6/54.3</b>	9.3/15.0	2.8/2.6
								<b>(0.042)</b>		
3-0	3	4.5	<b>30.8/117.0</b>	197/238	<b>10.7/39.3</b>	3.6/7.5	<b>18.5/37.0</b>	50.8/52.8	<b>5.1/15.2</b>	1.4/2.7
			<b>(&lt;0.001)</b>		<b>(&lt;0.001)</b>		<b>(0.021)</b>		<b>(&lt;0.001)</b>	
3/7/8-0 <sup>d</sup>	5	7.5	<b>28.2/120.0</b>	191.9/240.0	<b>8.3/40.4</b>	<b>2.4/7.7</b>	24.8/37.0	77.8/50.5	<b>6.0/15.5</b>	1.7/2.7
			<b>(&lt;0.001)</b>		<b>(&lt;0.001)</b>	<b>(0.037)</b>			<b>(&lt;0.001)</b>	
≥8-x <sup>e</sup>	11	16.5	40.1/60.0	70.4/129.3	15.8/20.7	5.5/3.5	13.7/27.8	<b>14.0/40.6</b>	<b>2.7/10.5</b>	0.9/1.4
								<b>(0.003)</b>	<b>(0.04)</b>	
2-2	1	1.5								
3-1	1	1.5								
3-2	1	1.5								
4-1	2	3.0								
5-2	1	1.5								
7-0	1	1.5								
8-0	1	1.5								
9-1	2	3.0								
11-1	2	3.0								

647 <sup>a</sup> Number of DNNN motifs followed by number of DHHNDHNNDNNN units648 <sup>b</sup> Number of samples bearing each genotype.

649 <sup>c</sup> Statistical analysis used Welsh two-sample t test to compare IC<sub>50</sub> for parasites bearing these  
650 genotypes versus those that do not have, and significant results (p<0.05) are in bold. The  
651 analysis was only done for genotypes with number of samples for each genotype >3.

652 <sup>d</sup> All the parasites without a DHHNDHNNNDNNN motif were compared with all others.

653 <sup>e</sup> All the parasites with a number of DNNN motif  $\geq 8$  were compared with all others.

654

655

656 **Table 4**

657 Association between the *pfmdr1* genotypes analyzed among the 67 clinical isolates from  
 658 Dakar, Senegal and *ex vivo* susceptibility to chloroquine (CQ), quinine (QN),  
 659 monodesethylamodiaquine (DQ), lumefantrine (LMF), mefloquine (MQ), piperazine (PPQ),  
 660 pyronaridine (PND) and dihydroartemisinin (DHA)

661

<i>Pfmdr1</i> Genotypes	No <sup>a</sup>	IC <sub>50</sub> mean in nM for parasites bearing the first genotype versus IC <sub>50</sub> mean in nM of those bearing the second (p is displayed only when p<0.05)							
		CQ	QN	DQ	LMF	MQ	PPQ	PND	DHA
N86/86Y	73/4	117.7/85.8	226.6/190	40.5/34.5	<b>9.9/2.1</b>	36.6/30.6	53.8/81.3	13/40.3	2.7/6.5
					<b>(0.002)</b>				
184/184F	31/44	111.3/116	227/223	38.2/39.4	6.2/11.6	42.4/31.8	57.5/54	13.3/15.6	2.6/3.2
N86-Y184	30/42	113.6/119	229/229	39.1/42	<b>6.4/12.4</b>	43/31.8	58.4/50	12.5/13.3	2.6/2.8
/N86-184F					<b>(0.049)</b>				

662 <sup>a</sup> Number of samples bearing each genotype; WT/Mutated

663 <sup>b</sup> Statistical analysis used Welsh two-sample t test to compare IC<sub>50</sub> for parasites bearing these  
 664 genotypes versus those bearing another, and significant results (p≤0.05) are in bold.

665

666

667 **Fig. 1.** Distribution of *ex vivo* responses of 67 *P. falciparum* clinical field isolates from Dakar,  
668 Senegal to piperazine (PPQ), monodesethylamodiaquine (DQ), mefloquine (MQ),  
669 pyronaridine (PND), chloroquine (CQ), quinine (QN), dihydroartemisinin (DHA) and  
670 lumefantrine (LMF).

671

672 **Fig. 2.** (A) Association between allele 8-1 of *pfmdr5* and increased susceptibility to  
673 piperazine (PPQ) (30.6 versus 54.3 nM;  $p=0.042$ ; Welch two-sample *t* test) and (B) between  
674 alleles with 8 and more than 8 motif repeats ( $\geq 8-x$ ) and increased susceptibility to PPQ (14.0  
675 versus 40.6 nM).

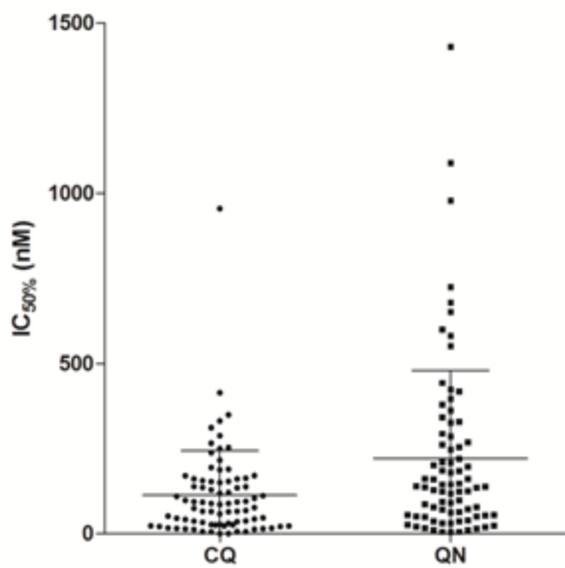
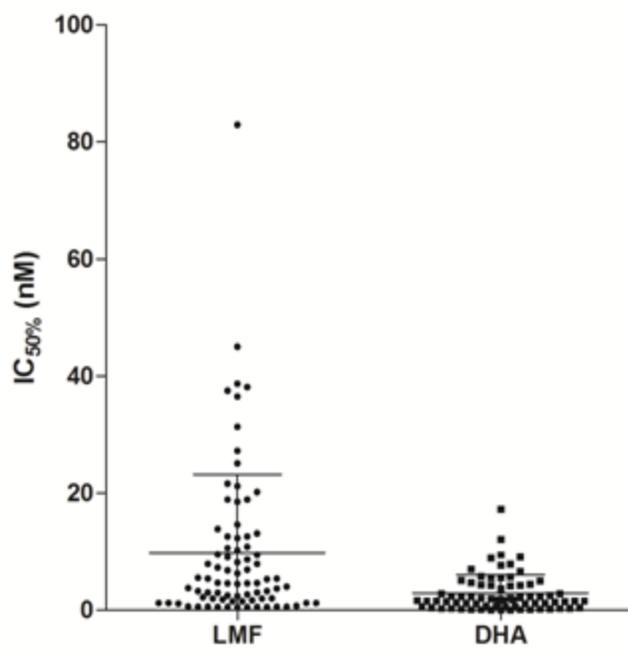
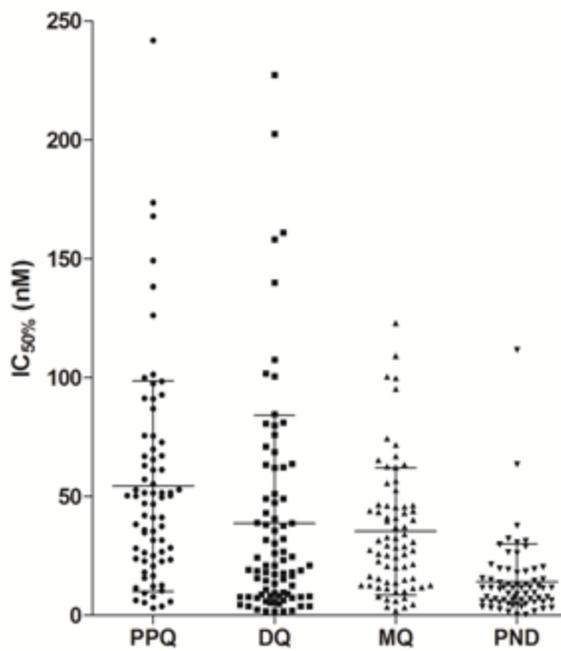
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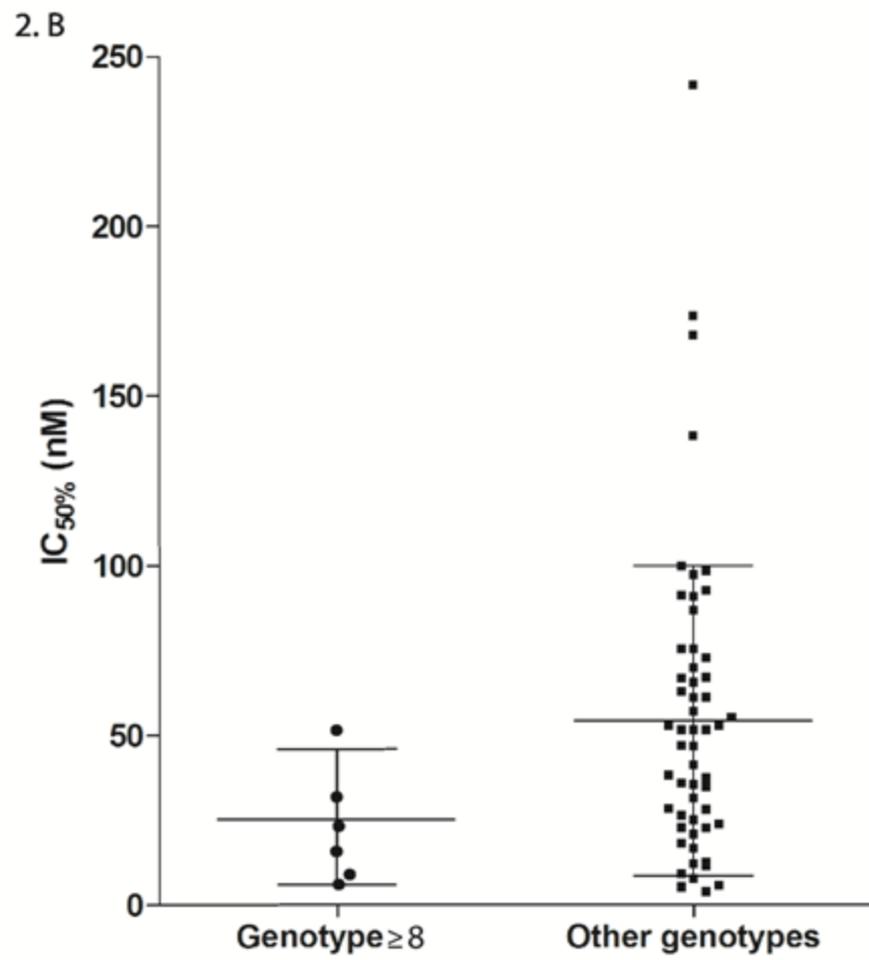
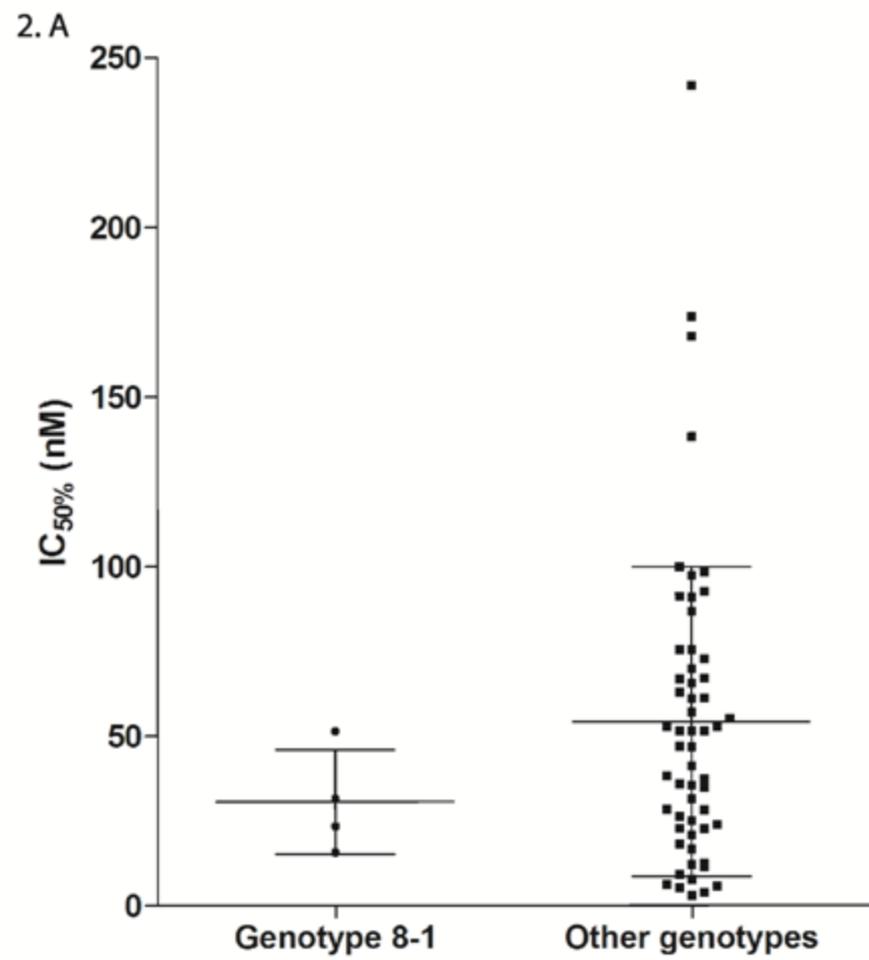
677 **Fig. 3.** Association between the *pfmdr1* N86-184F haplotype and decreased susceptibility to  
678 lumefantrine (LMF).

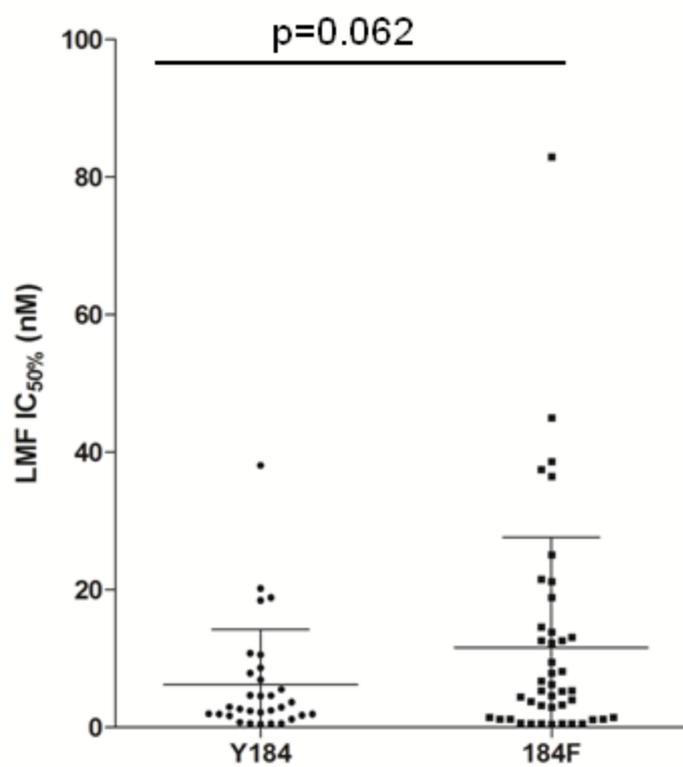
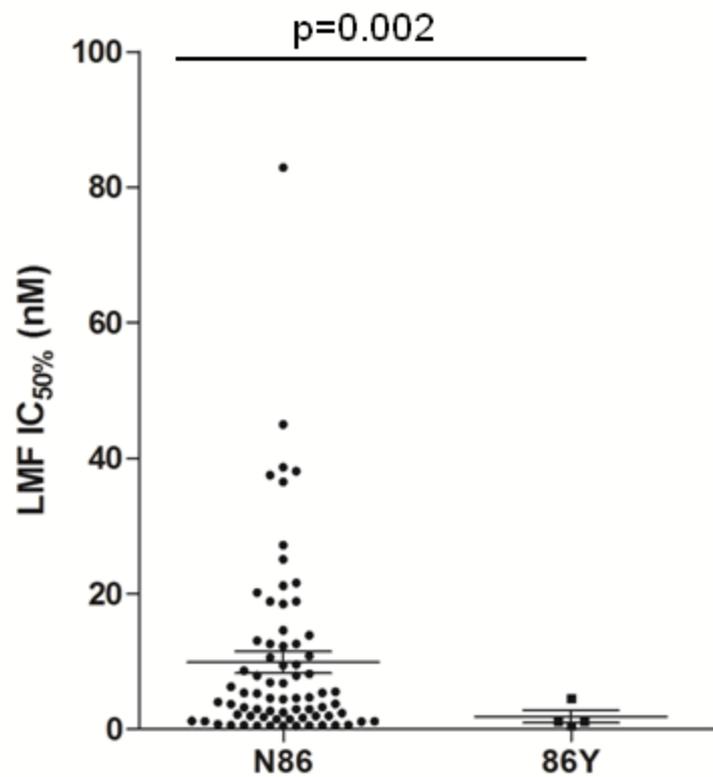
679

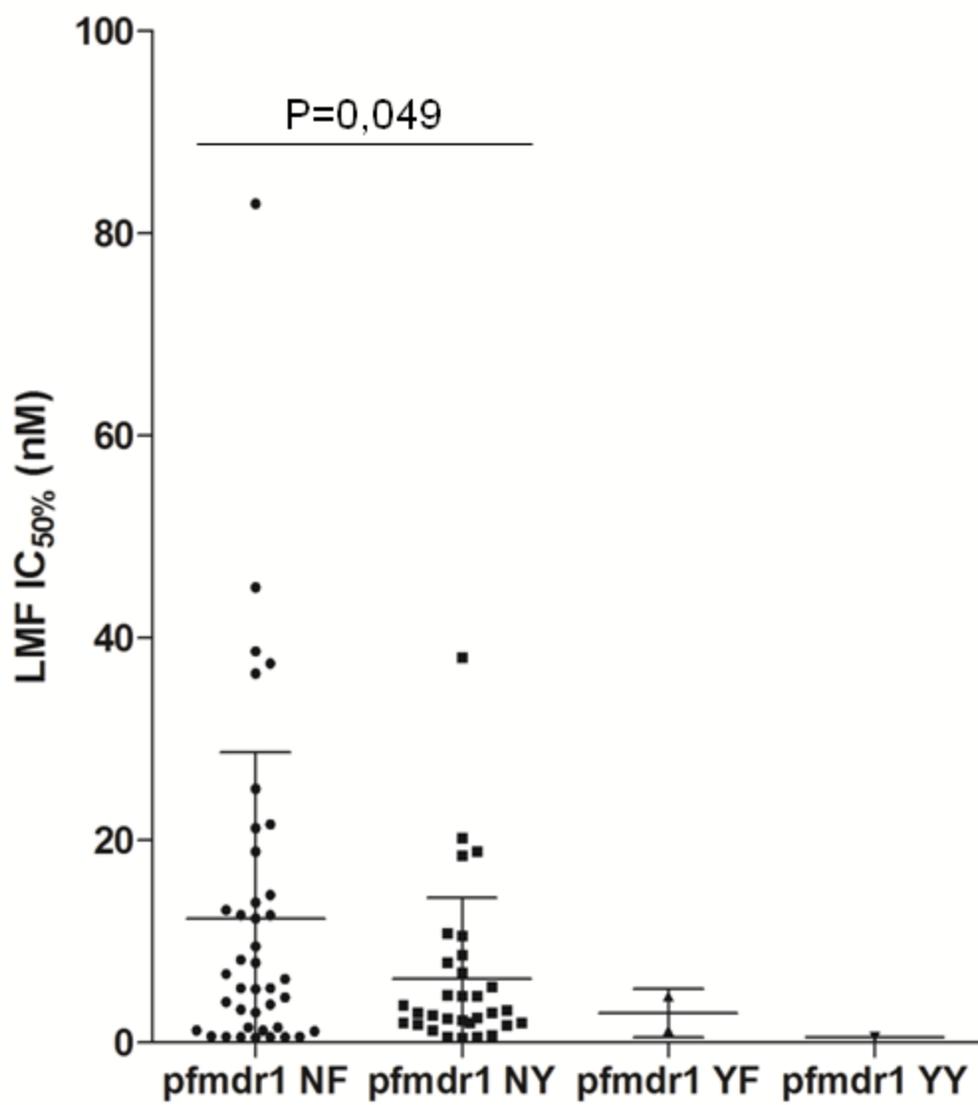
680 **Fig. 4.** Association between the *pfmdr1* N86Y and Y184F alleles with susceptibility to  
681 lumefantrine (LMF) (12.3 versus 6.3 nM;  $p=0.049$ ).

682

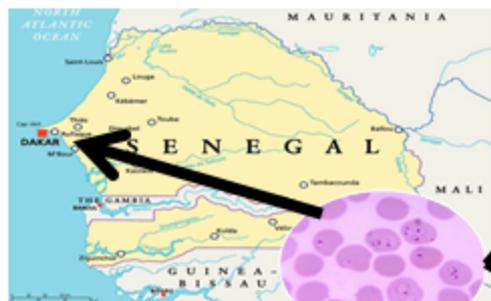
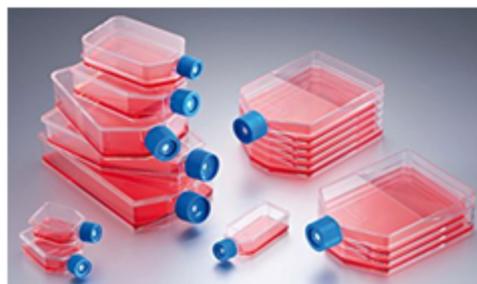




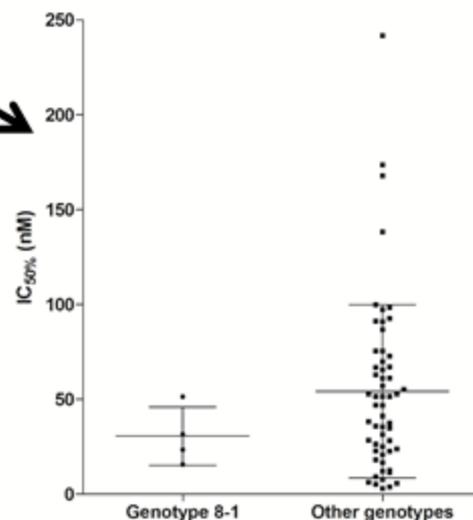




## Antimalarial drugs *in vitro* testing



*Plasmodium falciparum* isolates from Dakar



Sequencing of the ATP-binding cassette transporter genes *pfmdr1* and *pfmdr5*

The 8 repeats of DNNN motif associated with 1 repeat DHHNDHNNDNNN motif (8-1) allele in *pfmdr5* showed a significant association with increased susceptibility to piperazine