



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

Mathieu Gendrot, Mamadou Wague Gueye, Francis Tsombeng Foguim, Marylin Madamet, Khalifa Ababacar Wade, Mame Bou Kounta, Mansour Fall, Silman Diawara, Nicolas Benoit, Gora Lo, et al.

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2 *falciparum* ABC transporters (*pfmdr1* and *pfmdr5*)

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Abstract

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

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

1. Introduction

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

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

or artesunate plus mefloquine (Price et al., 2004). In Uganda, the use of dihydroartemisinin-piperaquine selected for the 86Y mutation (Nankabirwa et al., 2016). However, SNPs and overexpression of *pfmdr1* do not entirely explain responses observed in resistant *P. falciparum* parasites, suggesting that other polymorphisms may be involved in antimalarial drug susceptibility. The *P. falciparum* genome is rich in sequences encoding low-complexity regions which comprise repetitions of short motifs. Existence of these repeat polymorphisms has been identified in ABC transporters (Okombo et al., 2013; Veiga et al., 2014). But very few studies explored association between these repeat polymorphisms and parasite susceptibility to anti-malarial drugs. Interestingly, the presence of 7 and 9 Asn repeats in *pfmdr6* (PF3D7_1352100) were associated with modulation of *in vitro* lumefantrine and dihydroartemisinin drug responses in parasites from the China-Myanmar border area (Wang et al., 2012). Parasites with 6 Asn repeats were significantly less susceptible to lumefantrine, whereas those with 8 Asn repeats were less susceptible to piperaquine in parasites collected in Kenya (Okombo et al., 2013). The presence of 7 or 9 Asn repeats was also associated with reduced *in vitro* susceptibility to quinine in 77 Senegalese *P. falciparum* isolates (Gendrot et al., 2017).

Repetitive amino acid motifs in *pfmdr5* (PF3D7_1339900), which codes a protein localized at the plasma membrane of the parasite (Kavishe et al., 2009), were associated with reduced *in vitro* susceptibility to lumefantrine (Okombo et al., 2013). Additionally, deletion of *pfmdr5* in *P. falciparum* parasites resulted in a minor decrease in the inhibiting concentration 50% (IC₅₀) of artemisinin (van der Velden et al., 2015).

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

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

2. Materials and methods

2.1. Patients and sample collection

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

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

2.2. Drugs

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

(stock solution 40,000 nM), 0.1 to 107 nM for DHA (stock solution 26,750 nM) and 1.9 to 998 nM for PPQ (stock solution 10,000 nM). CQ and PND were resuspended and diluted in water to final concentrations ranging from 6 to 3,231 nM and 0.4 to 199 nM, respectively. LMF was resuspended and diluted in absolute ethanol to obtain final concentrations ranging from 0.6 to 310 nM (stock solution 3,000 nM). Each batch of plates were validated on the CQ-resistant W2 strain (isolated in Indochina; ref MRA-157 obtained from MR4, VA) in four independent experiments using the same conditions described below. The mean IC₅₀ values for the chloroquine-resistant W2 strain for the different batches used during the three years were 292 nM for CQ, 275 nM for QN, 72 nM 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 nM for DHA. A comparison of W2 susceptibility data for the nine anti-malarial drugs indicated that there was no significant difference in the responses to antimalarial drugs over the three years ($0.39 < p < 0.95$).

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

2.3. *Ex vivo* assay

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

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

2.4. Nucleic acid extraction

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

2.5. Gene sequence polymorphism analysis

Two genes, *pfmdr5* (PF3D7_1339900), and *pfmdr1* (PF3D7_0523000) were amplified by polymerase chain reaction using the oligonucleotide primer pairs described in Table 1. The primers used amplified a region of *pfmdr5* gene of about 900 bp. Two primer pairs were used to amplify the *pfmdr1* fragments carrying the five mutations in two regions of 610 pb and 890 pb. The reaction mixture consisted of 200 ng of genomic DNA, 0.32 μ M of each primer, 1X final of reaction buffer (750 mM Tris-HCl, 200 mM (NH₄)₂SO₄, 0.1 % (v/v) Tween 20 and stabilizer, pH 8.8), 200 μ M of deoxynucleoside triphosphate mixture (dGTP, dATP, dTTP and dCTP) (Euromedex, Souffelweyersheim, France), variable concentrations of MgCl₂ (Table 1) and 0.2 U of Red Diamond Taq® polymerase (Eurogentec, Liège, Belgium) in a final volume of 25 μ L. The thermal cycler (T3 Biometra, Biometra GmbH, Göttingen, Germany) was programmed as follows: 94°C for 10 min, then 40 cycles alternating among 94°C for 30 sec, hybridization temperature (Table 1) for 45 sec, and 72°C for extension at 1 min per 1,000 bp, followed by a 10-min final extension step at 72°C. The amplified products were sequenced using the PCR primers on an ABI Prism 3100 analyzer (Applied Biosystems) according to the manufacturer's instructions. The Vector NTI advance (TM) software (version 11, Invitrogen, Cergy Pontoise, France) was used to perform data analysis.

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

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

2.6. Statistical analysis

In this study, the association between the mutations in *pfmdr1* (N86Y, Y184F, S1034C, N1042D and D1246Y) or repetitive amino acid motifs in *pfmdr5* and the ex vivo susceptibility to antimalarial drugs was evaluated. The data were analyzed using the R software (version 3.0.2). Differences in the IC₅₀s of the isolates and the *pfmdr5* and *pfmdr1* polymorphisms were compared using the Kruskal-Wallis test and the Welch two-sample *t* test. The differences between susceptible and resistant *P. falciparum* parasites and polymorphisms were analyzed by logistic regression. The significance level was calculated using the correction of Bonferroni. The significance threshold retained for *pfmdr5* analysis was 0.007.

3. Results

The distribution and the average parameters of the IC₅₀ values for the eight antimalarial drugs are presented in Table 2 and Fig. 1.

3.1. *Pfmdr5*

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

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

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

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

3.2. *Pfmdr1*

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

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

3.3. *Pfmdr5/pfmdr1*

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

4. Discussion

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

ABC transporters have been involved in antimalarial drug resistance. PfMDR5 is a transporter that belongs to the ABC B family, like PfMDR1, PfMDR2 or PfMDR6. However, PfMDR5 is a half transporter and contains only one membrane domain (MD) with 6 putative α -helical transmembrane segments (TMS) and only one nucleotide-binding domain (NBD) containing the "Walker A" and the "Walker B" motifs and a short but highly conserve sequence which is typical for the members of the ABC family (Kavishe et al., 2009; Pradines et al., 2005; Pradines et al., 2009). The repetitive amino acid motifs analyzed in this study, DNNN and DHHNDDHNNDNNN, 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 DHHNDHNNDNNN 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 (IC₅₀>135 nM). Okombo and colleagues found that

275 allele 5-1 was significantly associated with an increased LMF IC₅₀ on Kenyan isolates, but

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

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

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

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

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

In this study, no association was highlighted between N86 and 86Y alleles with *in vitro* susceptibility modulation to DQ. However, this result should be taken with caution due to the low frequency of the 86Y SNP. The data on the 86Y mutation and its involvement in DQ susceptibility are contradictory. Previous works showed no association between the haplotypes, N86 and 86Y, and DQ IC₅₀ (Dahltröm et al., 2014), whereas other studies showed associations between 86Y mutation and increased (Wurtz et al., 2014) or decreased susceptibility to DQ (Folarin et al., 2011). The replacement by transfection of the 86Y mutation by the wild-type N86 resulted in increased susceptibility to DQ (Veiga et al., 2016). Nevertheless, the 86Y mutation was found to be associated with treatment failures after amodiaquine monotherapy (Holmgren et al., 2006; Danquah et al., 2010) or artesunate-amodiaquine (Venkatesan et al., 2014; Sondo et al., 2016; Yeka et al., 2016).

5. Conclusion

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

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

Ethics approval and consent to participate

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

Conflicts of interest

The authors declare that they have no competing interests.

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Table 1

Forward and reverse primers, hybridization temperature (T_m) and MgCl₂ concentration used for PCR

Gene	Forward and Reverse primers	T _m	[MgCl ₂]
<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'		
	MDR1-2F	56°C	2.5 mM
	5'-CAG GAA GCA TTT TAT AAT ATG CAT-3'		
	MDR1-2R		
	5'-CGT TTA ACA TCT TCC AAT GTT GCA-3'		

Table 2

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

Drugs	Quartile 1	Median	Quartile 3	Mean	Standard error	Cut off ¹	Reduced
							susceptibility (%) ²
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

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

²This column showed the proportion of isolates with IC₅₀ above the threshold value.

Table 3

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

Allele ^a	No ^b	Frequency (%)	IC ₅₀ mean in nM for parasites bearing these genotypes versus IC ₅₀ mean in nM of those that do not (p is displayed only when p<0.05) ^c							
			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	30.6/54.3	9.3/15.0	2.8/2.6
								(0.042)		
3-0	3	4.5	30.8/117.0	197/238	10.7/39.3	3.6/7.5	18.5/37.0	50.8/52.8	5.1/15.2	1.4/2.7
			(<0.001)		(<0.001)		(0.021)		(<0.001)	
3/7/8-0 ^d	5	7.5	28.2/120.0	191.9/240.0	8.3/40.4	2.4/7.7	24.8/37.0	77.8/50.5	6.0/15.5	1.7/2.7
			(<0.001)		(<0.001)	(0.037)			(<0.001)	
≥8-x ^e	11	16.5	40.1/60.0	70.4/129.3	15.8/20.7	5.5/3.5	13.7/27.8	14.0/40.6	2.7/10.5	0.9/1.4
								(0.003)	(0.04)	
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								

^a Number of DNN motifs followed by number of DHHNDHNNDNN units

^b Number of samples bearing each genotype.

649 ^c Statistical analysis used Welsh two-sample t test to compare IC₅₀ 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 ^d All the parasites without a DHHNDHNNNDNNN motif were compared with all others.

653 ^e All the parasites with a number of DNNN motif ≥ 8 were compared with all others.

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Table 4

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

<i>Pfmdr1</i>	No ^a	IC ₅₀ mean in nM for parasites bearing the first genotype versus IC ₅₀ mean in nM of those bearing the second (p is displayed only when p<0.05)							
Genotypes		CQ	QN	DQ	LMF	MQ	PPQ	PND	DHA
N86/86Y	73/4	117.7/85.8	226.6/190	40.5/34.5	9.9/2.1	36.6/30.6	53.8/81.3	13/40.3	2.7/6.5
					(0.002)				
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	6.4/12.4	43/31.8	58.4/50	12.5/13.3	2.6/2.8
/N86-184F					(0.049)				

^a Number of samples bearing each genotype; WT/Mutated

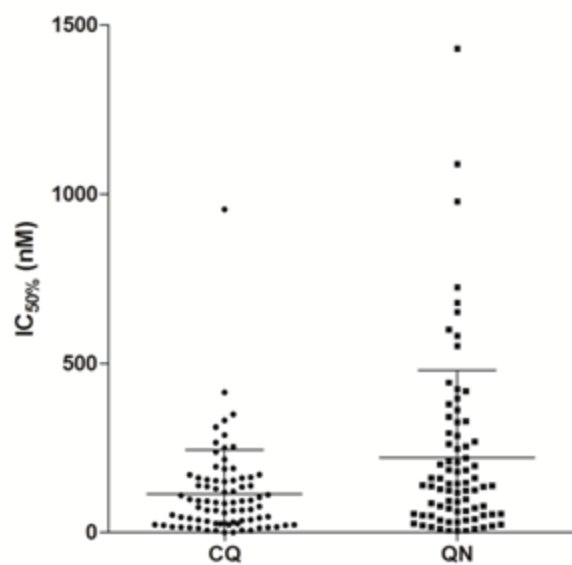
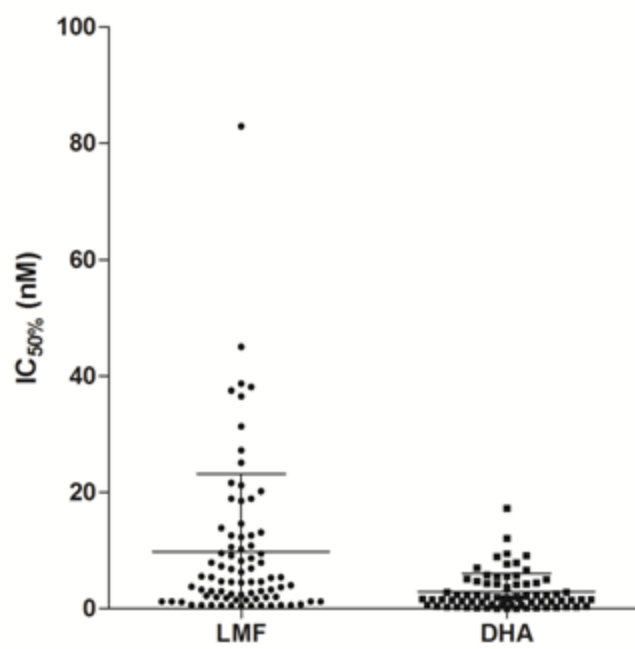
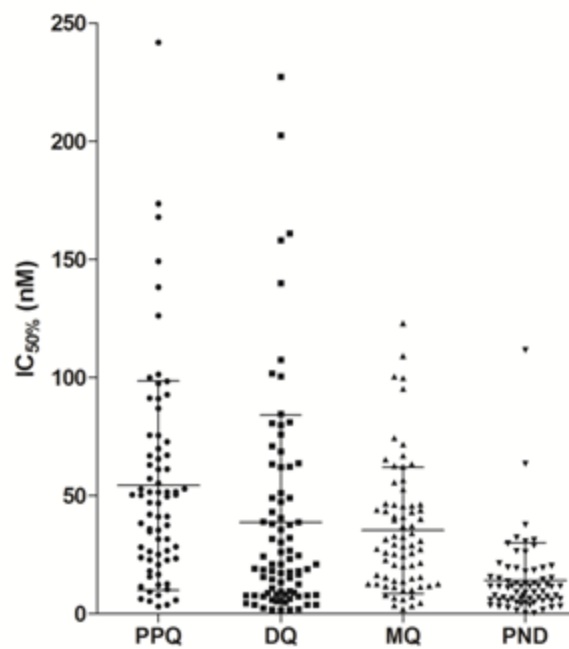
^b Statistical analysis used Welsh two-sample t test to compare IC₅₀ for parasites bearing these genotypes versus those bearing another, and significant results (p≤0.05) are in bold.

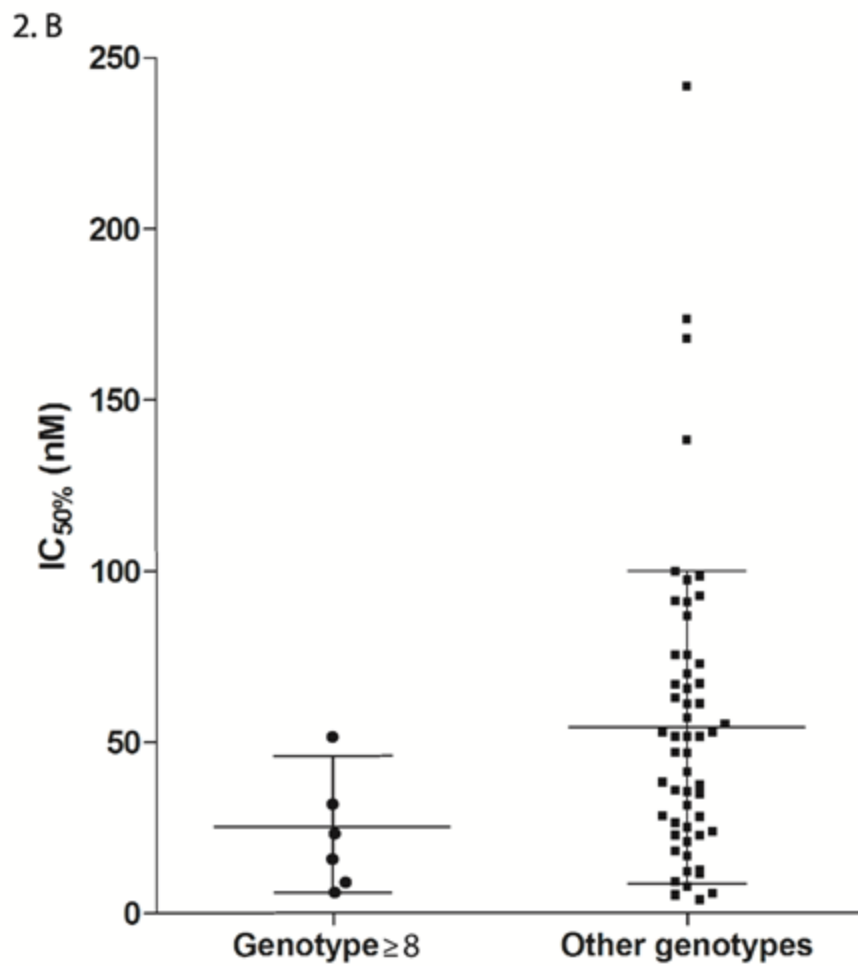
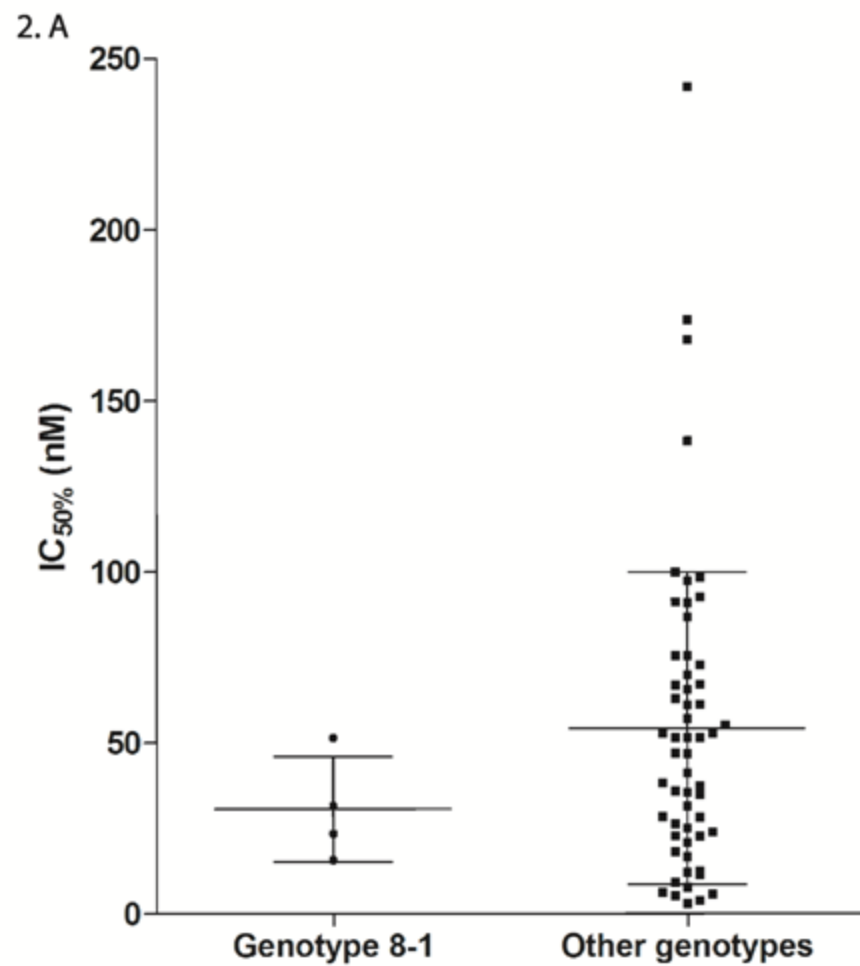
Fig. 1. Distribution of *ex vivo* responses of 67 *P. falciparum* clinical field isolates from Dakar, Senegal to piperazine (PPQ), monodesethylamodiaquine (DQ), mefloquine (MQ), pyronaridine (PND), chloroquine (CQ), quinine (QN), dihydroartemisinin (DHA) and lumefantrine (LMF).

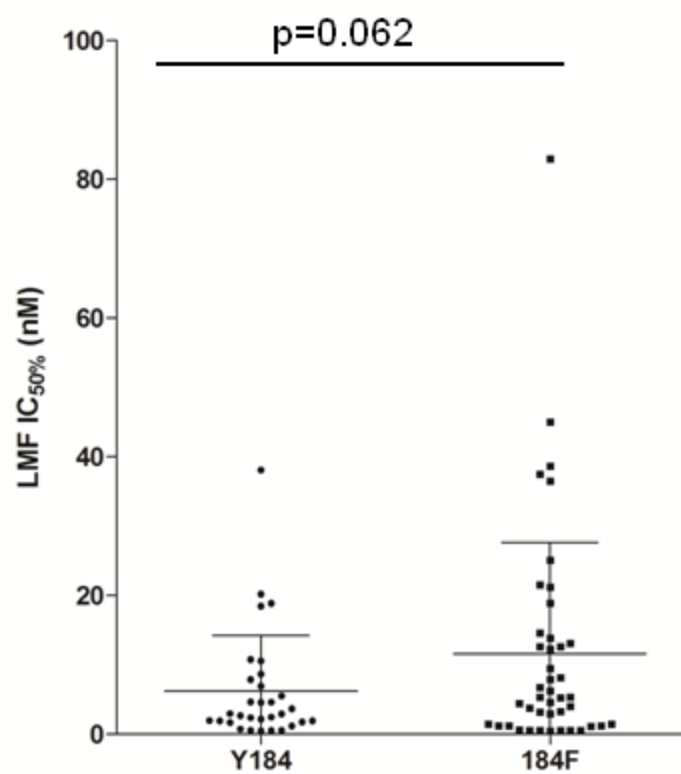
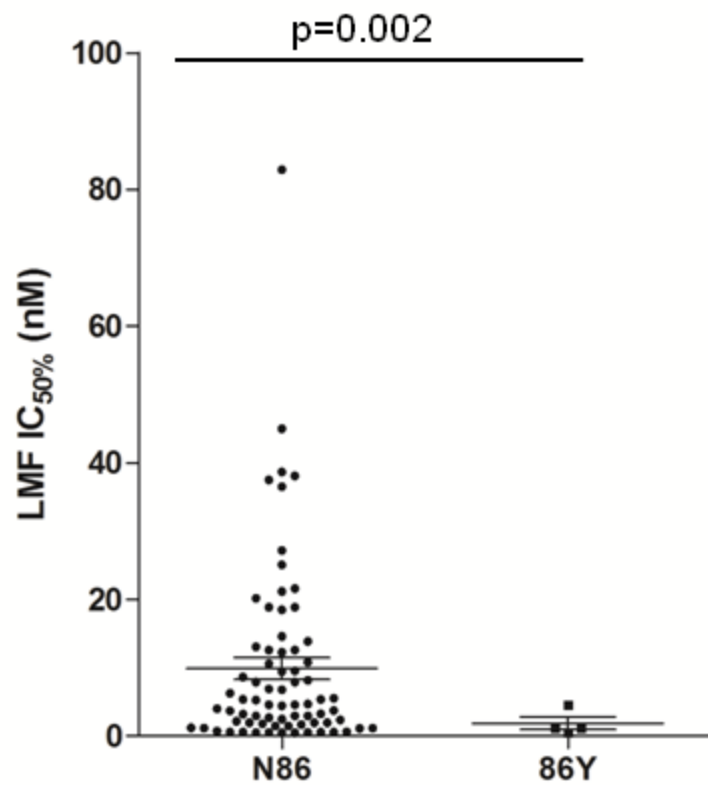
Fig. 2. (A) Association between allele 8-1 of *pfmdr5* and increased susceptibility to piperazine (PPQ) (30.6 versus 54.3 nM; $p=0.042$; Welch two-sample *t* test) and (B) between alleles with 8 and more than 8 motif repeats ($\geq 8-x$) and increased susceptibility to PPQ (14.0 versus 40.6 nM).

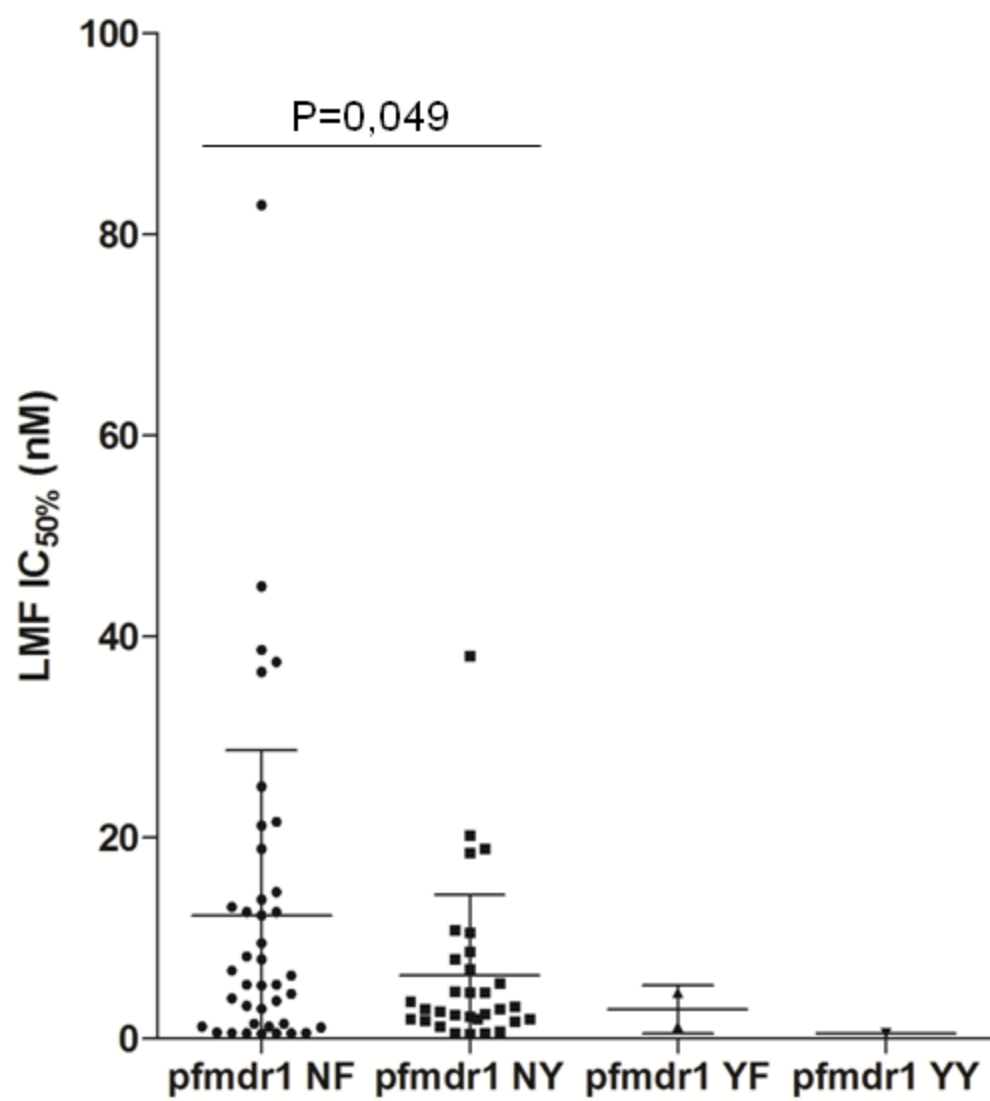
Fig. 3. Association between the *pfmdr1* N86-184F haplotype and decreased susceptibility to lumefantrine (LMF).

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

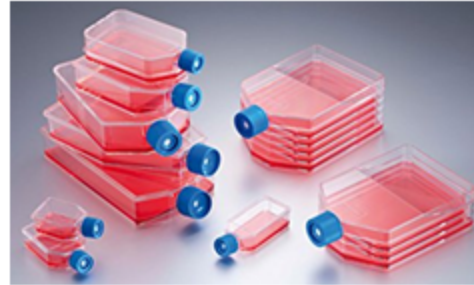








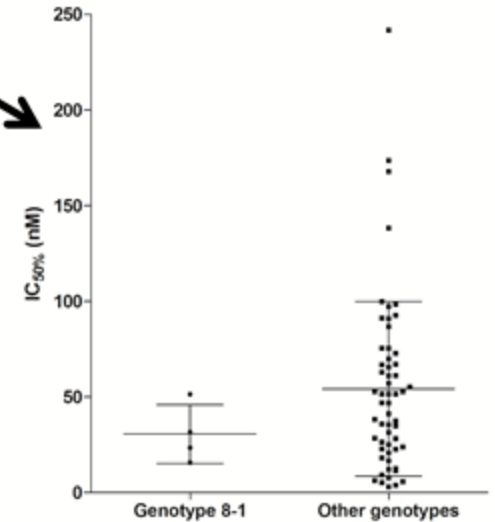
Antimalarial drugs *in vitro* testing



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