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African isolates show a high proportion of multiple copies of the *Plasmodium falciparum* *plasmepsin-2* gene, a piperazine resistance marker

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Abstract

Background: Today, the development of new and well-tolerated anti-malarial drugs is strongly justified by the emergence of *Plasmodium falciparum* resistance. In 2014–2015, a phase 2b clinical study was conducted to evaluate the efficacy of a single oral dose of Artefenomel (OZ439)–piperazine (PPQ) in Asian and African patients presenting with uncomplicated falciparum malaria.

Methods: Blood samples collected before treatment offered the opportunity to investigate the proportion of multidrug resistant parasite genotypes, including *P. falciparum* *kelch13* mutations and copy number variation of both *P. falciparum* *plasmepsin 2* (*Pfpm2*) and *P. falciparum* *multidrug resistance 1* (*Pfmdr1*) genes.

Results: Validated *kelch13* resistance mutations including C580Y, I543T, P553L and V568G were only detected in parasites from Vietnamese patients. In Africa, isolates with multiple copies of the *Pfmdr1* gene were shown to be more frequent than previously reported (21.1%, range from 12.4% in Burkina Faso to 27.4% in Uganda). More strikingly, high proportions of isolates with multiple copies of the *Pfpm2* gene, associated with piperazine (PPQ) resistance, were frequently observed in the African sites, especially in Burkina Faso and Uganda (> 30%).

Conclusions: These findings were considered to sharply contrast with the recent description of increased sensitivity to PPQ of Ugandan parasite isolates. This emphasizes the necessity to investigate in vitro susceptibility profiles to PPQ of African isolates with multiple copies of the *Pfpm2* gene and estimate the risk of development of PPQ resistance in Africa.

Trial registration Clinicaltrials.gov reference: NCT02083380. Study title: Phase II efficacy study of artefenomel and piperazine in adults and children with *P. falciparum* malaria. <https://clinicaltrials.gov/ct2/results?cond=&term=NCT02083380&cntry=&state=&city=&dist=>. FSFV: 23-Jul-2014; LSLV: 09-Oct-2015

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Background

Emergence of *Plasmodium falciparum* resistance to anti-malarial drugs is currently the primary rationale supporting the development of new and well-tolerated drugs. While the estimated number of malaria cases in the world decreased from 237 million (218–278 million) in 2010, to 211 million (192–257 million) in 2015, the morbidity and the mortality have stabilized in 2016 with estimates of 216 million cases (196–263 million) and 445,000 deaths (compared to 446,000 in 2015) as reported by the World Health Organization (WHO) [1–3].

Globally, the vast majority of deaths (>90%) caused by malaria is due to *P. falciparum* infections, occurring in Africa, in children under 5 years of age. Artemisinin-based combination therapy (ACT) which are currently recommended as first-line treatment of uncomplicated falciparum malaria, is less effective in Southeast Asia, particularly in Cambodia, where high rates of treatment failure associated with artemisinin and piperazine resistance are currently reported [4–16]. The containment and the elimination of these multidrug resistant parasites in Southeast Asia are a priority for the WHO to avoid their spread to Africa as was the case with previous generations of anti-malarial drugs (e.g. chloroquine, sulfadoxine–pyrimethamine) [17]. Fortunately, molecular markers associated with such resistance are available [10]. In particular, mutations in the propeller domain of a *kelch* gene located on the chromosome 13 (*kelch13*), and amplification of a cluster of genes encoding both *plasmepsin 2* (*Pfpm2*) and *plasmepsin 3* proteins, have been recently shown to be associated with artemisinin and PPQ resistance, respectively [18–20].

According to the latest WHO update on artemisinin resistance [21], to be validated a *kelch13* resistance mutant has to be correlated with delayed parasite clearance in clinical studies and reduced drug in vitro susceptibility with survival rate $\geq 1\%$ expressed by the Ring-stage Survival Assay, (RSA0–3 h) in fresh isolates (ex vivo assays), or culture-adapted field parasites or *kelch13* genome-edited parasites (in vitro assays) [22–25]. To date, only nine *kelch13* mutations have been shown to be validated (C580Y, Y493H, R539T, I543T, N458Y, P533L, M476I, R561H and F446I). The F446I mutant is highly prevalent in Myanmar as recently reported [26]. In Africa, a broad array of rare non-synonymous mutations in the *kelch13* gene have been described in *P. falciparum* isolates, but none of these mutants have been associated with artemisinin resistance [27], attesting that not all non-synonymous *kelch13* mutations confer resistance to artemisinin.

More recently, resistance to PPQ has been associated with an increase of survival rates of parasite exposed to 200 nM PPQ for 48 h in the piperazine survival assay

(PSA) and with the amplification of *plasmepsin 2–3* genes (*Pfpm2–3*) [6, 20]. In Cambodia, where high rates of treatment failure to dihydroartemisinin–piperazine (DHA–PPQ) are observed (i.e. >60% in some provinces), it has been demonstrated that amplification of *Pfpm2* gene and presence of validated *kelch13* mutations were highly predictive of DHA–PPQ treatment failure [20]. Most of these parasites harbour a single copy of *Pfmdr1* gene leading to the recovery of mefloquine sensitivity [4, 6] and suggesting a natural antagonism between PPQ resistance and mefloquine resistance. However, so far it was not understood whether *Pfmdr1* de-amplification (from multiple copies to single copy *Pfmdr1*) was due to the implementation of DHA–PPQ as first-line treatment or due to the release of mefloquine pressure and an increase in parasite fitness accompanying *Pfmdr1* gene de-amplification. To date, DHA–PPQ resistance was confined to Southeast Asia. So far, only few studies conducted on parasites from Mozambique and Mali have provided evidence of the presence (at low frequency, 1.1%, 10%) of parasites carrying multiple copies of *Pfpm2* [28, 29].

Facing the threat of losing all current artemisinin-based combinations front-line therapies due to resistance, a new generation of endoperoxides with more favourable pharmacokinetic profiles like the ozonide Artefenomel® (OZ439) have been developed [30]. The efficacy of this new chemical entity was evaluated in combination with PPQ in African and Southeast Asian (Vietnam) patients with uncomplicated falciparum malaria infection [30]. The primary objective of this phase 2b clinical study was to determine whether a single oral dose combination of artefenomel/PPQ was efficacious and safe [$\geq 95\%$ of patients cured on the basis of polymerase chain reaction (PCR)-adjusted adequate clinical parasitological response at day 28 (ACPR28)] in adults and children infected by *P. falciparum*. Blood samples collected in 2014–2015 from this clinical trial offered the opportunity to investigate the proportion of multidrug resistant parasites (i.e. *P. falciparum kelch13* mutants and gene copy number of both *Pfpm2* and *Pfmdr1*). Here, the occurrence of such genotypes from these samples is reported and a map of potential risk of emergence of resistance to the main front-line therapies currently used to treat malaria-infected patients and to the next generation of anti-malarial combinations is provided.

Methods

Study design, study sites and population

The study was a randomized, double-blind, single-dose design to investigate the efficacy, safety, tolerability and pharmacokinetics of artefenomel 800 mg in loose combination with three doses of PPQ phosphate (640 mg,

960 mg, 1440 mg) in male and female patients aged ≥ 6 months to < 70 years, with uncomplicated falciparum malaria in Africa and Southeast Asia (Vietnam), as previously described [31]. This study was conducted in 13 sites, including Burkina Faso (three sites, $N=127$), Uganda (one site, $N=124$), Benin (one site, $N=1$), the Democratic Republic of Congo (one site, $N=5$), Gabon (two sites, $N=94$), Mozambique (one site, $N=14$), and Vietnam (four sites, $N=83$). A total of 448 patients were randomized into each of three treatment arms: artefenomel 800 mg/PPQ 640 mg ($N=148$), artefenomel 800 mg/PPQ 960 mg ($N=151$) and artefenomel 800 mg/PPQ 1440 mg ($N=149$). Patients presenting *P. falciparum* mono-infection confirmed by microscopy to be in the range of 1000 to 100,000 asexual parasites/ μL of blood, and with fever (axillary temperature ≥ 37.5 °C) or reported fever episodes in the preceding 24 h, were included in the study after having submitted their written informed consent/assent. The following important exclusion criteria were considered: presence of severe malaria (WHO definition), haemoglobin below 8 g/dL, known history or evidence of clinically significant cardiac disorder, including QTcF or QTc B > 450 ms, or family history of sudden death or clinical conditions known to prolong QTc, clinically significant hepatic dysfunction and prior anti-malarial treatment within a specified time windows. After the drugs administration, patients were followed for 42 days or 63 days at some centres. Patients remained in the clinical unit for a minimum of 48 h (African patients > 5 years old) or 72 h (African patients ≤ 5 years old and all Asian patients) and were discharged on the basis of absence of detectable parasites and fever.

DNA extraction

Plasmodium falciparum DNA was extracted from dried blood spots using the QIAamp DNA Mini kit (Qiagen, Germany), according to the manufacturer's instructions. Samples were screened to confirm the presence of *P. falciparum* DNA using first a qualitative real-time PCR assay targeting the *Plasmodium cytochrome b* gene and secondly on positive samples, four real-time PCR assays specifically amplifying *P. falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* [32].

Detection of *kelch13* mutations

Plasmodium falciparum positive samples were tested for the presence of mutations in the propeller domain of the *kelch13* gene (PF3D7_1343700) that have been associated with artemisinin resistance [19]. Amplification of the Kelch-propeller domain (codons 440–680, 720 bp) was performed as previously described [27]. Cross-contamination was evaluated by adding no template samples (dried blood spots negative for *P. falciparum*) in

each PCR run. PCR products were sequenced by Macrogen (Seoul, Korea). Electropherograms were analysed on both strands, using PF3D7_1343700 as the reference sequence. The quality of the procedure was assessed by including dried blood spots with known *kelch13* mutations (wild-type, C580Y, R539T, I543T, Y493H) which were tested blindly in the same batches (each 96-well) with the test samples. Isolates with mixed alleles were considered as mutant. Following WHO recommendations, *kelch13* mutants were classified in three groups: (i) wild-type group (parasites with no synonymous or non-synonymous mutations compared to 3D7 sequence), (ii) *kelch13* validated (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, C580Y) and candidate mutation (P441L, G339A, V568G, P574L, A675V) group, and (iii) other *kelch13* mutants group (parasites with synonymous or non-synonymous mutations not present in the *kelch13* validated and candidate resistance mutation group).

Pfpm2 and *Pfmdr1* gene copy number variation assessment

Pfpm2 (PF3D7_1408000) and *Pfmdr1* (PF3D7_0523000) gene copy number were measured by qPCR using a CFX96 real-time PCR machine (Bio-Rad, France), relative to the single copy of the β -*tubulin* gene (used as reference gene), as previously described [20]. Amplification was carried out in triplicate. In each amplification run, six replicates using DNA from 3D7 parasite reference clone and three replicates without template (water) used as negative controls were included. Copy numbers were calculated using the formula: copy number = $2^{-\Delta\Delta C_t}$, with $\Delta\Delta C_t$ denoting the difference between ΔC_t of the unknown sample and ΔC_t of the reference sample (3D7). Specificities of *Pfpm2* and *Pfmdr1* amplification curves were evaluated by visualizing the melt curves. Multiple copies vs single copy, of both *Pfmdr1* and *Pfpm2*, were defined as copy numbers < 1.5 and ≥ 1.5 respectively.

Statistical analysis

Data were recorded and analyzed using Excel software and MedCalc (MedCalc Software, Belgium). Groups were compared using the Chi squared test or the Fisher's exact test. All reported *P*-values are two-sided and were considered statistically significant if < 0.05 .

Results

The *P. falciparum* samples collected from patients before treatment and yielding a successful result, by country and molecular assay, are presented in Table 1.

Global genotypes overview

Among the 68 Southeast Asian clinical isolates collected in Vietnam with available data, 67.6% (46/68) were found to harbour parasites with validated or candidate *kelch13*

Table 1 Number of isolates collected from each site in Southeast Asia (Vietnam) and Africa and number and proportion of successful molecular tests

Sites	No. isolates	No. of successful tests (%)		
		<i>kelch 13</i>	<i>Pfpm2</i>	<i>Pfmdr1</i>
Southeast Asia				
Gai Lai	18	13 (72)	18 (100)	18 (100)
Binh Phuoc	30	26 (87)	28 (93)	28 (93)
Quang Tri	1	1 (100)	1 (100)	1 (100)
Khanh Hao	34	28 (82)	32 (94)	32 (94)
Africa				
Benin	1	1 (100)	0 (0)	0 (0)
Burkina Faso	127	114 (90)	105 (83)	105 (83)
DR Congo	5	4 (80)	2 (40)	2 (40)
Gabon	94	83 (88)	71 (76)	72 (77)
Mozambique	14	14 (100)	8 (57)	12 (86)
Uganda	124	116 (94)	112 (90)	113 (91)
Total	448	400 (89)	377 (84)	383 (85)

resistance mutations (Table 2). Details regarding *kelch13* mutants according to the collection sites are presented in Table 3. By contrast, none of the 332 isolates collected from African patients and successfully tested were found to carry validated or candidate *kelch13* resistance mutations.

Significant difference in proportion of isolates with multiple copies *Pfmdr1* were found between Africa (21.1%, 64/304, 95% CI 16.2–26.9%) and Asia (6.3%, 5/79, 95% CI 2.0–14.8%, $P=0.002$, Table 2). Parasites with multiple copies of *Pfpm2* were observed in 11 Asian samples (13.9%, 11/79, 95% CI 6.9–24.9%) and unexpectedly at higher proportion in African isolates (26.8%, 80/298, 95% CI 21.3–33.4%, $P=0.02$, Table 2). However, multiple copies of *Pfpm2*/single copy *Pfmdr1*, hypothesized to favour resistance to PPQ, were found at similar proportion in a small cohort of 10 Asian isolates (12.7%, 10/79, 95% CI 6.1–23.3%) and 47 African samples (15.8%, 47/298, 95% CI 11.6–21.0%, $P=0.72$, Table 2 and Fig. 1).

In Asia, seven isolates (10.8%, 7/65, 95% CI 4.3–22.2%) had genotypes associated with both artemisinin and PPQ resistance (i.e. with *kelch13* validated and candidate resistance mutations, and multiple copy *Pfpm2*/single copy *Pfmdr1*) (Fig. 2a). In Africa, no clinical isolates had mutations conferring both artemisinin and PPQ resistance due to the absence of *kelch13* mutant-type parasites (Fig. 2b).

Southeast Asian (Vietnamese) genotypes

kelch13 validated and candidate mutations were detected in >60% of the isolates in all sites (from 61.1% in Gai Lai to 73.0% in Binh Phuoc) except Quang Tri (where only one sample was collected) (Table 3). C580Y was the most predominant *kelch13* validated and candidate mutation

Table 2 Distribution (number and proportion) of genotypes (*kelch13* mutations, *Pfmdr1* and *Pfpm2* gene copy numbers) detected in *Plasmodium falciparum* isolates collected from Southeast Asia and Africa in 2014–2015

Locus	Allele/haplotype	Number of isolates (%) detected in		P value
		Asia (N = 82)	Africa (N = 355)	
<i>kelch 13</i>	ART	46 (67.6)	0 (0.0)	< 10 ⁻⁴ ±
	OTH	1 (1.5)	10 (3.0)	
	WT	21 (30.9)	322 (97.0)	
<i>Pfpm2</i>	Single copy	68 (86.1)	218 (73.2)	0.02 ^Y
	Multiple copies	11 (13.9)	80 (26.8)	
<i>Pfmdr1</i>	Single copy	74 (93.7)	240 (78.9)	0.002 ^Y
	Multiple copies	5 (6.3)	64 (21.1)	
<i>Pfpm2</i> / <i>Pfmdr1</i>	Single copy/single copy	64 (81.0)	189 (63.4)	0.009±
	Single copy/multiple copies	4 (5.1)	29 (9.7)	
	Multiple copies/single copy	10 (12.7)	47 (15.8)	
	Multiple copies/multiple copies	1 (1.3)	33 (11.1)	
<i>kelch 13</i> / <i>Pfpm2</i> / <i>Pfmdr1</i>	ART/multiple copies/single copy	7 (10.8)	0 (0.0)	< 10 ⁻⁴ ±
	WT/multiple copies/single copy	2 (3.0)	43 (14.7)	
	ART/others	38 (57.5)	0 (0.0)	
	WT/others	18 (27.3)	241 (82.5)	

ART: validated or candidate *kelch 13* mutations; WT: *kelch 13* Wild type, OTH: *kelch 13* mutations with unknown association with artemisinin resistance (detailed in Tables 3 and 4)

Italic font denotes the allele or haplotype associate with drug resistance. P-value (Chi squared test± or Fischer exact test^Y)

Table 3 Distribution (number and proportion) of genotypes (*kelch13* mutations, *Pfmdr1* and *Pfpm2* gene copy numbers) detected in *Plasmodium falciparum* isolates collected in four sites located in Southeast Asia in 2014–2015

Locus	Allele/haplotype	Site							
		Gai Lai		Binh Phuoc		Quang Tri		Khanh Hao	
		N	%	N	%	N	%	N	%
<i>kelch 13</i>	ART								
	C580Y	3	23.1	16	61.5	0		6	21.4
	C580Y + P553L	0		0		0		2	7.1
	I543T	0		1	3.8	0		0	
	P553L	4	30.8	2	7.7	0		11	39.3
	V568G	1	7.7	0		0		0	
	OTH C469P	1	7.7	0		0		0	
<i>Pfpm2</i>	WT	4	30.8	7	26.9	1	100	9	32.1
	Single copy	15	83.3	20	71.4	1	100	32	100
<i>Pfmdr1</i>	Multiple copies	3	16.7	8	28.6	0		0	
	Single copy	16	88.9	27	96.4	1	100	30	93.8
<i>Pfpm2/Pfmdr1</i>	Multiple copies	2	11.1	1	3.6	0		2	6.3
	Single copy/single copy	14	77.8	19	67.9	1	100	30	93.8
	Single copy/multiple copies	1	5.6	1	3.6	0		2	6.3
	Multiple copies/single copy	2	11.1	8	28.6	0		0	
<i>kelch 13/Pfpm2/Pfmdr1</i>	Multiple copies/multiple copies	1	5.6	0		0		0	
	ART								
	Single copy/single copy	7	53.8	10	41.7	0		18	64.3
	Single copy/multiple copies	0		1	4.2	0		1	3.6
	Multiple copies/single copy	0		7	29.2	0		0	
	Multiple copies/multiple copies	1	7.7	0	–	0		0	
	OTH								
	Single copy/single copy	–		0		0		0	
	Single copy/multiple copies	1	7.7	0		0		0	
	Multiple copies/single copy	–		0		0		0	
	Multiple copies/multiple copies	–		0		0		0	
	WT								
	Single copy/single copy	2	15.4	6	25	1	100	8	28.6
Single copy/multiple copies	–		0		0		1	3.6	
Multiple copies/single copy	2	15.4	0		0		0		
Multiple copies/multiple copies	–		0		0		0		

ART: validated or candidate *kelch 13* mutations; WT: *kelch 13* Wild type, OTH: *kelch 13* mutations with unknown association with artemisinin resistance

Italic font denotes the allele or haplotype associate with drug resistance

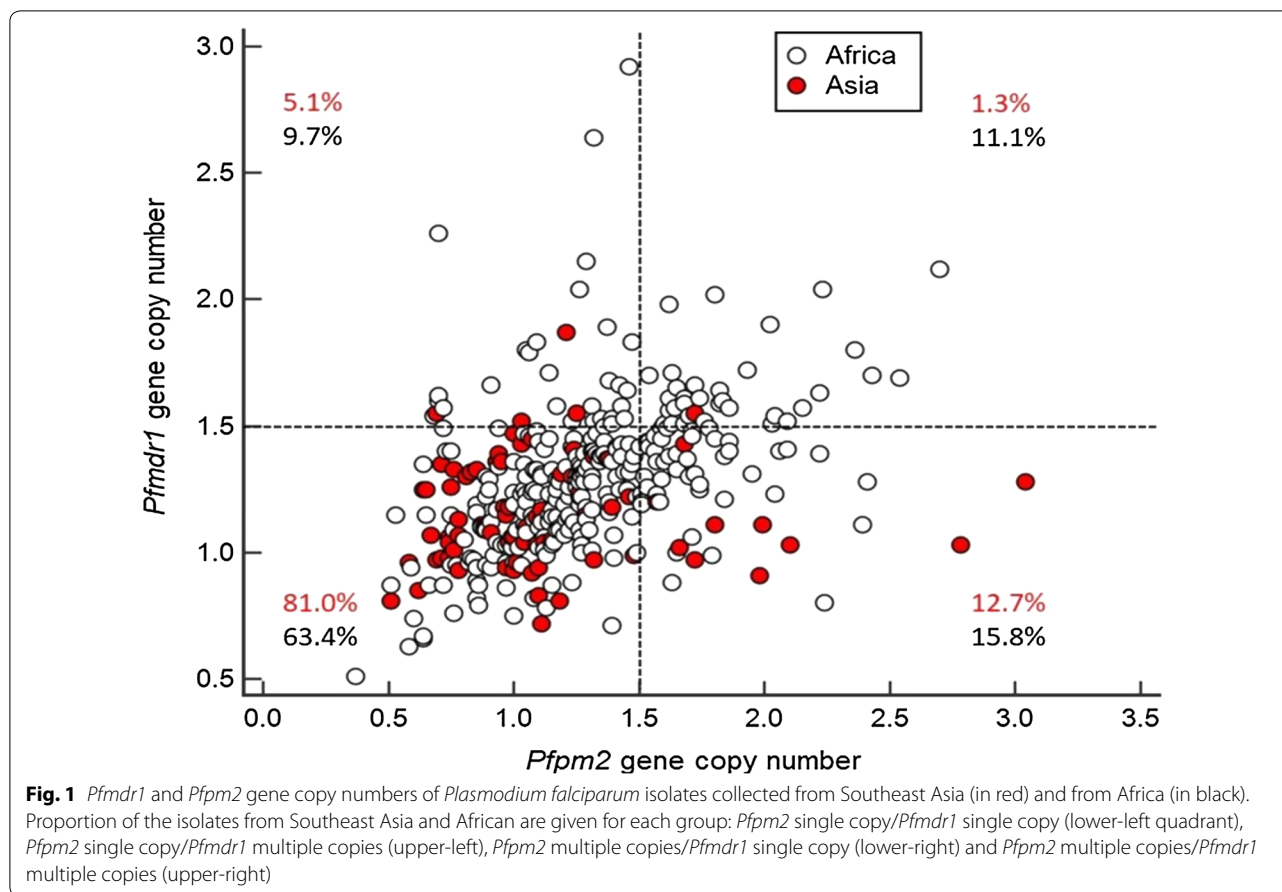
(54.3%, 25/46, 95% CI 25.2–80.2%) followed by P553L (37.0%, 17/46, 95% CI 21.5–59.2%), I543T (2.2%, 1/46, 95% CI 0.5–12.1%) and G568G (2.2%, 1/46, 95% CI 0.5–12.1%). In Khanh Hao, two isolates were found to have both C580Y and P553L single mutant parasites (likely from a polyclonal infection).

Isolates with multiple copies of *Pfpm2* were detected only in two sites located along the Cambodian border: in Gai Lai (16.7%, 3/18, 95% CI 3.4–48.7%) and in Binh Phuoc (28.6%, 8/28, 95% CI 12.3–56.3%). No parasites with multiple copies were detected out of 32 isolates in

Khanh Hao. Parasites with a single copy of *Pfmdr1* were frequent (>88%) in samples collected from all four study sites (from 88.9% in Gai Lai to 100% in Quang Tri).

Parasites with multiple copies *Pfpm2*/single copy *Pfmdr1* were observed in 10/79 (12.7%, 95% CI 6.1–23.3%) of the isolates collected from Vietnamese patients, representing in Gai Lai 11.1% (2/18, 95% CI 1.4–40.1%) and in Binh Phuoc 28.6% (8/28, 95% CI 12.3–56.3%).

Isolates with genotype conferring both artemisinin and PPQ resistance (i.e. with *kelch13* validated and candidate mutations, and multiple copy *Pfpm2*/single copy *Pfmdr1*)



were only observed in patients enrolled in Binh Phuoc (29.2%, 7/24, 95% CI 11.7–60.0%).

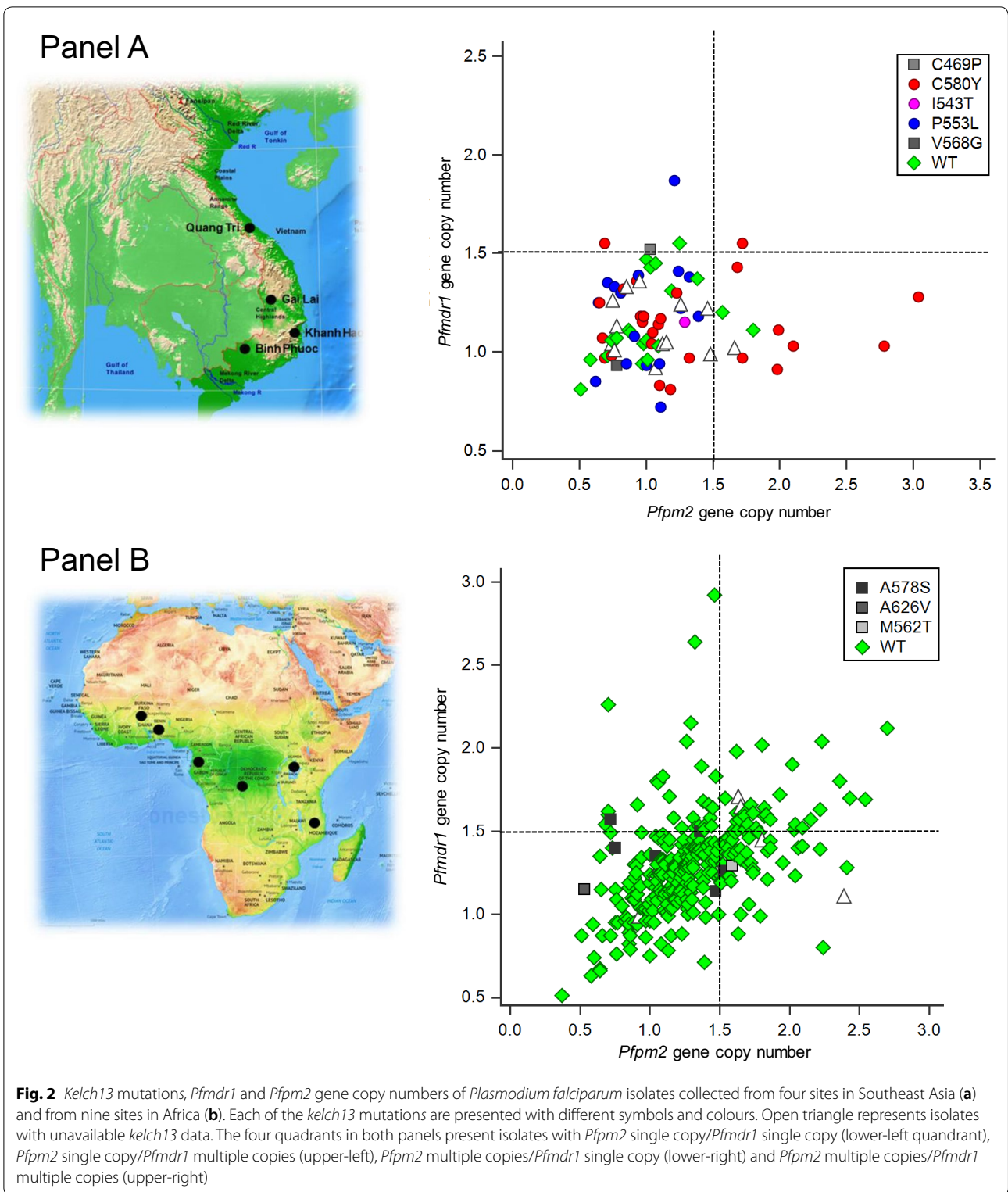
African genotypes

No *kelch13* validated and candidate mutations was detected at any site. Other non-synonymous mutations were observed: A578S was the most predominant *kelch13* mutation (7/10; 3 in Uganda, 2 in Gabon, 1 in Mozambique and 1 in Burkina Faso) followed by Y541F, M562T and A626V (only detected once in isolates from Burkina Faso) (Table 4).

Isolates from Uganda and Burkina Faso showed an unexpected high frequency of parasites with multiple copies of *Pfpm2* (33.9%, 38/112, 95% CI 24.0–46.6% and 30.5%, 32/105, 95% CI 20.9–43.0%, respectively). Samples from Gabon and Mozambique had a lower frequency of multiple copies of *Pfpm2* estimated at 11.3% (8/71, 95% CI 4.9–22.2%) and 12.5% (1/8, 95% CI 0.3–69.6%), respectively. Of note, in the Democratic Republic of Congo, results from two isolates were available and one isolate was found to carry parasites with multiple copies of *Pfpm2*.

Parasites with single copy *Pfmdr1* were detected in almost all isolates in patients enrolled across the six African sites, therefore, only 13/105 (12.4%, 95% CI 6.6–21.2%) isolates from Burkina Faso, 2/12 (16.7%, 95% CI 2.0–60.2%) from Mozambique, 17/72 (23.6%, 95% CI 13.8–37.8%) from Gabon and 31/113 (27.4%, 95% CI 18.6–38.9%) from Uganda had multiple copies of *Pfmdr1*. One out of two patients harboured parasites with multiple copies of *Pfmdr1* in DRC.

Parasites with multiple copies *Pfpm2*/single copy *Pfmdr1* were observed at a frequency of 20.9% (22/105, 95% CI 13.1–31.7%) in Burkina Faso, 18.8% (21/112, 95% CI 11.6–28.7%) in Uganda, 12.5% (1/8, 95% CI 0.3–69.7%) in Mozambique and 4.3% (3/71, 95% CI 0.9–12.5%) in Gabon. However, isolates with genotype conferring both artemisinin and PPQ resistance (i.e. with *kelch13* validated and candidate mutations, and multiple copy *Pfpm2*/single copy *Pfmdr1*) were not observed in patients enrolled in Africa since there were no *kelch13* validated and candidate mutations.



Discussion

The current phase 2b clinical study of artefenomel, an ozonide showing improved pharmacokinetics

properties compared to artemisinins, combined with PPQ was designed to assess the efficacy of single oral doses in patients with uncomplicated falciparum

Table 4 Distribution (number and proportion) of genotypes (*kelch13* mutations, *Pfmdr1* and *Pfpm2* gene copy numbers) detected in *Plasmodium falciparum* isolates collected in nine sites located in Africa in 2014–2015

Locus	Allele/haplotype	Site											
		BEN		BF		DRC		GAB		MOZ		UGA	
		N	%	N	%	N	%	N	%	N	%	N	%
<i>kelch 13</i>	ART	0		0		0		0		0		0	
	OTH												
	A578S	0		1	0.875	0		2	2.4	1	7.1	3	2.6
	Y541F	0		1	0.875	0		0		0		0	
	M562T	0		1	0.875	0		0		0		0	
	A626V	0		1	0.875	0		0		0		0	
	WT	1	100	110	96.5	4	100	81	97.6	13	92.9	113	97.4
<i>Pfpm2</i>	Single copy	0		73	69.5	1	50	63	88.7	7	87.5	74	66.1
	Multiple copies	0		32	30.5	1	50	8	11.3	1	12.5	38	33.9
<i>Pfmdr1</i>	Single copy	0		92	87.6	1	50	55	76.4	10	83.3	82	72.6
	Multiple copies	0		13	12.4	1	50	17	23.6	2	16.7	31	27.4
<i>Pfpm2/Pfmdr1</i>	Single copy/single copy	0		70	66.7	1	50	51	71.8	7	87.5	60	53.6
	Single copy/multiple copies	0		3	2.9	0		12	16.9	0		14	12.5
	Multiple copies/single copy	0		22	20.9	0		3	4.3	1	12.5	21	18.8
	Multiple copies/multiple copies	0		10	9.4	1	50	5	7	0		17	15.1
<i>kelch 13/Pfpm2/Pfmdr1</i>	ART												
	Single copy/single copy	0		0		0		0		0		0	
	Single copy/multiple copies	0		0		0		0		0		0	
	Multiple copies/single copy	0		0		0		0		0		0	
	Multiple copies/multiple copies	0		0		0		0		0		0	
	OTH												
	Single copy/single copy	0		0		0		0		1	12.5	2	1.9
	Single copy/multiple copies	0		1	1	0		1	1.5	0		1	0.9
	Multiple copies/single copy	0		2	1.9	0		0		0		0	
	Multiple copies/multiple copies	0		0		0		0		0		0	
	WT												
	Single copy/single copy	0		69	66.3	1	50	51	71.8	6	75	56	52.3
	Single copy/multiple copies	0		3	2.9	0		11	15.5	0		13	12.1
Multiple copies/single copy	0		20	19.2	0		3	4.2	1	12.5	19	17.8	
Multiple copies/multiple copies	0		9	8.7	1	50	5	7	0		16	15	

Countries: BEN: Benin; BF: Burkina Faso; DRC: Democratic Republic of Congo; GAB: Gabon; MOZ: Mozambique; UGA: Uganda. ART: validated or candidate *kelch13* mutations; WT: *kelch 13* Wild type, OTH: *kelch13* mutations with unknown association with artemisinin resistance

Italic font denotes the allele or haplotype associate with drug resistance

malaria in Southeast Asia (Vietnam) and Africa. In addition to the clinical outcome assessment, three molecular markers associated with drug resistance for mapping the potential risks of future treatment failures were investigated in isolates collected before treatment. The frequency of *kKelch13* mutations associated with artemisinin resistance, and *Pfmdr1* and *Pfpm2* genes copy number were measured in available isolates collected from all clinical sites. Artemisinin resistance was confirmed to be still confined in Southeast Asia. A high

proportion of *kelch13* validated and candidate resistance mutations were observed as well as a new unreported one (C469P) in Vietnamese parasites and the complete absence of these mutants in African isolates. As previously reported [27, 33, 34], a low proportion of *kelch13* mutations was detected in African samples and all these mutations have not been shown to be associated to artemisinin resistance [27].

However, a higher proportion (threefold) of parasites with multiple copies of *Pfmdr1*, a gene encoding a drug

efflux pump, was observed in African samples compared to Southeast Asian isolates. This observation contrasts with previous reports showing high frequency of parasites with multiple copies of *Pfmdr1* in Asia [35–37] compared to Africa [38–40]. These findings likely reflect the profiles of evolution of *P. falciparum* populations linked to anti-malarial drug pressure in both continents. Especially, the prevalence of high *Pfmdr1* amplification observed in Africa might be linked with the routine use of artemether–lumefantrine as first-line treatment for more than a decade. Indeed, increased *pfmdr1* copy number is known to modulate parasite responses to a wide range of drugs including lumefantrine [38, 41, 42]. Supporting this expectation, it seems feasible that such parasites exposed to lumefantrine as monotherapy for several days following clearance of artemether have been selected, while parasites with a single copy have been eliminated. In contrast, the low prevalence of *Pfmdr1* multiple copies observed in Southeast Asia could be due to the recent implementation of DHA–PPQ, the removal of the mefloquine drug pressure or both, as the case in Cambodia [18, 20, 43]. Resistance to piperazine and resistance to mefloquine have been shown to be mutually exclusive in South East Asia. A possible inverse susceptibility mechanism could be at the origin of this observation.

High frequency of isolates with multiple copies of the *Pfpm2* has already been reported in recent studies conducted in Cambodia [18, 20, 43]. As the Vietnamese clinical sites (Gai Lai and Binh Phuoc) are located alongside the Cambodian border (Fig. 2), data from this study might reflect an evolving situation where the amplification of *Pfpm2* is spreading beyond Cambodia, as described recently [5, 7]. To date, frequencies observed in Vietnamese isolates are not yet as high as the ones observed in Cambodia but might continue to increase in the future.

Unexpectedly, in African isolates, amplification of *Pfpm2* gene was shown to occur at a much higher frequency (~27% on average across clinical sites in Africa, reaching 30.5% in Burkina Faso and 33.9% in Uganda) than was recently described (from 11.1 to 13.8% in Uganda, 10% in Mali and 1.1% in Mozambique) [28, 29, 44]. Considering the geographical extent and the diversity of the clinical sites in Africa, the high frequency reported at sites distant to each other suggests that amplification of *Pfpm2* gene occurred independently in each site. More importantly, since in Southeast Asia most parasites with multiple copies of *Pfpm2* also display *kelch13* resistance mutations, which is not the case in African samples, it is likely that *Pfpm2* amplification originated in Africa, independently of Southeast Asia.

Unfortunately, *in vitro* or *ex vivo* drug susceptibility assays were not possible and association between

Pfpm2 amplification and clinical resistance to PPQ was not verifiable in the current study. An evaluation is currently ongoing to see whether, and if so to what extent, these markers of artemisinin and PPQ resistance affected the parasite clearance half-life (PCT_{1/2}) and PCR-adjusted 28 days follow-up in patients treated with artemether/PPQ (study MMV OZ439 13,003). However, it was recently reported that compared to drug sensitivities measured on Ugandan isolates from 2010 to 2013 (from the same site, namely Tororo), those measured in 2016 to chloroquine, amodiaquine, and PPQ were increased by 7.4, 5.2 and 2.5-fold, respectively [44]. This longitudinal study showed that rather than drug resistance developing to these three anti-malarial drugs, an increase in sensitivity was observed that was correlated with low prevalence of the polymorphisms recently associated with resistance to artemisinins or PPQ. Indeed, clinical resistance to DHA–PPQ has not yet been reported in Africa [45]. Although, the possibility that parasites showing amplification of *Pfpm2* observed in the current study are resistant to PPQ without confirmation of *in vitro* or *ex vivo* phenotypes cannot be excluded, data reported by Rasmussen et al. [44] suggest that significant occurrence of clinical resistance to PPQ is unlikely. In other words, in Africa it is unclear whether the amplification of *Pfpm2* is necessary and/or sufficient for the development of resistance to PPQ. The ongoing analysis relating the markers of resistance to clinical outcome may provide some insights regarding this question. Until very recently, it was still debated whether additional genetic modifications in the *P. falciparum* chloroquine resistance transporter (*Pfcr*) gene are required to confer such resistance [46, 47]. Recent genomic and biological investigations have revealed a rapid increase in the prevalence of novel *Pfcr* mutations in Cambodia (H97Y, F145I, M343L, and G353V). These mutants (from culture-adapted Cambodian field isolates or Dd2 gene-edited clones) were confirmed to confer PPQ resistance as determined using the PSA^{0–3h} [6, 48].

Conclusions

At present, several artemisinin-based combinations are used in Africa and Asia to treat patients with uncomplicated malaria. Artemether–lumefantrine (AL), artesunate–amodiaquine (AS–AQ), artesunate–mefloquine (AS–MQ), artesunate–sulfadoxine–pyrimethamine (AS–SP), dihydroartemisinin–piperazine (DHA–PPQ) and pyronaridine–artesunate (PA) achieve more than 95% efficacy in clinical trials based on PCR-adjusted Day 28 ACPR. Due to the long post treatment prophylaxis of the well-tolerated PPQ, DHA–PPQ is currently under evaluation in a number

of interventions, such as intermittent preventive treatment in pregnant women or in infants (IPTp, IPTi) and mass drug administration campaigns (MDA) in Africa. As a key surveillance goal, it is therefore of particular importance to continue following the evolution of *Pfpm2* amplification along with mutations in the *Pfprt* gene and to investigate whether these genetic signatures are associated with PPQ resistance in Africa.

Authors' contributions

FM, DL, BL, RK, NK, EL and DM substantially contributed to analysis and interpretation of data. DM, RK, NK, and EL substantially contributed to acquisition of data. YA, SO, AB, GMN, JMNN, RV, YD, AKT, TTD and PBQ were responsible for the prospective collection of biological samples for this study. FM substantially contributed to conception and design. DL and DM drafted the manuscript. All authors contributed to critically reviewing of the manuscript. The authors agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

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Competing interests

FM and DL are employees of Medicines for Malaria Venture. None of the other authors declare competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request.

Consent for publication

No details relating to individual participants are presented in this manuscript.

Ethics approval and consent to participate

The study (MMV OZ439 13 003) conformed to the Declaration of Helsinki and Standard Operating Procedures that meet current regulatory requirements and guidelines laid down by the International Conference on Harmonization for Good Clinical Practice in Clinical Studies, and approved by the relevant

Independent Ethics Committees (IEC), National Institutional Review Boards and where relevant, local regulatory authorities at each of the participating sites. Patients and parents or guardians of participants provided written informed consent prior to enrollment. Study related information was provided in the participants' local languages. The study protocol was registered and the study results are reported on clinicaltrials.gov (NCT02083380).

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