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

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

Marie Gladys Robert,[a,b] Francis Foguim Tsombeng,[a,b] Mathieu Gendrot,[a,b] Joel Mosnier,[a,b,c] Rémy Amalvict,[a,b,c] Nicolas Benoit,[a,b,c] Marylin Torrentino-Madamet,[a,b,c] Bruno Pradines[a,b,c] and the French National Reference Centre for Imported Malaria Study Group

Unité Parasitologie et entomologie, Département des maladies infectieuses, Institut de Recherche Biomédicale des Armées, Institut hospitalo-universitaire (IHU) Méditerranée Infection, Marseille, France[a]; Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, IHU-Méditerranée Infection[b]; Centre National de Référence du Paludisme, Institut hospitalo-universitaire (IHU) Méditerranée Infection, Marseille, France[c]

Address for correspondence: Bruno Pradines, Unité Parasitologie et entomologie, Institut de recherche biomédicale des armées, Institut hospitalo-universitaire (IHU) Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France; Phone: 33 413 732 231; email: bruno.pradines@gmail.com

Abstract

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

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

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

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

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

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

Piperaquine (PPQ) for the *ex vivo* drug susceptibility assay was obtained from Shin Poong Pharm Co (Seoul, Korea). PPQ was first dissolved in methanol and later diluted in water to final concentrations that ranged from 1.9 to 998 nM. The isolates were incubated for 72 hours in a controlled atmosphere set at 85% N_2, 10% O_2, 5% CO_2 and 37°C (maximum final parasitemia at 0.5% and final hematocrit of 1.5%). The isolates with parasitemia above 0.5% were diluted to 0.5% with fresh uncontaminated erythrocytes. The drug susceptibility assay was revealed by the HRP2 ELISA-based assay implemented in the Malaria Ag Celisa kit (ref KM2159, Cellabs PTY LDT, Brookvale, Australia) as previously described (*14*). IC_{50} values were validated only if the optical density (OD) ratio (OD at zero concentration / OD at maximum concentration of drug) was above 1.6 and the 95% confident interval of the IC_{50} estimation was below 2.0. Each batch of plates was validated on the CQ-resistant W2 clone of Indochina strain (obtained from MR4, VA, USA) in four independent experiments using the same conditions as described below. The mean PPQ 50% inhibitory concentration (IC_{50}) values for the chloroquine-resistant W2 clone for the different batches used during the study was 54.1 ± 5.4 nM. There was no significant difference in the responses of the strains to PPQ according to the different batches ($p = 0.770$).

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

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

The *ex vivo* IC_{50} curves of PPQ were satisfactorily fit to a sigmoidal function. Unlike the cultured-adapted Cambodian parasites (16), none of our isolates exhibited a bimodal dose-response curve when exposed to PPQ. The PPQ IC_{50} of the 175 isolates ranged from 0.94 to 137.5 nM (Figure 1). A wide range of the PPQ responses was already observed in several studies and whatever the methodology used. PPQ IC_{50} values, assessed by a 72 hrs incubation 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 IC_{50} 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 IC_{50}, 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. IC_{50} and cutoff values for *in vitro* resistance are
115 specific to the methodology. The in vitro effects and the IC_{50} 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 IC_{50}, 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 IC_{50}.
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 (IC_{50} > 135 nM) (19). Another possible source of
129 variation in the PPQ drug responses could result from the range of parasitemias used in the

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

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

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

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

harbouring a single copy of *plasmepsin 2* gene for which a large range of PPQ IC_{50} was observed as well as some parasites with high IC_{50} (12). The use of the standard *ex vivo* assay and not the PPQ survival assay (PSA) can be questionable (11, 26). However, Amato et al identified the same association between *ex vivo* PPQ resistance and the amplification of the *plasmepsin 2* gene by using a standard susceptibility drug assay (12). The resistant parasites showed *ex vivo* IC_{50} values between 89.3 and 159.6 nM and *in vitro* IC_{50} values between 55.9 and 79.7 nM after cultures had adapted, reflecting a high PSA % survival that ranged from 52.2 to 74.9% (12). Additionally, a novel mutation (F145I) on the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) was recently identified and described to have an association with the PPQ IC_{90} values in Cambodian isolates by the standard *in vitro* assay (27). The influence of this mutation will have to be evaluated in African *P. falciparum* isolates.

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

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

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

Acknowledgments

The authors thank the patients and the staff of the hospitals of the French National Reference Center for Imported Malaria network.

The authors would like to thank Pr. Sandrine Houzé (Centre national de reference du Paludisme, Paris) for providing the clinical sample with two copies of *plasmepsin 2* gene.

This study was funded by the French Institute for Public Health Surveillance (Santé Publique France, grant CNR paludisme). Francis Foguim was supported by the Foundation Méditerranée Infection.

The authors have declared that they have no competing interests.

French National Reference Centre for Imported Malaria Study Group

D Basset (Centre Hospitalier Universitaire de Montpellier, Montpellier), P Bastien (Centre Hospitalier Universitaire de Montpellier, Montpellier), F Benoit-Vical (Centre Hospitalier

Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Universitaire de Rangueil, Toulouse), A Berry (Centre Hospitalier Universitaire de Rangueil, Toulouse), P Brouqui (Institut Hospitalo-Universitaire Méditerranée Infection, Marseille), M Cividin (Centre Hospitalier du Pays d'Aix, Aix en Provence), P Delaunay (Centre Hospitalier Universitaire de l'Archet, Nice), L Delhaes (Hôpital Pellegrin, Bordeaux), M Drancourt (Institut Hospitalo-Universitaire Méditerranée Infection, Marseille), T Gaillard (Hôpital d'Instruction des Armées Saint-Anne, Toulon), A Genin (Centre Hospitalier du Pays d'Aix, Aix en Provence), E Garnotel (Hôpital d'Instruction des Armées Laveran, Marseille), E Javelle (Hôpital d'Instruction des Armées Laveran, Marseille), C L'Ollivier (Institut Hospitalo-Universitaire Méditerranée Infection, Marseille), M Leveque (Centre Hospitalier Universitaire de Montpellier, Montpellier), D Malvy (Hôpital Pellegrin, Bordeaux), P Marty (Centre Hospitalier Universitaire de l'Archet, Nice), G Ménard (Hôpital d'Instruction des Armées Saint-Anne, Toulon), P Millet (Hôpital Pellegrin, Bordeaux), P Minodier (Hôpital Nord, Marseille), A Mottard (Hôpital de Fréjus-Saint Raphael, Fréjus), P Parola (Institut Hospitalo-Universitaire Méditerranée Infection, Marseille), R Piarroux (Hôpital la Timone, Marseille), C Pomares-Estran (Centre Hospitalier Universitaire de l'Archet, Nice), M-C Receveur (Hôpital Pellegrin, Bordeaux), A Robin (Centre Hospitalier du Pays d'Aix, Aix en Provence), E Sappa (Centre Hospitalier du Pays d'Aix, Aix en Provence), H Savini (Hôpital d'Instruction des Armées Laveran, Marseille), F Simon (Hôpital d'Instruction des Armées Laveran, Marseille), Y Sterkers (Centre Hospitalier Universitaire de Montpellier, Montpellier), C Surcouf (Hôpital d'Instruction des Armées Laveran, Marseille), E Varlet (Centre Hospitalier Universitaire de Montpellier, Montpellier), A Wolff (Hôpital d'Instruction des Armées Laveran, Marseille).

Ethics approval and consent to participate

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. Informed consent was not required for this study because the sampling procedures and testing are part of the French national recommendations for the care and surveillance of malaria.

References

1. Mita T, Venkatesan M, Ohashi J, Culleton R, Takahashi N, Tsukahara T, Ndounga M, Dysoley L, Endo H, Hombhanje F, Ferreira MU, Plowe CV, Tanabe K. 2011. Limited geographical origin and global spread of sulfadoxine-resistant *dhps* alleles in *Plasmodium falciparum* populations. J Infect Dis 204:1980-1988.
2. Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J, Baruch DI, Magill AJ, Su XZ. 2002. Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. Nature 418:320-323.
3. Dondorp AM., Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ. 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455-467.
4. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Seng S, Anderson JM, Mao S, Sam B, Sopha C, Chuor CM, Nguon C, Sovannaroth S, Pukrittayakamee S, Jttamala P, Chotivanich K, Chutasmit K, Suchatsoonthorn C, Runcharoen R, Hien TT, Thuy-Nhien NT, Thanh NV, Phu NH, Htut Y, Han KT, Aye KH, Mokuolu OA,

Olaosebikan RR, Folaranmi OO, Mayxay M, Khanthavong M, Hongvanthong B, Newton PN, Onyamboko MA, Fanello CI, Tshefu AK, Mishra N, Valecha N, Phyo AP, Nosten F, Yi P, Tripura R, Borrmann S, Bashraheil M, Peshu J, Faiz MA, Ghose A, Hossain MA, Samad R, Rahman MR, Hasan MM, Islam A, Miotto O, Amato R, MacInnis B, Stalker J, Kwiatkowski D, Bozdech Z, Jeeyapant A, Cheah PY, Sakulhaew T, Chalk J, Intharabut B, Silamut K, Lee SJ, Vihokhern B, Kunasol C, Imwong M, Tarning J, Taylor WJ, Yeung S, Woodrow CJ, Flegg JA, Des D, Smith J, Venkatesan M, Plowe CV, Stepniewska K, Guerin PJ, Dondorp AM, Day NP, White NJ. 2015. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 371:411-423.

5. Spring MD, Lin JT, Manning JE, Vanachayangkul P, Somethy S, Bun R, Se Y, Chann S, Ittiverakul M, Sia-ngam P, Kuntawunginn W, Arsanok M, Buathong N, Chaorattanakawee S, Gosi P, Ta-aksorn W, Chanarat N, Sundrakes S, Kong N, Heng TK, Nou S, Teja-isavadharm P, Pichyangkul S, Phann ST, Balasubramanian S, Juliano JJ, Meshnick SR, Chour CM, Prom S, Lanteri CA, Lon C, Saunders DL. 2015. Dihydroartemisinin-piperaquine failure associated with a triple mutant including kelch13 C580Y in Cambodia: an observational cohort study. Lancet Infect Dis 15:683-691.

6. Amaratunga C, Lim P, Suon S, Sreng S, Mao S, Sopha C, Sam B, Dek D, Try V, Amato R, Blessborn D, Song L, Tullo GS, Fay MP, Anderson JM, Tarning J, Fairhurst RM. 2016. Dihydroartemisinin-piperaquine resistance in *Plasmodium falciparum* malaria in Cambodia: a multisite prospective cohort study. Lancet Infect Dis 16:357-365.

7. Phuc BQ, Rasmussen C, Duong TT, Dong LT, Loi MA, Ménard D, Traning J, Bustos D, Ringwald P, Galappaththy GL, Thieu NQ. 2017. Treatment failure of dihydroartemisinin/piperaquine for *Plasmodium falciparum* malaria, Vietnam. Emerg Infect Dis 23:715-717.

278 8. Thanh NV, Thuy-Nhien N, Tuyen NT, Tong NT, Nha-Ca NT, Dong LT, Quang HH,
279 Farrar J, Thwaites G, White NJ, Wolbers M, Hien TT. 2017. Rapid decline in the
280 susceptibility of *Plasmodium falciparum* to dihydroartemisinin-piperaquine in the south
281 of Vietnam. Malar J 16:27.
282 9. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V,
283 Bouchier C, Ma L, Lim P, Leang R, Duong S, Sreng S, Suon S, Chuor CM, Bout DM,
284 Ménard S, Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A,
285 Barale JC, Fiarhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Ménard D. 2014. A
286 molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature
287 505:50-55.
288 10. Ménard D, Khim N, Beghain J, Adegnika AA, Alam MS, Amodu O, Awab Gr, Barnadas
289 C, Berry A, Boum Y, Bustos MD, Cao J, Chen JH, Collet L, Cui L, Das Thakur G, Dieye
290 A, Djalle D, Dorkenoo MA, Eboumbou Moukoko CE, Espino E, Fandeur T, Ferreira-Da-
291 Cruz Mf, Fola AA, Fuehrer HP, Hassan Am, Herrera S, Hongvanthong B, Houze **S**,
292 Karim MJ, Jiang L, Kano S, Khan WA, Khanthavong M, Kremsner PG, Lacerda M,
293 Leang R, Leelawong M, Li M, Lin K, Laminou IM, Mazarati JB, Menard S, Morlais I,
294 Muhindo Mavoko H**,** Musset L, Na-Bangchang K, Nambozi M, Niare K, Noedl H,
295 Ouedraogo JB, Pillai DR, Pradines B, Quang Phuc B, Ramharter M, Randrianarivelojosia
296 M, Sattabongkot J, Sheikh Omar R, Silue KD, Sirima SB, Sutherland C, Syafruddin D,
297 Tahar R, Tang LH, Toure OA, Tshibangu P, Vigan-Womas I, Warsame M, Wini L,
298 Zakeri S, Kim S, Eam R, Berne L, Khean C, Chy S, Ken M, Loch K, Canier L, Duru V,
299 Legrand E, Barale JC, Stokes B, Straimer J, Witkowski B, Fidock DA, Rogier C,
300 Ringwald P, Ariey F, Mercereau-Puijalon O; for the K13 Artemisinin Resistance
301 Multicenter Assessment consortium (KARMA). 2016. A worldwide map of *Plasmodium*
302 *falciparum* K13-propeller polymorphisms. N Engl J Med 374:2453-2464.

11. Witkowski B, Duru V, Khim N, Ross LS, Saintpierre B, Beghain J, Chy S, Kim S, Ke S, Kloeung N, Eam R, Khean C, Ken M, Loch K, Bouillon A, Domergue A, Ma L, Bouchier C, Leang R, Huy R, Nuel G, Barale JC, Legrand E, Ringwald P, Fidock DA, Mercereau-Puijalon O, Ariey F, Ménard D. 2017. A surrogate marker of piperaquine-resistant *Plasmodium falciparum* malaria: a phenotype-genotype association study. Lancet Infect Dis 17:174-183.
12. Amato R, Lim P, Miotto O, Amaratunga C, Dek D, Pearson RD, Almagro-Garcia J, Neal AT, Sreng S, Suon S, Drury E, Jyothi D, Stalker J, Kwiatkowski DP, Fairhurst RM. 2017. Genetic markers associated with dihydroartemisinin-piperaquine failure in *Plasmodium falciparum* malaria in Cambodia: a genotype-phenotype association study. Lancet Infect Dis 17:164-173.
13. Wurtz N, Fall B, Bui K, Pascual A, Fall M, Camara C, Diatta B, Ba Fall K, Saliou Mbaye P, Diémé Y, Bercion R, Wade B, Briolant S, Pradines B. 2013. *Pfhrp2* and *pfhrp3* polymorphisms in *Plasmodium falciparum* isolates from Dakar, Senegal: impact on rapid malaria diagnostic tests. Malar J 12:34.
14. Fall B, Madamet M, Camara C, Amalvict R, Fall M, Nakoulima A, Diatta B, Diémé Y, Wade B, Pradines B. 2016. *Plasmodium falciparum* in vitro resistance to monodesethylamodiaquine, Dakar, Senegal, 2014. Emerg Infect Dis 22:841-845.
15. Price RN, Uhlemann AC, Brockman A, McReady R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S. 2004. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet 364:438-447.
16. Bopp S, Magistrado P, Wong W, Schaffner SF, Mukherjee A, Lim P, Dhorda M, Amaratunga C, Woodrow CJ, Ashley EA, White NJ, Dondorp AM, Fairhurst RM, Ariey F, Menard D, Wirth DF, Volkman SK. 2018. *Plasmepsin II-III* copy number accounts for

bimodal piperaquine resistance among Cambodian *Plasmodium falciparum*. Nat Commun 9:1769.

17. Fall B, Camara C, Fall M, Nakoulima A, Dionne P, Diatta B, Diémé Y, Wade B, Pradines B. 2015. *Plasmodium falciparum* susceptibility to standard and potential anti-malarial drugs in Dakar, Senegal, during the 2013-2014 malaria season. Malar J 14:60.
18. Gendrot M, Diawara S, Madamet M, Kouta MB, Briolant S, Wade KA, Fall M, Benoit N, Nakoulima A, Amalvict R, Diémé Y, Fall B, Wade B, Diatta B, Pradines B. 2017. Association between polymorphisms in the *Pfmdr6* gene and ex vivo susceptibility to quinine in *Plasmodium falciparum* parasites from Dakar, Senegal. Antimicrob Agents Chemother 61:e01183-16.
19. Pascual A, Madamet M, Briolant S, Gaillard T, Amalvict R, Benoit N, Travers D, Pradines B. 2015. Multinormal *in vitro* distribution of *Plasmodium falciparum* susceptibility to piperaquine and pyronaridine. Malar J 14:49.
20. Tumwebase P, Conrad MD, Walakira A, LeClair N, Byaruhanga O, Nakazibwe C, Kozak B, Bloome J, Okiring J, Kakuru A, Bigira V, Kapisi J, Legac J, Gut J, Cooper RA, Kamya MR, Havlir DV, Dorsey G, Greehouse B, Nsobya SL, Rosenthal PJ. 2015. Impact of antimalarial treatment and chemoprevention on the drug sensitivity of malaria parasites isolated from Ugandan children. Antimicrob Agents Chemother 59:3018-30.
21. Rasmussen SA, Ceja FG, Conrad MD, Tumwebaze PK, Byaruhanga O, Katairo T, Nsobya SL, Rosenthal PJ, Cooper RA. 2017. Changing antimalarial drug sensitivities in Uganda. Antimicrob Agents Chemother 61:e01516-17.
22. Pradines b, Rogier C, Fusai T, Mosnier J, Daries W, Baret E, Parzy D. 2001. In vitro activities of antibiotics against *Plasmodium falciparum* are inhibited by iron. Antimicrob Agents Chemother 45:1746-1750.

23. Briolant S, Parola P, Fusai T, Madamet-Torrentino M, Baret E, Mosnier J, Delmont JP, Parzy D, Minodier P, Rogier C, Pradines B. 2007. Influence of oxygen on asexual blood cycle and susceptibility of *Plasmodium falciparum* to chloroquine: requirement of a standardized in vitro assay. Malar J 6:44.

24. Pascual A, Basco LK, Baret E, Amalvict R, Travers D, Rogier C, Pradines B. 2011. Use of the atmospheric generators for capnophilic bacteria Genbag-CO2 for the evaluation of in vitro *Plasmodium falciparum* susceptibility to standard anti-malarial drugs. Malar J 10:8.

25. Wein S, Maynadier M, Tran Van Ba C, Cerdan R, Peyrottes S, Fraisse L, Vial H. 2010. Reliability of antimalarial sensitivity tests depends on drug mechanisms of action. J Clin Microbiol 48:1651-1660.

26. Duru V, Khim N, Leang R, Kim S, Domergue A, Kloeung N, Ke S, Chy S, Eam R, Khean C, Loch K, Ken M, Lek D, Beghain J, Ariey F, Guerin PJ, Huy R, Mercereau-Puijalon O, Witkowski B, Ménard D. 2015. *Plasmodium falciparum* dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations. BMC Med 13:305.

27. Agrawal S, Moser KA, Morton L, Cummings MP, Parihar A, Dwivedi A, Shetty AC, Drabek EF, Jacob CG, Henrich PP, Parobek CM, Jongsakul K, Huy R, Spring MD, Lanteri CA, Chaorattanakawee S, Lon C, Fukuda MM, Saunders DL, Fidock DA, Lin JT, Juliano JJ, Plowe CV, Silva JC, Takala-Harrison S. 2017. Association of a novel mutation in the *Plasmodium falciparum* chloroquine transporter with decreased piperaquine sensitivity. J Infect Dis 216:468-476.

28. Conrad MD, Mota D, Foster M, Tukwasibwe S, Legac J, Tumwebaze P, Whalen M, Kakuru A, Nayebare P, Wallender E, Havlir DV, Jagannathan P, Huang L, Aweeka F,

376 Kamya MR, Dorsey G, Rosenthal PJ. 2017. Impact of intermittent preventive treatment
377 during pregnancy in *Plasmodium falciparum* drug resistance-mediating polymorphisms
378 in Uganda. J Infect Dis 216:1008-17.
379 29. Malvy D, Torrentino-Madamet M, L'Ollivier C, Receveur MC, Jeddi F, Delhaes L,
380 Piarroux R, Millet P, Pradines B. 2018. *Plasmodium falciparum* recrudescence two years
381 after treatment of an uncomplicated infection without return to an area where malaria is
382 endemic. Antimicrob Agents Chemother 62:e01892-17.
383 30. Gupta H, Macete E, Bulo H, Salvador C, Warsame M, Carvalho E, Ménard D, Ringwald
384 P, Bassat Q, Enosse S, Mayor A. 2018. Drug-resistant polymorphisms and copy numbers
385 of *Plasmodium falciparum*, Mozambique, 2015. Emerg Infect Dis 24:40-48.
386 31. Madamet M, Kouta MB, Wade KA, Lo G, Diawara S, Fall M, Bercion R, Nakoulima A,
387 Fall KB, Benoit N, Gueye MW, Fall B, Diatta B, Pradines B. 2017. Absence of
388 association between polymorphisms in the K13 gene and the presence of *Plasmodium*
389 *falciparum* parasites at day 3 after treatment with artemisinin derivatives in Senegal. Int J
390 Antimicrob Agents 49:754-756.
391 32. Dieye B, Affara M, Sangare L, Joof F, Ndiaye YD, Gomis JF, Ndiaye M, Mbaye A,
392 Diakite M, Sy N, Mbengue B, Deme AB, Daniels R, Ahouidi AD, Dieye T, Abdullahi A,
393 Doumbia S, Ndiaye JL, Diarra A, Ismaela A, Coulibaly M, Welty C, Ngwa AA, Shaffer
394 J, D'Alessandro U, Volkman SK, Wirth DF, Krogstad DJ, Koita O, Nwakanma D,
395 Ndiaye D. 2016. West Africa international centers of excellence for malaria research:
396 drug resistance patterns to artemether-lumefantrine in Senegal, Mali, and The Gambia.
397 Am J Trop Med Hyg 95:1054-1060.
398 33. Plucinski MM, Dimbu PR, Macaia AP, Ferreira CM, Samutondo C, Quivinja J, Afonso
399 M, Kiniffo R, Mbounga E, Kelley JS, Patel DS, He Y, Talundzic E, Garrett DO, Halsey
400 ES, Udhayakumar V, Ringwald P, Fortes F. 2017. Efficacy of artemether-lumefantrine,

artesunate-amodiaquine, and dihydroartemisinin-piperaquine for treatment of uncomplicated *Plasmodium falciparum* malaria in Angola, 2015. Malar J 16:62.

34. Plucinski MM, Talundzic E, Morton L, Dimbu PR, Macaia AP, Fortes F, Goldman I, Lucchi N, Stennies G, MacArthur JR, Udhayakumar V. 2015. Efficacy of artemether-lumefantrine and dihydroartemisinin-piperaquine for treatment of uncomplicated malaria in children in Zaire and Uíge Provinces, Angola. Antimicrob Agents Chemother 59:437-443.

35. Sutherland CJ, Lansdell P, Sanders M, Muwanguzi J, van Schalkwyk DA, Kaur H, Nolder D, Tucker J, Bennett HM, Otto TD, Berriman M, Patel TA, Lynn R, Gkrania-Klotsas E, Chiodina PL. 2017. pfk13-independent treatment failure in four imported cases of *Plasmodium falciparum* malaria treated with artemether-lumefantrine in the United Kingdom. Antimicrob Agents Chemother 61:e02382-16.

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 piperaquine

418

419 **Figure 2.** Copy number of the *plasmepsin 2* gene as a function of piperaquine IC_{50} of 175

420 African *Plasmodium falciparum* isolates

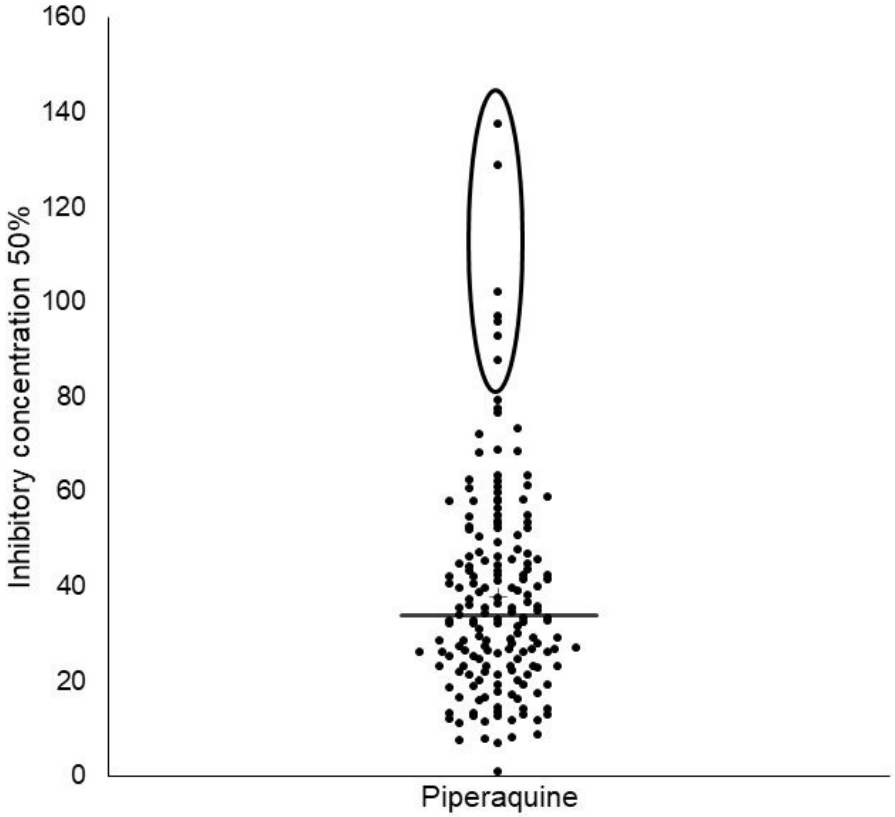

Figure 1. Distribution of *ex vivo* responses (IC_{50}) of 175 African *Plasmodium falciparum* isolates to piperaquine

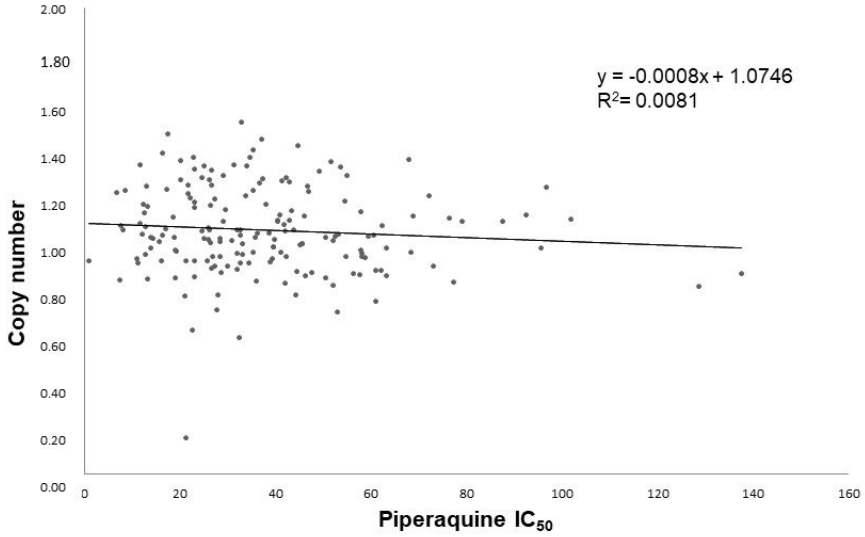

Figure 2. Copy number of the *plasmepsin 2* gene as a function of piperaquine IC_{50} of 175 African *Plasmodium falciparum* isolates