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A surrogate marker of piperaquine-resistant *Plasmodium falciparum* malaria: a phenotype–genotype association study

Benot Witkowski*, Valentine Duru*, Nimol Khim, Leila S Ross, Benjamin Saintpierre, Johann Beghain, Sophy Chy, Saarin Kim, Sophieavatey Ke, Nimol Kloeung, Rotha Eam, Chanra Khean, Malen Ken, Kaknika Loch, Anthony Bouillon, Anais Domergue, Laurence Ma, Christiane Bouchier, Ritheo Leang, Rekol Huy, Grégory Nuel, Jean-Christophe Barale, Eric Legrand, Pascal Ringwald, David A Fidock, Odile Mercereau-Puijalon, Frédéric Ariey, Didier Ménard

**Summary**

**Background** Western Cambodia is the epicentre of *Plasmodium falciparum* multidrug resistance and is facing high rates of dihydroartemisinin–piperaquine treatment failures. Genetic tools to detect the multidrug-resistant parasites are needed. Artemisinin resistance can be tracked using the K13 molecular marker, but no marker exists for piperaquine resistance. We aimed to identify genetic markers of piperaquine resistance and study their association with dihydroartemisinin–piperaquine treatment failures.

**Methods** We obtained blood samples from Cambodian patients infected with *P falciparum* and treated with dihydroartemisinin–piperaquine. Patients were followed up for 42 days during the years 2009–15. We established in-vitro and ex-vivo susceptibility profiles for a subset using piperaquine survival assays. We determined whole-genome sequences by Illumina paired-reads sequencing, copy number variations by qPCR, RNA concentrations by qRT-PCR, and protein concentrations by immunoblotting. Fisher’s exact and non-parametric Wilcoxon rank-sum tests were used to identify significant differences in single-nucleotide polymorphisms or copy number variants, respectively, for differential distribution between piperaquine-resistant and piperaquine-sensitive parasite lines.

**Findings** Whole-genome exon sequence analysis of 31 culture-adapted parasite lines associated amplification of the *plasmepsin 2–plasmepsin 3* gene cluster with in-vitro piperaquine resistance. Ex-vivo piperaquine survival assay profiles of 134 isolates correlated with *plasmepsin 2* gene copy number. In 725 patients treated with dihydroartemisinin–piperaquine, multicopy *plasmepsin 2* in the sample collected before treatment was associated with an adjusted hazard ratio (aHR) for treatment failure of 20·4 (95% CI 9·1–45·5, *p*<0·0001). Multicopy *plasmepsin 2* predicted dihydroartemisinin–piperaquine failures with 0·94 (95% CI 0·88–0·98) sensitivity and 0·77 (0·74–0·81) specificity. Analysis of samples collected across the country from 2002 to 2015 showed that the geographical and temporal increase of the proportion of multicopy *plasmepsin 2* parasites was highly correlated with increasing dihydroartemisinin–piperaquine treatment failure rates (r=0·89 [95% CI 0·77–0·95], *p*<0·0001, Spearman’s coefficient of rank correlation). Dihydroartemisinin–piperaquine efficacy at day 42 fell below 90% when the proportion of multicopy *plasmepsin 2* parasites exceeded 22%.

**Interpretation** Piperaquine resistance in Cambodia is strongly associated with amplification of *plasmepsin 2–3* encoding haemoglobin-digesting proteases, regardless of the location. Multicopy *plasmepsin 2* constitutes a surrogate molecular marker to track piperaquine resistance. A molecular toolkit combining *plasmepsin 2* with *K13* and *mdr1* monitoring should provide timely information for antimalarial treatment and containment policies.

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**Introduction** Antimalarial efficacy of artemisinin-based combination therapies, the first-line treatment for uncomplicated *Plasmodium falciparum* malaria, relies on both fast-acting artemisinin derivatives and long-lasting partner drugs. Resistance to artemisinin, which is now fixed in western Cambodia and observed across southeast Asia, increases the proportion of parasites surviving a 3 day course of an artemisinin-based combination therapy. Resistance to the partner drug is a greater risk when more parasites survive artemisinin treatment. The reduced efficacy of artemisinin derivatives and partner drugs translates into late treatment failures and prolonged parasite carriage.
Dihydroartemisinin–piperaquine failure rates have increased in western Cambodia since 2010 and in eastern Cambodia since 2014. They are caused by parasites that are resistant to both artemisinin and piperaquine. Combined analysis of K13 polymorphisms and plasmpesin 2 copy number represents the first informative molecular signature for dihydroartemisinin–piperaquine failures. These molecular markers can now be used to track emergence and dissemination of resistance to artemisinin and piperaquine in field populations, especially in areas where piperaquine is being or will be recommended in combination with artemisinin derivatives as first-line treatment or in preventive treatment for infants or pregnant women, as developed in African settings.

**Added value of this study**

We identified amplification of the plasmpesin 2–3 gene cluster encoding proteases involved in haemoglobin degradation as the most significant molecular signature associated with in-vitro resistance to piperaquine assessed using the piperaquine survival assay. Using a large longitudinal collection of samples collected during clinical efficacy studies of dihydroartemisinin–piperaquine done across Cambodia since 2009, we examined 725 P falciparum isolates and found that an increased plasmpesin 2 gene copy number was strongly associated with dihydroartemisinin–piperaquine treatment failures. Patients harbouring multicopy plasmpesin 2 parasites had a 20 times higher risk of recrudescence during the 42-day post-treatment follow-up (94% sensitivity and 77% specificity). Our retrospective analysis of samples collected in Cambodia during the last decade before and after implementation of dihydroartemisinin–piperaquine as first-line treatment showed that the proportion of multicopy plasmpesin 2 parasites correlated with the increase of dihydroartemisinin–piperaquine treatment failure rates, from 2009 to 2015 in western Cambodia and during 2014–15 in eastern Cambodia. In areas of artemisinin resistance, the clinical efficacy of dihydroartemisinin–piperaquine at day 42 fell under 90% when the local proportion of multicopy plasmpesin 2 parasites rose above 22%.

**Research in context**

**Evidence before this study**

We searched PubMed for studies on piperaquine resistance using the term “resistance” in combination with “falciparum” and “piperaquine” on May 19, 2016, without any date or language restrictions, and identified 74 publications. These publications included clinical trials done in 11 countries evaluating the efficacy of dihydroartemisinin–piperaquine for the treatment of uncomplicated Plasmodium falciparum malaria (26 reports) or asymptomatic infections (one report) and for intermittent preventive treatment of pregnant women (three reports) or infants (five reports). In all studies, cure rates were above 90%, except studies done in Cambodia after the year 2010, for which cure rates ranging from 85% to 40% were recorded. Overall, 26 publications reported susceptibility of parasites collected in 15 countries, studied using in-vitro or ex-vivo assays. Virtually all isolates tested by standard dose–response susceptibility assays (with parasite quantification based on isolates, Sybr Green, or HRP2) were susceptible to piperaquine (<100 nmol/L), except those collected in Cambodia after 2010 and samples collected in China before 1998 (when piperaquine monotherapy was intensively used). Piperaquine resistance at present appears confined to Cambodia. Resistance is a major concern because alternative therapeutic options are scarce and the reduced cure rates translate into prolonged parasite carriage and increased transmission potential of resistant parasites. To map the geographical extension of piperaquine resistance and deploy containment measures to prevent its further spread, rapid detection tests are needed but are lacking at present. Potential molecular signatures associated with piperaquine resistance were investigated in 11 studies. The only consistently recorded finding was an increased proportion of single copy mdr1 parasites in piperaquine-resistant areas. This marker is not informative for piperaquine resistance because wild-type susceptible parasites can also have a single-copy mdr1 locus.

**Added value of this study**

We identified amplification of the plasmpesin 2–3 gene cluster encoding proteases involved in haemoglobin degradation as thereby increasing the transmission potential of drug-resistant infections. In Cambodia, artesunate–mefloquine was chosen as the first-line drug in 2001. By 2008, the high frequency of treatment failures in western provinces, the epicentre of P falciparum multidrug resistance, led to its replacement with dihydroartemisinin–piperaquine in those areas in 2008, and later throughout Cambodia in 2010. In recent years the spread of artemisinin-resistant P falciparum, from western Cambodia to neighbouring provinces, has been followed by a substantial increase in dihydroartemisinin–piperaquine failure rates. Failures are estimated to reach 60%, indicating a dramatic expansion of piperaquine resistance. Until now, the detection of piperaquine resistance has been based on logistically demanding 42-day follow-up studies of patients treated with dihydroartemisinin–piperaquine. The in-vitro piperaquine survival assay (PSA) has been shown with in-vitro culture-adapted parasites and freshly collected ex-vivo patient isolates to detect piperaquine resistance and treatment failure more reliably than classic dose–response assays. The in-vitro PSA therefore provides a reliable tool to identify molecular signatures associated with resistance.

Here, we used the phenotypic information from the PSA to identify genetic marker(s) of piperaquine resistance and study their association with dihydroartemisinin–piperaquine treatment failures.
Methods
Overview
First, the exomes of culture-adapted artemisinin-resistant Cambodian P falciparum lines defined as piperaquine-susceptible or piperaquine-resistant based on their PSA survival rates were compared for single-nucleotide polymorphisms (SNPs) and copy number variations (CNVs). This process identified an increased copy number of the plasmepsin 2–plasmepsin 3 gene cluster as a putative genetic signature associated with in-vitro piperaquine resistance. Increased plasmepsin 2 gene copy number was then assessed as a candidate resistance marker in isolates with documented ex-vivo PSA survival rates and in blood samples collected during the years 2009–15 from Cambodian patients treated with dihydroartemisinin–piperaquine and followed up for 42 days. Finally, we investigated the geographical and temporal distribution of multicopy plasmepsin 2 parasites in the country from 2002 to 2015 and its correlation with dihydroartemisinin–piperaquine treatment failures.

Study sites and patients
Patients with P falciparum malaria were enrolled in clinical studies done at health centres located across Cambodia during the years 2009–15 (table 1, figure 1). After obtaining written informed consent, patients were treated with dihydroartemisinin–piperaquine (Duo-Cotexin [dihydroartemisinin 40 mg and piperaquine 320 mg], Zhejiang Holley Nanhu Pharmaceutica Co Ltd, Jiaxing City, Zhejiang Province, China) and followed up for 42 days, as previously described. The endpoint to assess the efficacy of dihydroartemisinin–piperaquine was the proportion of PCR-corrected recrudescent P falciparum infections at day 42. All studies were approved by the Ethical Committee for Health Research of the Cambodian Ministry of Health. Clinical trials were registered at the Australian New Zealand Clinical Trials Registry (numbers ACTRN 12615000793516, 12612000184875, 12612000183886, 12612000181808, and 12614000344695).

Procedures
Blood samples were collected into acid-citrate-dextrose tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) before treatment and sent to Institut Pasteur in Cambodia within 24 h. A subset of freshly collected samples was used to do the ex-vivo PSA. All samples were cryopreserved in glycerolyte. Red cell pellets were stored at −20°C for molecular studies. Blood spots were prepared on day 0 and when applicable on the day of recrudescence.

Cryopreserved parasites were culture-adapted as described. Susceptibility to piperaquine was investigated using in-vitro PSA for culture-adapted parasites and ex-vivo PSA for fresh isolates. Survival rates were assessed microscopically and parasites with a survival rate of at least 10% were considered piperaquine-resistant. msp1, msp2, and glurp polymorphisms were determined to distinguish recrudescent from new infections. Sequencing of the K13-propeller domain was used to screen for artemisinin resistance. Whole-genome sequencing was done with Illumina paired-reads sequencing. Data were integrated into the Whole-genome Data Manager database and exomes of piperaquine-resistant and piperaquine-sensitive lines were compared after excluding low-coverage positions (ie, lower than 25% of the genome-wide mean coverage). Genes from highly variable multigene families (var, rifin, phist, and stevor) were excluded. SNPs and CNVs were investigated using PlasmoCNVScan and the Phen2gen software (appendix). Plasmepsin 2 and mdr1

| Year | Site Location | Number of isolates with in-vitro PSA survival data (n=31) | Number of isolates with ex-vivo PSA survival data (n=134) |
|------|--------------|-------------------------------------------------|-------------------------------------------------|
| 2009 | Palin         | 32                                               | 0                                               |
|      | Preah Vihear  | 30                                               | 0                                               |
| 2010 | Palin         | 21                                               | 4 (19%)                                         |
|      | Pursat        | 32                                               | 3 (9.4%)                                        |
|      | Rattanakiri   | 30                                               | 0                                               |
| 2011 | Kratié        | 51                                               | 2 (3.9%)                                        |
|      | Preah Vihear  | 34                                               | 2 (5.9%)                                        |
|      | Pursat        | 41                                               | 7 (17.1%)                                       |
| 2012 | Battambang    | 39                                               | 12 (30.8%)                                      |
|      | Kampong Speu  | 22                                               | 0                                               |
|      | Kampong Thom  | 38                                               | 0                                               |
|      | Pursat        | 23                                               | 2 (8.7%)                                        |
| 2013 | Kampot        | 17                                               | 1 (5.9%)                                        |
|      | Kratié        | 22                                               | 0                                               |
|      | Preah Vihear  | 16                                               | 1 (6.3%)                                        |
|      | Rattanakiri   | 31                                               | 1 (3.2%)                                        |
| 2014 | Mondulkiri    | 39                                               | 4 (10.3%)                                       |
|      | Siemneap      | 40                                               | 25 (62.5%)                                      |
|      | Stungtreng    | 33                                               | 11 (33.3%)                                      |
|      | Rattanakiri   | 34                                               | 5 (14.7%)                                       |
| 2015 | Mondulkiri    | 16                                               | 4 (25.0%)                                       |
|      | Rattanakiri   | 54                                               | 16 (29.6%)                                      |
|      | Siemneap      | 17                                               | 10 (58.8%)                                      |
|      | Stungtreng    | 13                                               | 6 (46.1%)                                       |

Site location and years of collection are provided for isolates with in-vitro and ex-vivo piperaquine survival assay (PSA) profiles (see figure 1 for a map of the study site locations). Data are n or n (%)
copy number was determined by qPCR (appendix). Steady-state plasmepsin 2 mRNA concentrations were measured by RT-qPCR (appendix) and plasmepsin 2 protein expression by immunoblotting (appendix).

Statistical analysis
Data were analysed with MedCalc version 12 (Mariakerke, Belgium). Kruskal-Wallis or Mann-Whitney tests were used for non-parametric comparisons and Student’s t test or one-way ANOVA were used for parametric comparisons. For proportions (expressed with percentages and 95% CIs), we used $\chi^2$ or Fisher’s exact tests. Manhattan plots were generated using the SNPEVG software.11 We did an SNP-wise analysis using a homemade script developed by FA and BS and used Fisher’s exact test to identify significant SNP differences between piperaquine-resistant and piperaquine-sensitive parasite lines. We tested CNVs for differential distribution between piperaquine-resistant and piperaquine-sensitive parasite lines using a non-parametric Wilcoxon rank-sum test. The Bonferroni and the Benjamini-Hochberg corrections were used to assess genome-wide significance and adjust p values when statistical tests were done simultaneously on a single dataset (appendix). Relative risks were estimated using the Mantel-Haenszel test. Associations between a cumulative risk of failure at day 42 and molecular signatures associated with piperaquine resistance were assessed by survival analysis. Curves were compared with the Mantel-Haenszel log-rank test. The Cox proportional-hazards regression model was used to assess the association between parasite genotypes ($K13$ mutations, plasmepsin 2, and $mdr1$ copy number), sampling locations, and treatment responses. A linear regression analysis was used to assess the association between the efficacy of dihydroartemisinin–piperaquine and the proportion of parasites with multicopy plasmepsin 2. We deemed p values of less than 0.05 as significant.

Role of the funding source
The funders of this study had no role in study design, data collection, data analysis, data interpretation, writing of the report, and the decision to submit. The corresponding author had full access to all data in the study and final responsibility for the decision to submit for publication.

Results
From Sept 15, 2009, to Feb 23, 2015, 725 patients were enrolled in clinical studies to assess the efficacy of the standard 3-day dihydroartemisinin–piperaquine treatment. By 2015, the cumulative proportion of $P$ falciparum recrudescence at day 42 after PCR correction was 16.4% (119 of 725 patients), ranging from 0% to 62.5% depending on the site and the year of study (table 1, figure I). Whole-genome sequences were obtained from 31 artemisinin-resistant ($K13$ C580Y mutant) culture-adapted parasite lines collected in Cambodia.
in 2012, including 23 piperazine-resistant and eight piperazine-sensitive lines as defined by their in-vitro PSA survival rates (table 2). We recorded 120691 exomic (coding sequence) SNPs. Genome-wide association analyses of SNPs identified significant differences between resistant and sensitive lines at two positions located in adjacent genes on chromosome 4: position 896588 of PF3D7_0420000 (encoding a putative zinc-finger protein; p<0·56×10⁻⁹, Fisher’s exact test; p=0·042 after Bonferroni correction) and position 908385 of PF3D7_0420100 (encoding a Rio2 Ser–Thr protein kinase; p<0·35×10⁻⁷, Fisher’s exact test; p=0·042 after Bonferroni correction). However, these positions (and indeed the sequences of both genes) were ambiguous with variable proportions of wild-type and mutant nucleotides, precluding identification of specific resistance-associated mutations, and were not studied further (appendix).

By contrast, signals of gene amplification were detected in the piperazine-resistant group for two adjacent genes from the cluster located on chromosome 14 that encode haemoglobin-digesting proteases known as plasmepsins (p=0·01795 Wilcoxon test with Benjamini-Hochberg correction; figure 2, table 3). Irrespective of piperazine susceptibility, all plasmepsin 3 sequences were wild type and all plasmepsin 2 sequences had a Q442H plasmepsin 2 polymorphism, which has been frequently recorded in reference laboratory lines or wild isolates. The correlation between in-vitro PSA survival rates and plasmepsin 2–3 copy number was highly significant (r=0·83 [95% CI 0·67–0·91], p<0·0001 for plasmepsin 2 copy number and r=0·85 [0·71–0·93], p<0·0001 for plasmepsin 3 copy number). We recorded three different DNA expansion profiles (table 2, appendix). In-vitro PSA survival rates were significantly lower in parasites harbouring DNA expansion type 2 (n=6, median PSA survival rate 34·1% [IQR 25–84–40–0]) compared with those harbouring DNA expansion type 1 (n=13, median PSA survival rate 51·8% [IQR 45·6–61·7], p=0·006, Mann-Whitney test) or type 3 (n=3, median PSA survival rate 58·7%, p=0·02, Mann-Whitney test).

Conversely, a cluster of five genes on chromosome 5 (PF3D7_0531700, PF3D7_0522900, PF3D7_0523000, PF3D7_0523100, and PF3D7_0523200), which included mdr1, had increased copy numbers in sensitive lines. mdr1 was amplified in five of eight piperazine-sensitive lines but in none of the 23 piperazine-resistant lines (p=0·015, Wilcoxon test; appendix).

To confirm the association between plasmepsin CNV and ex-vivo PSA survival rate, we used plasmepsin 2 as an amplicon reporter. First, we optimised a qPCR method to assess plasmepsin 2 gene copy number (appendix). Plasmepsin 2 copy number detected by qPCR was 100% concordant with the whole-genome sequencing estimates for the 31 culture-adapted parasites (p=0·0001, Fisher’s test). From a set of 134 isolates with known ex-vivo PSA profiles, plasmepsin 2 was amplified in 67 of 69 piperazine-resistant parasites (50, 15, and two isolates with two, three, or four plasmepsin 2 copies, respectively), and zero of 65 piperazine-susceptible parasites (figure 3). The median ex-vivo PSA survival rate was significantly higher in isolates with at least two plasmepsin 2 copies compared with those with unamplified plasmepsin 2 (51·7% [IQR 29·7–75·1] vs 0·004% [0·003–0·39]; p<0·0001, Mann-Whitney test). An increased plasmepsin 2 copy number predicted ex-vivo piperazine resistance with a sensitivity of 0·97 (95% CI

| Year | Site location | In-vitro PSA survival rate (%) | In-vitro susceptibility to PPQ* | DNA expansion type† |
|------|---------------|-------------------------------|-------------------------------|-------------------|
| 6241 | 2012 Pursat    | 64·4                          | Resistant                      | Type 1            |
| 6431 | 2012 Battambang | 61·5                          | Resistant                      | Type 1            |
| 6220 | 2012 Battambang | 62·1                          | Resistant                      | Type 1            |
| 6261 | 2012 Pursat    | 70·5                          | Resistant                      | Type 1            |
| 6411 | 2012 Battambang | 71·6                          | Resistant                      | Type 1            |
| 6427 | 2012 Battambang | 77·4                          | Resistant                      | Type 3            |

The last column lists the DNA expansion types recorded in the region of chromosome 14 encoding the plasmepsin 1–4 haemoglobinases. Threshold used to define in-vitro susceptibility to PPQ: sensitive if survival rates were less than 10% and resistant if survival rates were 10% or more. See appendix for details.

Table 2: Details of the 31 K13-C580Y mutant, piperazine (PPQ)-resistant and PPQ-sensitive culture-adapted parasites analysed by whole-genome sequencing and compared with the 3D7 reference line.

For more on the Q442H PfPM2 polymorphism see http://plasmodb.org/plasmo

Figure 2: Manhattan plot showing the significance of copy number variations between whole-genome exome sequences of 23 piperazine-resistant and eight piperazine-sensitive culture-adapted lines collected in Cambodia in 2012 and phenotyped using the in-vitro piperazine survival assay.

Each dot represents a gene in the set of 31 culture-adapted parasites, according to chromosome. The x axis represents genomic location, and the y axis represents the log₂-transformed Wilcoxon test p values. Wilcoxon test p<0·0193, after Benjamini-Hochberg correction, only two genes, PF3D7_1408000 (plasmepsin 2) and PF3D7_1408100 (plasmepsin 3) achieved genome-wide significance (p<0·03795).
This finding is consistent with increased protein concentrations in the multicopy plasmepsin 2 lines studied. However, further work is required to expand this analysis to additional lines.

We then explored the association between plasmepsin 2 CNV and dihydroartemisinin–piperquine treatment outcome in the isolates from 725 patients collected before dihydroartemisinin–piperquine treatment, of whom 119 experienced recrudescence between day 12 and day 42 (figure 4). Plasmepsin 2 was unamplified in 476 (65·7%) of 725 isolates, had two copies in 153 (21·1%) of 725 isolates, and three or more copies in 96 (13·2%) of 725 isolates. Only seven (1·5%) of 476 patients with unamplified plasmepsin 2 parasites had recrudesced by day 42 compared with 112 (45·0%) of 249 patients infected with multicopy plasmepsin 2 parasites (relative risk [RR] 22·8 [95% CI 10·7–48·6], p<0·0001). Recrudescence was more frequent for isolates with three or more plasmepsin 2 copies compared with those with two copies (52 [54·2%] of 96 vs 60 [39·2%] of 153, p=0·02). The cumulative incidence of dihydroartemisinin–piperquine treatment failure increased with increasing plasmepsin 2 gene copies: unamplified versus two copies, hazard ratio (HR) 32·2 [95% CI 17·9–58·0], p<0·0001; unamplified versus three copies, HR 49·0 [23·0–104·2], p<0·0001; or two copies versus three or more copies, HR 1·53 [1·04–2·25], p=0·017 (figure 5A). The mean time to recrudescence decreased with increasing plasmepsin 2 copy number: 41·8–42·0) for patients with unamplified plasmepsin 2 copies.

### Table 3: List of genes with copy number variation most strongly associated with in-vitro piperquine resistance expressed by the piperquine survival assay

| Gene description | Chromosome number | Unadjusted Wilcoxon p value | Bonferroni* | Benjamini-Hochberg* |
|-------------------|-------------------|-----------------------------|-------------|---------------------|
| PF3D7_1408000     | Plasmepsin 2      | 14                          | 2·43×10⁻¹   | 0·1139              | 0·03795           |
| PF3D7_1408100     | Plasmepsin 3, histo-aspartic protease (HAP) | 14                          | 2·43×10⁻¹   | 0·1139              | 0·03795           |
| PF3D7_0422000     | Steroid dehydrogenase, putative | 4                          | 6·54×10⁻¹   | 0·306               | 0·0765            |
| PF3D7_0708000     | Pfmc-2TM Mauree's cleft two transmembrane protein (MC-2TM) | 7                          | 4·22×10⁻⁴   | 1                    | 0·2468            |
| PF3D7_1353000     | Plasmid exported protein, unknown function | 13                         | 4·22×10⁻⁴   | 1                    | 0·2468            |
| PF3D7_0713000     | Pfmc-2TM Mauree's cleft two transmembrane protein (MC-2TM) | 7                          | 6·65×10⁻⁴   | 1                    | 0·3112            |
| PF3D7_0605000     | Ser-Thr protein kinase (ARK1) | 6                          | 6·65×10⁻⁴   | 1                    | 0·3112            |
| PF3D7_0508000     | Transcription factor lfb, putative | 5                          | 1·02×10⁻¹   | 1                    | 0·4143            |
| PF3D7_0715000     | Conserved Plasmidum protein, unknown function | 7                          | 1·06×10⁻¹   | 1                    | 0·4143            |
| PF3D7_1211000     | Kinesin-like protein, putative | 12                         | 1·25×10⁻¹   | 1                    | 0·4208            |
| PF3D7_1304500     | Small heat shock protein, putative | 13                         | 1·52×10⁻¹   | 1                    | 0·4208            |
| PF3D7_1120100     | Phosphoglycerate mutase, putative (PGM1) | 11                         | 1·52×10⁻¹   | 1                    | 0·4208            |
| PF3D7_0315600     | Conserved Plasmidum protein, unknown function | 3                          | 1·52×10⁻¹   | 1                    | 0·4208            |
| PF3D7_0800700     | Surface-associated interspersed gene 8,3 (SURF8,3) (SURF8,3) | 8                          | 1·52×10⁻¹   | 1                    | 0·4208            |
| PF3D7_1117700     | GTP-binding nuclear protein ran/tc4 (RAN) | 11                         | 1·85×10⁻¹   | 1                    | 0·4572            |
| PF3D7_0909500     | Subpellicular microtubule protein 1, putative (SPM1) | 9                          | 1·85×10⁻¹   | 1                    | 0·4572            |
| PF3D7_1120200     | Conserved Plasmidum protein, unknown function | 13                         | 2·24×10⁻¹   | 1                    | 0·4995            |
| PF3D7_0925900     | Conserved Plasmidum protein, unknown function | 9                          | 2·24×10⁻¹   | 1                    | 0·4995            |
| PF3D7_0322000     | Peptidyl-prolyl cis-trans isomerase (CYP19A) | 3                          | 2·69×10⁻¹   | 1                    | 0·5254            |

*Based on 4422 genes included in the analysis (total of 4678 screened genes; 256 genes with <500 bp were excluded from the final analysis).
Among the 725 patients treated with dihydroartemisinin–piperaquine, K13 mutants were detected in 443 (61.1%) of 725 day 0 isolates (figure 5B). Of these, 116 (26.2%) were from patients who failed dihydroartemisinin–piperaquine treatment by day 42 compared with three (1.1%) of 282 from patients who withdrew or were lost to follow-up. K13 mutation was significantly more common among patients who failed dihydroartemisinin–piperaquine (aHR 20.4 [95% CI 9.1–45.5], p < 0.0001), followed by mdr1 mutation (K13 wild-type parasites: aHR 2.05 [0.95–4.42], p = 0.06). The cumulative incidence of dihydroartemisinin–piperaquine treatment failure (adjusted HR [aHR] 3.0 [95% CI 1.4–6.3], p = 0.003). The cumulative incidence of dihydroartemisinin–piperaquine treatment failure did not increase with increasing age (stratified in three classes: 0–15 years, 16–30 years, and >30 years; p = 0.1809, log-rank test) or with increasing parasite numbers in isolates collected before dihydroartemisinin–piperaquine treatment (stratified in four classes: <5000 parasites per μL, 5001–20000 parasites per μL, 20001–50000 parasites per μL, and >50000 parasites per μL; p = 0.4612, log-rank test).

After controlling for K13 and mdr1 genotypes in a Cox proportional-hazards regression model, *plasmspin 2* copy number (any increase compared with non-amplification) was the most significant molecular signature associated with dihydroartemisinin–piperaquine treatment failure (adjHR [aHR] 20.4 [95% CI 9.1–45.5], p < 0.0001), followed by K13 mutation (aHR 5.5 [1.7–18.3], p = 0.005), then mdr1 single copy (aHR 2.05 [0.95–4.42], p = 0.06). The cumulative incidence of dihydroartemisinin–piperaquine treatment failure among patients harbouring artemisinin-resistant parasites (ie, an artemisinin resistance-associated K13 mutation) increased significantly with *plasmspin 2* copy number (unamplified vs two or more copies, seven [3·3%] vs 18·4%, p < 0.0001).
of 208 vs 109 [46·4%] of 235; HR 17·5 [95% CI 6·9–45·2]).

CNVs in *plasmepsin* 2 were investigated in 1252 samples collected across Cambodia from 2002 to 2015 (ie, before and after the introduction of dihydroartemisinin–piperaquine). This sample included 527 archived isolates in addition to the 725 studied above (appendix). A longitudinal sampling was done in Pailin (western Cambodia) and Ratanakiri (eastern Cambodia), where dihydroartemisinin–piperaquine was introduced in 2008 and 2010, respectively. In Pailin, the proportion of multicopy *plasmepsin* 2 parasites increased from 27·9% (19 of 68) in 2008–09 to 91·2% (52 of 57) in 2014–15. In Ratanakiri, multicopy *plasmepsin* 2 parasites were infrequent until 2012–13 (3·2% [one of 31]) but increased to 45·5% (40 of 88) in 2014–15 (appendix). A steady increase of multicopy *plasmepsin* 2 parasites after introduction of dihydroartemisinin–piperaquine was recorded in other provinces as well (Preah Vihear, Pursat; appendix).

In the 12 sites where dihydroartemisinin–piperaquine efficacy studies were done in 2009–15, the proportion of multicopy *plasmepsin* 2 isolates was negatively correlated with day 42 cure rates (r=0·89 [95% CI 0·77–0·95], p<0·0001; appendix). A Cox regression model showed that the risk of recrudescence following a dihydroartemisinin–piperaquine 3-day course was significantly associated (p<2×10−16) with the presence of multicopy *plasmepsin* 2 parasites on day 0 irrespective of the site of enrolment (appendix). A linear regression model showed that the clinical efficacy of dihydroartemisinin–piperaquine at day 42 fell below 90% when the proportion of multicopy *plasmepsin* 2 parasites on K13-mutant genetic background rose above 22%.

**Discussion**

Following reports of increasing failure of artemisinine–mefloquine in western Cambodia, dihydroartemisinin–piperaquine was adopted in 2008 in the western provinces and implemented nationwide in 2010. Resistance to this combination has recently accelerated to levels that render it widely ineffective.1 The dearth of alternatives creates a perilous situation whereby these multi-drug-resistant infections might become untreatable and spread to other regions with endemic malaria.

The strategy used here to search for genetic associations with piperaquine resistance relied on genome-wide sequence comparisons of a set of artemisinin-resistant parasite lines collected in Cambodia in 2012, all harbouring the C580Y artemisinin resistance mutation and presenting in-vitro PSA survival rates indicative of piperaquine resistance or susceptibility. We reasoned that such a focused sampling in a geographically restricted population would reduce the genetic noise of artemisinin responses and population structure. Results identified amplification of the *plasmepsin* 2–3 cluster as a putative genetic event associated with piperaquine resistance. To confirm this association across the country, we focused on *plasmepsin* 2, located in the centre of the amplicon. *Plasmepsin* 2 amplification strongly correlated with ex-vivo PSA survival rates irrespective of artemisinin susceptibility and was highly predictive of dihydroartemisinin–piperaquine failures in all geographical areas of Cambodia. *Plasmepsin* 2 amplification thus represents an informative marker for piperaquine resistance.

The strong association between K13 polymorphisms and *plasmepsin* 2 amplification in the Cambodian parasites of 208 vs 109 [46·4%] of 235; HR 17·5 [95% CI 12·2–25·2]).
studied herein most likely reflects the history of drug selection in Cambodia. The proportion of isolates with different K13–plasmepsin 2 combinations (appendix) is consistent with a stepwise selection first for artemisinin resistance then for piperaquine resistance. This scenario is in line with the delayed appearance of multicycopy plasmepsin 2 parasites in eastern provinces where the emergence of artemisinin resistance was delayed compared with western provinces (appendix). Dihydro-artemisinin–piperaquine treatment failures were rare in eastern Cambodia by 2013, confirming observations by others but increased steadily in 2014 to reach a high frequency by 2015. Most treatment failures had a single gene copy of mdr1 (112 [94-1%] of 119), confirming earlier reports of failures. The presence of single copy mdr1 is consistent with data reported for in-vitro-selected piperaquine-resistant Dd2 parasites and analysis of field samples from Cambodia, suggesting opposing resistance mechanisms against these molecules. We did not observe the ccr C101F mutation recorded in a piperaquine-pressured parasite line selected in vitro. Thus, our data show that although the most informative marker for piperaquine resistance is plasmepsin 2 copy number, mutation of K13 alongside a single mdr1 gene copy contributes to the dihydroartemisinin–piperaquine failure phenotype. This finding does not exclude the possibility that additional genes contribute to piperaquine resistance. In particular, the significance of the mutations observed for PF3D7_0420000 and PF3D7_0420100 is unclear. Whether the notable sequence heterogeneity of both genes reflected ongoing purifying selection associated with piperaquine resistance or loss of mefloquine resistance is uncertain. Analysis of a larger number of isolates with documented phenotypes for both mefloquine and piperaquine is needed to address this question.

Drug-selected gene amplification is a well-known phenomenon in malaria parasites. The size of the amplicons on chromosome 14 varied depending on the isolate, as reported for mdr1. Gene amplification, which is more frequent than point mutation in P falciparum parasites, is consistent with the remarkably rapid rise and spread of piperaquine resistance in Cambodia. Conversely, mdr1 de-amplification, consistent with regained susceptibility to mefloquine, occurred in Cambodia in recent years and, as shown here, is associated with the emergence of piperaquine-resistant strains.

Plasmepsins are expressed during the intra-erythrocytic asexual blood stage cycle and by sexual stage gametocytes that can be transmitted to the mosquito vector. All four plasmepsins are located in the digestive vacuole of intra-erythrocytic developmental forms where they engage in different steps of haemoglobin degradation. Studies of parasites disrupted in the plasmepsin genes pointed to redundancy in the haemoglobin degradation machinery. To our knowledge, there are no reported studies about the consequences of overexpressing these proteases. We show here that plasmepsin 2 amplification is associated with a notable increase of steady-state mRNA and protein concentrations in two culture-adapted isolates. This observation needs to be confirmed with additional isolates. A reasonable hypothesis is that the amplification of plasmepsins overcomes the inhibitory effect of piperaquine on haemoglobin degradation and haem detoxification, possibly by reducing concentrations of reactive haem species that are preferred substrates for piperaquine binding. Piperaquine-treated trophozoites have been shown to possess large digestive vacuoles containing membrane-bound packets of undigested haemoglobin. The observation that piperaquine-resistant parasites have a single mdr1 copy is consistent with this scenario, since maintenance of a single mdr1 copy (or reversion to a single copy) might avoid importing excessive amounts of piperaquine into the digestive vacuole (appendix).

We note that the association of piperaquine resistance with amplification of the plasmepsin 2–3 cluster on chromosome 14 is not proof of causality. The structured populations of P falciparum parasites in Cambodia might confound the robustness of the association and additional loci might also contribute to piperaquine resistance. The present findings should be complemented with laboratory investigations of the cellular consequences of this amplification on the parasite response to piperaquine and on parasite fitness and transmissibility. Nonetheless, our data are timely in providing a molecular tool that predicts the appearance of piperaquine resistance in endemic settings.

Piperaquine is a well-tolerated partner drug used in combination with artemisinin derivatives or the ozonide compound artemolane (OZ277). The mechanism of piperaquine resistance in the specific context of Cambodia, where artemisinin resistance is nearly fixed and drug pressure is high, might not extrapolate to areas where artemisinin resistance has not yet been documented. Nevertheless, we propose to extend the assessment of plasmepsin 2 gene copy number to areas where piperaquine is being used in artemisinin-based combination therapies at a very large scale, and to combine this assay with K13 sequencing to localise areas of parasite resistance to both components. In Cambodia, where the rapid failure of first-line artemisinin-based combination therapies is jeopardising elimination efforts and accelerating the emergence and spread of resistance, the opposing susceptibility between mefloquine and piperaquine could be used to implement new strategies based on artemisinin-based combination drug rotation, sequential administration, or triple combinations including both artemisinin-based combination partner drugs. Although challenging to implement, these alternative strategies will help to ensure long-term efficacy of antimalarials to reach the elimination goal.

Contributors

BW, VD, LSR, FA, J-CB, DAF, OM-P, and DM contributed to study design. BS, JB, LM, CB, and FA analysed the whole-genome sequencing

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data. NKh, SC, RE, CK, MK, KL, and EL did qPCR and RT-qPCR and LSR and AB did the antibody analysis. NKI, SKE, AD, VD, and BW did the in-vitro and ex-vivo drug assays. RL, RH, SKi, and PR gathered the clinical data. GN did the statistical analysis. VD, BW, FA, and DM analysed the data. OM-P, FA, LSR, DAF, and DM wrote the first manuscript. All authors read and approved the final manuscript.

Declaration of interests
All authors declare no competing interests. PR is a staff member of WHO. The authors are responsible for the views expressed in this publication, which do not necessarily represent the decisions, policy, or views of WHO.

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