

1 **Absence of High Level of Duplication of the *plasmepsin 2* Gene in Africa**

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3 Running title: *P. falciparum* plasmepsin 2 copy number

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19

20 Abstract

21 Resistance to piperazine has been associated with the amplification of the *plasmepsin 2* gene
22 in Cambodia. None of the 175 African isolates that we analyzed had *plasmepsin 2* gene
23 amplification (piperazine inhibitory concentration 50% ranged from 0.94 to 137.5 nM),
24 suggesting a low level of piperazine reduced susceptibility prevalence in Africa.
25 Additionally, the few isolates with reduced susceptibility to piperazine did not harbor
26 amplification of the *plasmepsin 2* gene.

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28 **Keywords:** malaria, *Plasmodium falciparum*, antimalarial drug, resistance, in vitro, molecular
29 marker, plasmepsin 2, piperazine

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32 *Plasmodium falciparum* resistance to most antimalarial drugs has emerged in Southeast Asia
33 and spread to Africa (1,2). Since 2005, the World Health Organization (WHO) recommended
34 artemisinin-based combination therapies (ACT) as the first-line treatment for uncomplicated
35 malaria followed by artesunate for the treatment of severe malaria. However, artemisinin
36 derivatives-resistant *P. falciparum* strains emerged in western Cambodia, Myanmar and
37 Thailand and eventually in all of Southeast Asia (3,4). As soon as the last marketed ACT,
38 dihydroartemisinin-piperaquine, was used, resistance emerged in Cambodia and later in
39 Vietnam (5-8). In this context, it is essential to have markers of resistance to monitor the
40 emergence and spread of resistance to dihydroartemisinin-piperaquine. Mutations (Y493H,
41 F446I and C580Y) in the propeller domain of the Kelch 13 (K13) gene (PF3D71343700)
42 were associated with *in vivo* and *in vitro* resistance to artemisinin in Southeast Asia (9,10).
43 Two recent studies showed that *in vitro* and *in vivo* resistance to piperaquine was associated
44 with the amplification of the copy number of the *plasmepsin 2* gene (PF3D7_1408000)
45 (11,12). However, these data were validated only on Cambodian isolates. The objective of the
46 present study was to evaluate the copy number of the gene in African *P. falciparum* isolates
47 and its association with *in vitro* susceptibility to piperaquine.

48 A total of 175 *P. falciparum* isolates were successfully evaluated for the copy number of the
49 *plasmepsin 2* gene and assessed for *ex vivo* susceptibility to piperaquine. The isolates were
50 collected from patients hospitalized in France from January 2015 to April 2017 with imported
51 malaria from a malaria-endemic country and, more particularly, from African French-
52 speaking countries, such as Côte d'Ivoire, Cameroon, Central African Republic, Republic of
53 Congo, Guinea, Burkina Faso, Togo, Gabon and Senegal (Table 1). The samples were sent
54 from different civilian or military hospitals of the French National Reference Center for
55 Imported Malaria Network (Aix en Provence, Bordeaux, Marseille, Montpellier, Nice, Toulon

56 and Toulouse) to the French National Reference Center for Malaria (IRBA, IHU Méditerranée
57 Infection Marseille).

58 The parasitemia, which ranged from 0.005% to 9.5%, was estimated on thin blood smears
59 that were stained by eosin and methylene blue using a RAL[®] kit (Réactifs RAL, Paris,
60 France). The diagnosis of *P. falciparum* mono-infection was confirmed by real time PCR
61 (LightCycler 2.0, Roche Group, Switzerland), as previously described (13).

62 Piperaquine (PPQ) for the *ex vivo* drug susceptibility assay was obtained from Shin Poong
63 Pharm Co (Seoul, Korea). PPQ was first dissolved in methanol and later diluted in water to
64 final concentrations that ranged from 1.9 to 998 nM. The isolates were incubated for 72 hours
65 in a controlled atmosphere set at 85% N₂, 10% O₂, 5% CO₂ and 37°C (maximum final
66 parasitemia at 0.5% and final hematocrit of 1.5%). The isolates with parasitemia above 0.5%
67 were diluted to 0.5% with fresh uncontaminated erythrocytes. The drug susceptibility assay
68 was revealed by the HRP2 ELISA-based assay implemented in the Malaria Ag Celisa kit (ref
69 KM2159, Cellabs PTY LDT, Brookvale, Australia) as previously described (14). IC₅₀ values
70 were validated only if the optical density (OD) ratio (OD at zero concentration / OD at
71 maximum concentration of drug) was above 1.6 and the 95% confident interval of the IC₅₀
72 estimation was below 2.0. Each batch of plates was validated on the CQ-resistant W2 clone of
73 Indochina strain (obtained from MR4, VA, USA) in four independent experiments using the
74 same conditions as described below. The mean PPQ 50% inhibitory concentration (IC₅₀)
75 values for the chloroquine-resistant W2 clone for the different batches used during the study
76 was 54.1 ± 5.4 nM. There was no significant difference in the responses of the strains to PPQ
77 according to the different batches (p = 0.770).

78 The *plasmepsin 2* copy number was estimated by TaqMan real-time PCR (Lightcycler[®] 2.0,
79 Roche) using the single-copy gene *β-tubulin* (PF10_0084) as control housekeeping gene. The
80 following primers and probes were used: 5'- GGA GAT AAC CAA CAA CCA TTT AC -3',

81 5'- GTT GTA CAT TTA ACA CTT GGG A-3' and 5'- FAM-CCC ATA AAT TAG CAG
82 ATC CTG TAT C-TAMRA-3' for *plasmepsin 2* and 5'- TGA TGT GCG CAA GTG ATC C-
83 3', 5'- TCC TTT GTG GAC ATT CTT CCT C-3' and 5'- FAM-TAG CAC ATG CCG TTA
84 AAT ATC TTC CAT GTC T-TAMRA-3' for β -*tubulin* (Eurogentec, Angers, France). PCR
85 reactions were carried out using 1 \times LightCycler® TaqMan® Master (ROCHE, Allemagne),
86 900 nM forward primer, 900 nM reverse primer, 250 nM Taqman probe and 3 μ l of template
87 DNA. The thermal cycling conditions were 45 cycles of 95°C for 15 s and 60°C for 1 min.
88 Each sample was assayed in duplicate. The $2^{-\Delta\Delta C_t}$ method of relative quantification (where C_t
89 indicates cycle threshold) was used and adapted to estimate the copy number of
90 the *plasmepsin 2* gene, by using the formula $\Delta\Delta C_t = (C_{t_{plasmepsin\ 2}} - C_{t_{\beta-tubulin}})_{sample} - (C_{t_{plasmepsin\ 2}} - C_{t_{\beta-tubulin}})_{calibrator}$. Genomic DNA extracted from the *P. falciparum* 3D7 strain, which has a
91 single copy of each gene, was used as calibrator, and β -*tubulin* served as the control
92 housekeeping gene in all experiments. We previously verified that the PCR efficiency was
93 identical for the two genes. Samples were evaluated twice if the C_t standard deviation was
94 above 0.5 and if the copy number was above 1.5. Isolates with a copy number ≥ 1.6 were
95 classified as isolates with 2 copies (11,15). A control parasite isolate harboring at least two
96 copies of the *plasmepsin 2* gene and sampled in patient with malaria after returning from
97 Cambodia was used to validate the quantitative PCR assay (sample kindly provided by Pr.
98 Houzé).

100 The *ex vivo* IC_{50} curves of PPQ were satisfactorily fit to a sigmoidal function. Unlike the
101 cultured-adapted Cambodian parasites (16), none of our isolates exhibited a bimodal dose-
102 response curve when exposed to PPQ. The PPQ IC_{50} of the 175 isolates ranged from 0.94 to
103 137.5 nM (Figure 1). A wide range of the PPQ responses was already observed in several
104 studies and whatever the methodology used. PPQ IC_{50} values, assessed by a 72 hrs incubation
105 with atmospheric generators for capnophilic bacteria and HRP2 ELISA in isolates from Dakar

106 in 2013-2014 and 2013-2015, ranged from 2.5 to 168 nM and 3.9 to 241.9nM, respectively
107 (17,18). The distribution of PPQ IC₅₀ from 313 isolates obtained between 2008 and 2012 from
108 patients hospitalized in France for imported malaria and assessed by a 42 hrs isotopic test
109 ranged from 9.8 to 217.3 nM (19). A wide range of PPQ responses (3.1 to 188.9 nM) was also
110 observed in isolates collected in 2010-2013 in Uganda and assessed using HRP2 ELISA
111 detection (20). Only the isolates collected in 2016 in Uganda and assessed using a 72 hrs
112 fluorescence assay with SYBR green I detection presented a narrow range for PPQ IC₅₀, from
113 1.8 to 26.6 nM (21). In the absence of standardized *ex vivo* and *in vitro* tests, it is difficult to
114 compare data from different laboratories. IC₅₀ and cutoff values for *in vitro* resistance are
115 specific to the methodology. The *in vitro* effects and the IC₅₀ values for antimalarial drugs
116 depend on incubation and gas conditions and methodology (22-25). The isolates collected in
117 Uganda in 2016 and performed using a 72 hrs fluorescence assay with SYBR green I
118 detection showed a narrow range for PPQ IC₅₀, unlike those collected in 2010-2013 and
119 assessed using HRP2 ELISA that presented a wide range of PPQ responses (20,21). Only the
120 isolates that were assessed with SYBR green I detection showed a narrow range for PPQ IC₅₀.
121 Another hypothesis as a source of variation in drug responses may be the storage and the time
122 between sample collection from patients and the completion of the *ex vivo* test. Blood was
123 collected before therapy in ethylenediaminetetraacetic acid tube. Drug susceptibilities were
124 assessed immediately or from samples stored at 4°C, even during transport, for a maximum of
125 48 hrs and without short-term culture. After the collection from patients, the storage
126 temperature of the samples was controlled by sensor. Additionally, Senegalese isolates, which
127 were assessed immediately, presented the same wide range of *in vitro* responses (17,18). One
128 isolate had a reduced susceptibility to PPQ (IC₅₀ > 135 nM) (19). Another possible source of
129 variation in the PPQ drug responses could result from the range of parasitemias used in the

130 assay. This is because samples below 0.5% parasitemia were included in the assays, and thus
131 could not be adjusted to the standardized assay parasitemia.

132 Significant cross-susceptibilities were found between PPQ and mefloquine (coefficient of
133 correlation, $r = 0.453$; $p < 0.0001$), pyronaridine ($r = 0.406$; $p < 0.0001$), quinine ($r = 0.247$; p
134 $= 0.0011$) and monodesethylamodiaquine ($r = 0.189$; $p = 0.0138$). Associations between PPQ
135 and chloroquine or lumefantrine were not significant ($r = 0.137$, $p = 0.0755$ and $r = 0.114$; $p =$
136 0.1413 , respectively). The values of the coefficient of determination (r^2) of the different
137 associations were too low to explain the wide range of PPQ responses.

138 The copy number values ranged from 0.16 to 1.51 with a mean of 1.04 (Figure 2). None of
139 the isolates had more than one copy of the *plasmepsin 2* gene.

140 We did not observe any *plasmepsin 2* gene amplification in the African isolates analyzed in
141 this study. There was no association between the copy number of the *plasmepsin 2* gene and
142 susceptibility to PPQ. The most representative countries in terms of numbers were the
143 Cameroon and Côte d'Ivoire (47 and 37 isolates, respectively). Seven isolates ($IC_{50} > 90$ nM)
144 were outside the main scatter points representing the distribution of the *in vitro* responses to
145 PPQ (Figure 1) and could be considered as parasites with reduced susceptibility to PPQ,
146 although below 135 nM for six of them. The Cambodian *P. falciparum* strains, which were
147 resistant *ex vivo* to PPQ and showed amplification of the *plasmepsin 2* gene, presented IC_{50}
148 ranged from 89.3 to 159.6 nM (12). Among these seven isolates, four came from Cameroon
149 and two from Côte d'Ivoire, suggesting the presence of few *P. falciparum* strains with
150 reduced susceptibility to PPQ. The primary limitation of the present study is the low number
151 of parasite isolates with reduced susceptibility to PPQ, probably due to the low use of
152 dihydroartemisinin-piperaquine in Africa compared to Southeast Asia. Additionally, none of
153 the seven isolates with reduced susceptibility to PPQ ($IC_{50} > 90$ nM) harbored more than one
154 copy of the *plasmepsin 2* gene. This phenomenon was also shown in Cambodian isolates

155 harbouring a single copy of *plasmepsin 2* gene for which a large range of PPQ IC₅₀ was
156 observed as well as some parasites with high IC₅₀ (12). The use of the standard *ex vivo* assay
157 and not the PPQ survival assay (PSA) can be questionable (11, 26). However, Amato et al
158 identified the same association between *ex vivo* PPQ resistance and the amplification of the
159 *plasmepsin 2* gene by using a standard susceptibility drug assay (12). The resistant parasites
160 showed *ex vivo* IC₅₀ values between 89.3 and 159.6 nM and *in vitro* IC₅₀ values between 55.9
161 and 79.7 nM after cultures had adapted, reflecting a high PSA % survival that ranged from
162 52.2 to 74.9% (12). Additionally, a novel mutation (F145I) on the *P. falciparum* chloroquine
163 resistance transporter gene (*pfcr1*) was recently identified and described to have an association
164 with the PPQ IC₉₀ values in Cambodian isolates by the standard *in vitro* assay (27). The
165 influence of this mutation will have to be evaluated in African *P. falciparum* isolates.

166 The amplification of the *plasmepsin 2* gene does not fully explain the *in vitro* susceptibility
167 decreased to PPQ of some *P. falciparum* parasites in Africa. Indeed no correlation was
168 observed between the copy number of the *plasmepsin 2* gene and *ex vivo* susceptibility to
169 piperazine in *P. falciparum* Ugandan isolates (21). The use of dihydroartemisinin-
170 piperazine as intermittent preventive treatment during pregnancy (IPTp) did not select for
171 genotypes associated with amplification of the *plasmepsin 2* gene in Uganda (28).
172 Additionally, no amplification of the *plasmepsin 2* gene was found in Cameroonian
173 recrudescence *Plasmodium falciparum* parasites two years after treatment by
174 dihydroartemisinin-piperazine (29). Gupta et al. reported that 1.1% of the *Plasmodium*
175 *falciparum* isolates circulating in Mozambique in 2015 harbored multiple copies of the
176 *plasmepsin 2* gene (30). However, none of these isolates were compared with *in vitro* data or
177 clinical responses. These data do not allow assess the possible association between
178 amplification of the *plasmepsin 2* gene and reduced susceptibility to PPQ. Additionally,
179 Amato et al showed that more than half of isolates harbouring multicopies of the *plasmepsin 2*

180 gene presented low susceptibility to PPQ, suggesting that amplification of the *plasmepsin 2*
181 gene did not alone explain reduced susceptibility to PPQ (12).

182 These finding suggest that copy number variation of the *plasmepsin 2* gene may not alone
183 predict PPQ resistance in Africa. It seems that over resistance mechanisms and therefore other
184 molecular markers may exist in Africa compared to Asia. This phenomenon has been also
185 observed with resistance to artemisinin which is associated with *pfk13* polymorphisms in
186 Asia, while none polymorphism is observed in most of the cases of clinical failure of ACT in
187 Africa (10,31-35). To overcome the limitation of this study, i.e., the low number of samples
188 with reduced susceptibility, it is imperative to further assess more isolates from different
189 geographical areas of Africa, and especially more *P. falciparum* strains resistant to PPQ from
190 Africa.

191

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227

228 **Ethics approval and consent to participate**

229 Bio-banking of human clinical samples used for malaria diagnostics and secondary uses for
230 scientific purposes is possible as long as the corresponding patients are informed and have not
231 indicated any objections. This requirement was fulfilled here by giving verbal information to
232 the patients, and no immediate or delayed patient opposition was reported to the hospital
233 clinicians. Informed consent was not required for this study because the sampling procedures
234 and testing are part of the French national recommendations for the care and surveillance of
235 malaria.

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- 413

414 **Table 1.** Geographical repartition of the 175 *Plasmodium falciparum* isolates

Country	Isolates number	Percentage
Cameroon	47	26,9
Cote d'Ivoire	37	21,1
Central African Republic	18	10,3
Guinea	15	8,6
Republic of the Congo	14	8,0
Togo	8	4,6
Burkina Faso	7	4,0
Gabon	7	4,0
Benin	5	2,9
Chad	2	1,1
Comores	2	1,1
Ghana	1	0,6
Madagascar	2	1,1
Mali	2	1,1
Nigeria	2	1,1
Angola	1	0,6
Democratic Republic of the Congo	1	0,6
Djibouti	1	0,6
Senegal	1	0,6

415

416 **Figure 1.** Distribution of *ex vivo* responses (IC_{50}) of 175 African *Plasmodium falciparum*
417 isolates to piperazine
418
419 **Figure 2.** Copy number of the *plasmepsin 2* gene as a function of piperazine IC_{50} of 175
420 African *Plasmodium falciparum* isolates

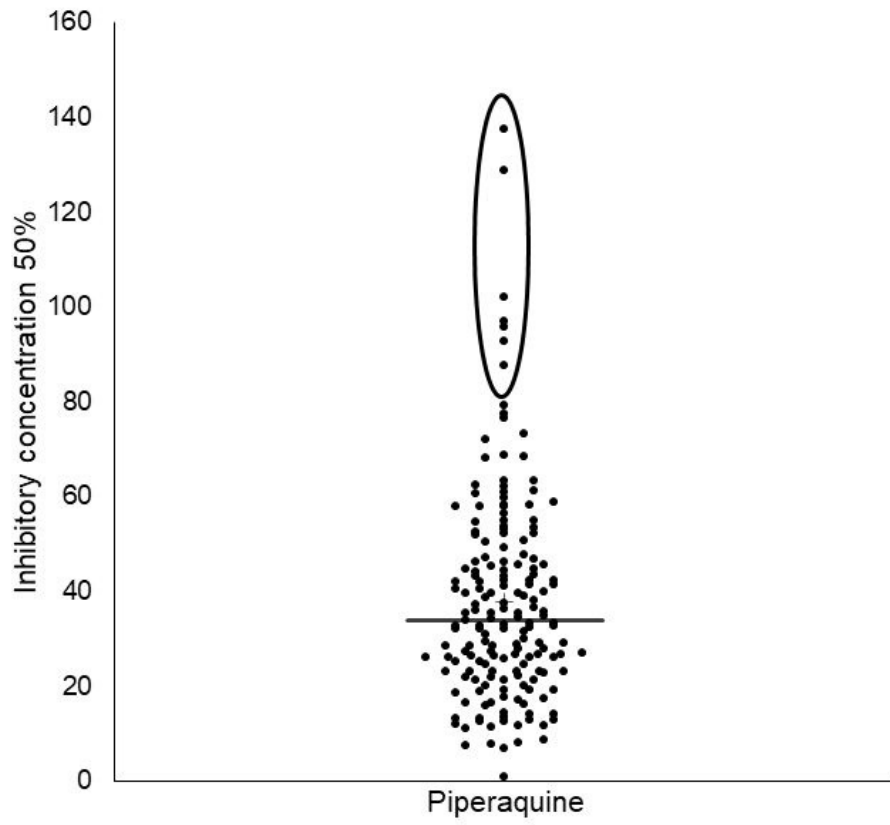


Figure 1. Distribution of *ex vivo* responses (IC₅₀) of 175 African *Plasmodium falciparum* isolates to piperazine

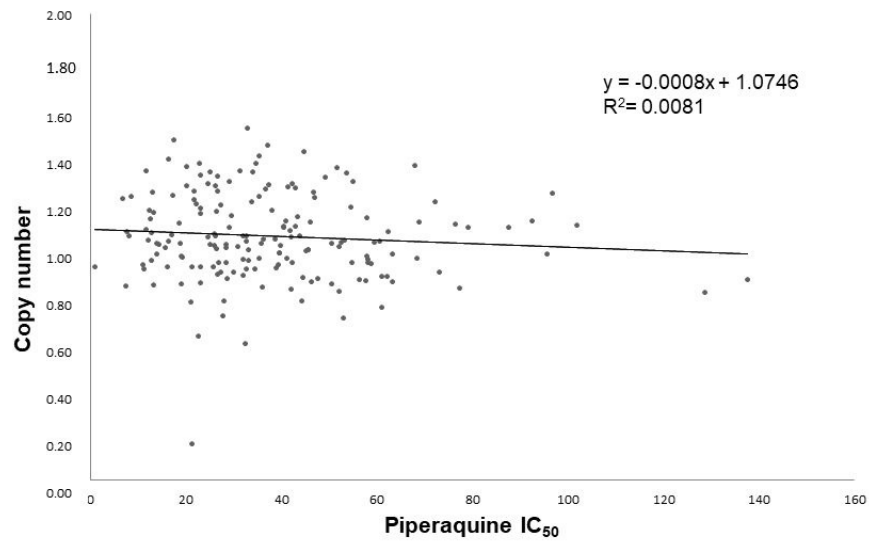


Figure 2. Copy number of the *plasmepsin 2* gene as a function of piperaquine IC₅₀ of 175 African *Plasmodium falciparum* isolates