Impact of piperaquine resistance in *Plasmodium falciparum* on malaria treatment effectiveness in The Guianas: a descriptive epidemiological study

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Summary

Background ***Plasmodium falciparum*** **is an apicomplexan parasite responsible for lethal cases of malaria. According to WHO recommendations,** ***P falciparum*** **cases are treated with artemisinin-based combination therapy including dihydroartemisinin–piperaquine. However, the emergence of resistant parasites against dihydroartemisinin–piperaquine was reported in southeast Asia in 2008 and, a few years later, suspected in South America.**

Methods To characterise resistance emergence, a treatment efficacy study was performed on the reported patients infected with ***P falciparum*** **and treated with dihydroartemisinin–piperaquine in French Guiana (n=6, 2016–18). Contemporary isolates collected in French Guiana were genotyped for** ***P falciparum*** **chloroquine resistance transporter (*pfCRT*; n=845) and *pfpm2* and *pfpm3* copy number (n=231), phenotyped using the in vitro piperaquine survival assay (n=86), and analysed through genomic studies (n=50). Additional samples from five Amazonian countries and one outside the region were genotyped (n=1440).**

Findings In field isolates, 40 (47%) of 86 (95% CI 35·9–57·1) were resistant to piperaquine in vitro; these phenotypes were more associated with *pfCRT*^{C350R} (ie, Cys350Arg) and *pfpm2* and *pfpm3* amplifications (Dunn test, p<0·001). Those markers were also associated with dihydroartemisinin–piperaquine treatment failure (n=3 [50%] of 6). A high prevalence of piperaquine resistance markers was observed in Suriname in 19 (83%) of 35 isolates and in Guyana in 579 (73%) of 791 isolates. The *pfCRT*^{C350R} mutation emerged before *pfpm2* and *pfpm3* amplification in a temporal sequence different from southeast Asia, and in the absence of artemisinin partial resistance, suggesting a geographically distinctive epistatic relationship between these genetic markers.

Interpretation The high prevalence of piperaquine resistance markers in parasite populations of the Guianas, and the risk of associated therapeutic failures calls for caution on dihydroartemisinin–piperaquine use in the region. Furthermore, greater attention should be given to potential differences in genotype to phenotype mapping across genetically distinct parasite populations from different continents.

Funding Pan American Health Organization and WHO, French Ministry for Research, European Commission, Santé publique France, Agence Nationale de la Recherche, Fundação de Amparo à Pesquisa do Estado do Amazonas, Ministry of Health of Brazil, Oswaldo Cruz Foundation, and National Institutes of Health.

Introduction

Plasmodium falciparum is responsible for hundreds of millions of malaria cases worldwide each year. Since 2001, WHO has recommended the use of artemisinin-based combination therapies (ACTs), which combine a fast-acting drug (artemisinin and its derivatives) with long-acting partner drugs, to limit drug resistance. Nonetheless, artemisinin partial resistance, as defined by WHO, has arisen first in southeast Asia in the mid-2000s and now in South America and Africa, threatening the long-term efficacy of ACTs by facilitating selection for partner drug resistance.[1]

Among the six WHO-recommended ACTs, dihydroartemisinin–piperaquine has been widely used in southeast Asia and has been approved in Europe and several African countries.[1] 1 year after its deployment in Cambodia, artemisinin–piperaquine double-resistant parasites emerged.[2–4] Copy number amplifications of the *plasmepsin 2* (*pfpm2*) and *plasmepsin 3* (*pfpm3*) genes were first identified as putative piperaquine resistance markers. These plasmepsins are aspartic proteases with multifaceted functions, including haemoglobin degradation, but their exact role in piperaquine resistance is unclear.[3] Subsequently, single-point mutations (*Thr93Ser*, *His97Tyr/Leu*, *Phe145Ile*, *Ile218Phe*, *Met343Leu/Ile*, and *Gly353Val*) in the *P falciparum* chloroquine resistance transporter (*pfCRT*) were identified as key drivers of resistance to piperaquine.[5,6]

Lancet Infect Dis 2024; 24: 161–71

Published Online October 16, 2023 https://doi.org/10.1016/S1473-3099(23)00502-9

This online publication has been corrected. The corrected version first appeared at thelancet.com/infection on December 18, 2023

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For the Portuguese translation of the abstract see Online for appendix 2

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Research in context

Evidence before this study

Since 2008, in southeast Asia, piperaquine resistance emerged in *Plasmodium falciparum* strains already partly resistant to artemisinin. Previous studies published in 2017 identified *pfCRT* mutations as well as *plasmepsin 2* and *plasmepsin 3* gene amplifications as being responsible for the piperaquine resistance phenotype in this region. Different single-point mutations on *pfCRT* were suggested to be the main drivers of piperaquine resistance. We searched PubMed for articles published before May 25, 2023, with no language restrictions, using the terms "malaria", "piperaquine", "resistance", and "South America". Our search yielded 10 results including, in 2015, the first documented association between *pfCRT*^{C350R} (ie, Cys350Arg) mutation and chloroquine resistance reversion as well as a slight decrease of *P falciparum* susceptibilities to piperaquine, in French Guiana. This mutation was later reported in Surinamese samples in 2017. To date, piperaquine resistance has not been documented outside southeast Asia.

Added value of this study

This study highlighted a high prevalence of piperaquine resistance in the parasite population of the Guiana Shield, which hinders dihydroartemisinin-piperaquine efficacy in the absence of artemisinin partial resistance. This resistance is associated with two genetic markers, a unique single-point mutation on *pfCRT*, *pfCRT*^{C350R}, and *plasmepsin 2* and *plasmepsin 3* gene amplifications. Additionally, a retrospective analysis of isolates from the Amazonian basin countries collected across a period of 20 years, shows an original evolutionary path of the parasites from the Guiana Shield. Since the early 2000s, the parasite population experienced multiple events of de novo emergence of the same *pfCRT*^{C350R} mutation and preceding plasmepsin amplification. Overall, it appears that within the same period, piperaquine resistance emerged independently in southeast Asia and South America but followed a distinct path of selection, which appeared independent of the *P falciparum* genetic background.

Implications of all the available evidence

Given this high prevalence and spread of piperaquine resistance in South America, dihydroartemisinin–piperaquine to treat *P falciparum* malaria is no longer an alternative for the region.

This transmembrane protein, located on the parasite digestive vacuole, exports host-derived peptides out of the digestive vacuole and regulates drug traffic.[7] In southeast Asia, *pfCRT* mutations and *pfpm2* and *pfpm3* amplifications emerged on a *pfkelch13* (*pfk13*) mutant background responsible for artemisinin partial resistance.[5,8] This joint artemisinin partial resistance–piperaquine resistance lineage rapidly spread throughout the Cambodian parasite population.[4,8]

In South America, artemether–lumefantrine is the predominant first-line therapy; however French Guiana sporadically also prescribes dihydroartemisinin–piperaquine. Additionally, another ACT including piperaquine (ie, Artecom, dihydroartemisinin–piperaquine–trimethoprim, Chongqing Tonghe Pharmaceutical, Chongqing, China) is used in remote mining regions by self-medicating gold miners. They represent the main population infected with *P falciparum* in the region.[9] In South America, following intense chloroquine drug pressure in the 20th century, a specific *pfCRT* haplotype was selected at positions 72–76 (SVMNT).[10] Although the removal of chloroquine pressure selected for the return of the ancestral *K76* allele and chloroquine susceptibility on other continents, the *K76T* allele appears to have been fixed in the Guiana Shield region (encompassing Guyana, Suriname, French Guiana, and parts of Brazil, Venezuela, and Colombia). Instead, chloroquine susceptibility was restored by a new mutation, *pfCRT*^{C350R} (ie, Cys350Arg). Its spread was initially hypothesised to be driven by selection to restore a fitness deficit caused by chloroquine resistance. However, this mutation also appeared to modulate in vitro piperaquine sensitivity. The uncertain relationship between the in vitro isotopic testing method for this drug and in vivo treatment failures precluded confident identification of *C350R* as a selective driver of piperaquine resistance.[11] Subsequently, a gene-editing study on the South American *7G8* reference strain showed an increase in piperaquine survival rate.[12]

Here, we aimed to describe the prevalence of piperaquine resistance in *P falciparum* populations from the Guiana Shield, associated with the *pfCRT*^{C350R} mutation and *pfpm2* and *pfpm3* amplifications.

Methods

Treatment follow-up

Between 2016 and 2018, a treatment follow-up was conducted among the military personnel infected with *P falciparum* during operations in gold-mining sites and treated with a standard 3-day regimen of dihydroartemisinin–piperaquine (Eurartesim, Sigma Tau, Alfasigma, Bologna, Italy) according to patients' weights. Treatments were supervised and their efficacy was evaluated according to the WHO protocol (appendix 3 p 11).

Sample collection

This retrospective cross-sectional study evaluating the *pfCRT* genotype at position 350 and *pfpm2* and *pfpm3* gene copy number was performed on *P falciparum* isolates collected in French Guiana (1997–2018), Brazil (1997–2014, including Acre State; Amazonas State; and Pará State), Suriname (2010–13), Guyana (2014–18),

Venezuela (2017–18), and Colombia (2013–14; appendix 3 pp 6, 14–27). Isolates from Afghanistan (2012–13) were included as a control for strains outside the Amazonian region as they also carry the SVMNT *pfCRT* haplotype following amodiaquine pressure.[13] Samples were collected in accordance with ethical requirements (appendix 3 p 3). Participants provided informed consent in accordance with the ethical regulations of the countries.

Genotyping and genomic analysis

DNA was extracted from blood samples (from French Guiana and Brazil) and dried blood spots (from Guyana, Suriname, Venezuela, Colombia, and Afghanistan) using the QIAamp DNA mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Targeted fragments of the *pfCRT* gene of each sample (exons 2 and 10) were amplified by PCR and sequenced using the Sanger method.[11] *Pfpm2* and *pfpm3* and *pfmdr1* gene copy numbers were evaluated by Taqman qPCR (Applied Biosystems, Waltham, MA, USA) using β-tubulin as a single copy gene reference,[14] and performed on a StepOnePlus thermocycler (Applied Biosystems, Waltham, MA, USA). Genotyping analysis was performed as described in appendix 3 (p 3).

Whole-genome sequencing was conducted on 52 cultured French Guianese parasites collected between December, 2008, and April, 2018, using Illumina technology (San Diego, CA, USA). Alignment and variant calling followed best practices established by the Pf3k Consortium.[15] 50 samples had sufficient coverage for downstream analysis (>30% of the genome at 5 times the coverage). Relatedness among monoclonal samples was estimated with identity-by-descent measurements in the program hmmIBD. We clustered samples with the R package iGraph based on their identity-by-descent relationships to form groups at 0·99 identity-by-descent (clonal clusters) and 0·85 identity-by-descent (highly related clusters).[15] For haplotype block construction of the 200 kb region surrounding *pfCRT*, we filtered sites that were within five nucleotides of an indel or called in fewer than 50% of samples. For both the identity-by-descent analysis and the haplotype construction, we used only monoclonal samples and, for haplotype construction, we additionally excluded samples from patients with travel history outside French Guiana and included only one representative sample per highly related cluster.

A genome-wide association study using the GMMAT package in R,[15] was conducted with a set of 4600 high-quality single nucleotide polymorphisms with a minor allele frequency of greater than 0·1 (appendix 3 p 4). Separate models were constructed using binary resistant or susceptible phenotype measurements, only a single sample per clonal cluster, and only monoclonal samples. Single-marker effect size estimates (β, the slope of the generalised linear mixed model generated by GMMAT) were calculated with a Wald test in GMMAT (appendix 3 p 4).

In vitro drug sensitivity assays (ring-stage survival assay)

86 cultured parasites were phenotyped using the ring-stage survival assay and piperaquine survival assay methods in vitro as previously described.[16,17] Briefly, synchronised parasites at ring stage (0–3 h) were exposed to dihydroartemisinin 700 nM (ring-stage survival assay) or piperaquine 200 nM (piperaquine survival assay) for 6 h or 48 h, respectively. Survival was assessed 66 h or 24 h later, respectively. Ring-stage survival assay$_{0–3h}$ and piperaquine survival assay$_{0–3h}$ were interpretable if the initial parasitaemia was greater than 0·25% and if the growth rate of the control was greater than two-fold per 72 h. Tests were replicated between one to four times. Statistical significance between survival rates of the different isolates were calculated using Student's *t* test.

Statistical analysis

Statistical analysis was performed through R, with the comparison of multiple groups done using the ggstasplot package.[18] Trend hypothesis was evaluated through Mann-Kendall test. Welch's *t* test was performed to compare means between two independent groups and *Z* test was used to compare two populations on one variable. Non-parametric independent parameters (more than two) were compared by the Kruskal-Wallis test; if significant Dunn's test was performed. Bonferroni-Holm correction was performed for multiple comparisons. A p value of less than 0·05 was considered statistically significant.

Role of the funding source

The funders of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

Results

In vitro piperaquine survival assays of randomly cultured French Guianese *P falciparum* isolates identified 40 (47%) of 86 (95% CI 35·9–57·1) of them to be piperaquine resistant based on a 10% survival rate threshold (appendix 3 p 7). Resistant isolates exhibited a mean survival rate of 24% (IQR 16·6–30·4). All isolates carrying the wild-type *pfCRT*^{C350} allele were piperaquine sensitive (mean survival rate of 0·8% [IQR 0·0–0·6]) whereas 40 (71%) of 56 *pfCRT*^{C350R} mutant isolates were piperaquine resistant (figure 1). A significant association was observed between piperaquine resistance and the single nucleotide polymorphism coding for *pfCRT*^{C350R} (Welch's *t* test, p<0·001, figure 1). Furthermore, we found that the frequency of the *pfCRT*^{C350R} allele evolved over time and rapidly increased among isolates in French Guiana after its first observation in 2002, reaching 16 (84%) of

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See Online for appendix 3

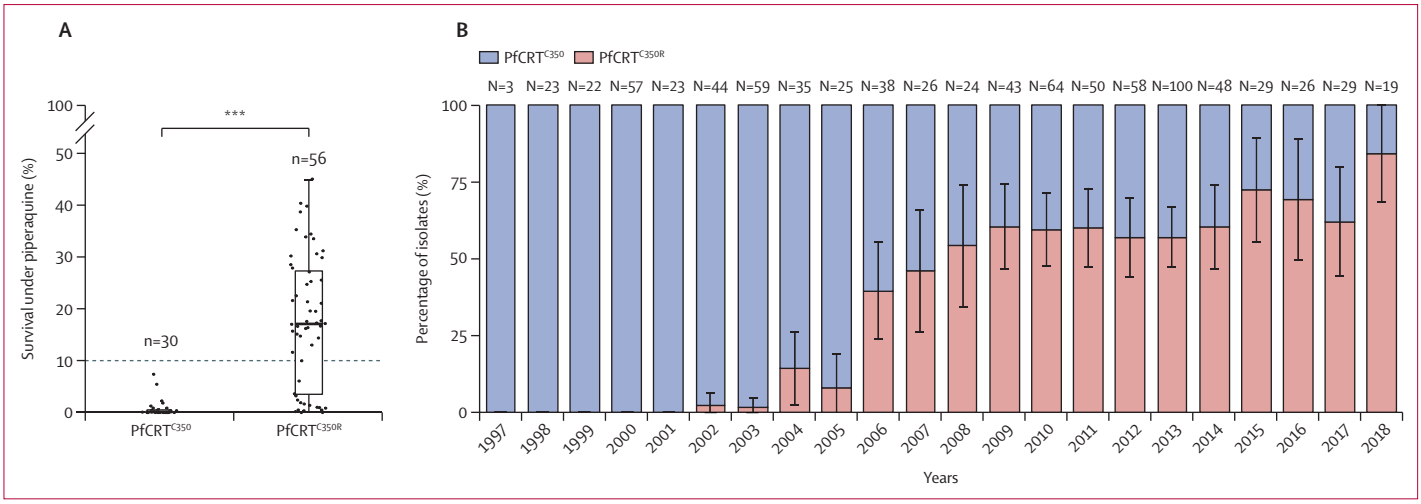

Figure 1: **Piperaquine resistance and *pfCRT*^{C350} allele status in French Guiana, 1997–2018**
(A) The *pfCRT*^{C350R} mutation is associated with higher parasite survival rates after 48 h of piperaquine exposure (piperaquine survival assay). The dashed line marks the resistance threshold for piperaquine. (B) The *pfCRT*^{C350R} mutation was first detected in 2002, and then rapidly increased in frequency. Between 2005 and 2006, the prevalence of this mutation rose from 2 (8%) of 25 patients (95% CI 0–18·9%) to 15 (40%) of 38 (23·9–55·0%). Since 2008, *pfCRT*^{C350R} isolates have accounted for more than half of collected isolates each year. The blue bars represent *pfCRT*^{C350} wild-type isolates and the red bars, *pfCRT*^{C350R} mutants. Error bars represent the 95% CIs. *pfCRT*^{C350R}=*plasmodium falciparum* chloroquine resistance transporter (ie, Cys350Arg). ***p<0·001.

19 (95% CI 67·5–100·0) in 2018 (Mann-Kendall test, τ=0·84, p<0·001; figure 1).

To determine the potential effect of the *pfCRT*^{C350R} allele on drug efficacy, we systematically investigated treatment response in patients treated by dihydroartemisinin–piperaquine in French Guiana. Between Nov 28, 2016 and March 30, 2018, six patients infected with *P falciparum* were treated with a supervised full course of dihydroartemisinin–piperaquine (appendix 3 p 11). Two patients exhibited adequate clinical and parasitological responses and for four patients the treatment did not work with parasite recrudescence observed between day 23 and day 29. Of the four patients for whom the treatment did not work, one patient exhibited subtherapeutic piperaquine exposure. The other three were associated with correct piperaquine intake, ie, piperaquine resistance. Parasites within these three infections were *pfCRT*^{C350R} mutant, with or without a *pfpm2* or *pfpm3* amplification (figure 2; appendix 3 p 11). In summary, the presence of the *pfCRT*^{C350R} mutation led to the treatment not working after dihydroartemisinin–piperaquine treatment in three (75%) of four patients (95% CI 32·6–100·0) and in vitro parasite resistance in 40 (71%) of 56 (59·6–83·3; figure 1) of the patients. Treatment failure occurred solely through piperaquine resistance, without artemisinin partial resistance (assessed both in vitro and through *Pfk13* genotyping). The two patients with adequate clinical and parasitological response were associated with parasites harbouring a single copy for *pfpm2* and *pfpm3*. One patient was associated with parasites *pfCRT*^{C350R} however, piperaquine-resistance status in vitro was unavailable due to an absence of material. The second patient showed in vitro susceptibility to piperaquine associated with wild-type C350.

To further confirm the association between *pfCRT*^{C350R} and piperaquine resistance, we performed whole-genome sequencing and a genome-wide association analysis on a subset of 50 samples including 25 piperaquine-sensitive and 25 piperaquine-resistant parasites. *pfCRT*^{C350R} was the only locus reaching statistical significance (figure 3). These results support the conclusion that *pfCRT*^{C350R} is a major driver of piperaquine resistance, although it does not account for the complete resistance phenotype.

Genomic data further support the hypothesis that the *pfCRT*^{C350R} mutation experienced a soft, rather than a hard, selective sweep.[19] First, pairwise fractional identity-by-descent around the *pfcrt* locus is not high between *pfCRT*^{C350R} individuals (60th percentile; figure 3). Second, we found evidence consistent with the occurrence of multiple de novo mutation events at *pfCRT*^{C350}. Among the six clonal clusters whose members are separated by mutation but not recombination, we found one four-member clonal cluster that contains both *pfCRT*^{C350} and *pfCRT*^{C350R} individuals (appendix 3 p 8). A complementary analysis of the 200 kb surrounding *pfCRT* showed 12 unique haplotypes, three of which contain both *pfCRT*^{C350} and *pfCRT*^{C350R} alleles (figure 3).

We expanded our genotyping pool of isolates from French Guiana to a larger panel of 1374 additional isolates collected between July, 1997, and October, 2018, in Colombia, Venezuela, Guyana, Suriname, and Brazil (ie, Pará, Amazonas, and Acre states). After 2006, the *pfCRT*^{C350R} allele was at a high frequency in three countries of the Guiana Shield, namely Suriname (29 [83%] of 35 isolates [95% CI 67·6–93·5%]), Guyana (579 [73%] of 791 [69·9–76·1%]), and French Guiana (326 [59%] of 554 [54·7–62·9%]; figure 4). Notably, we also observed a high prevalence of *pfpm2* and *pfpm3*

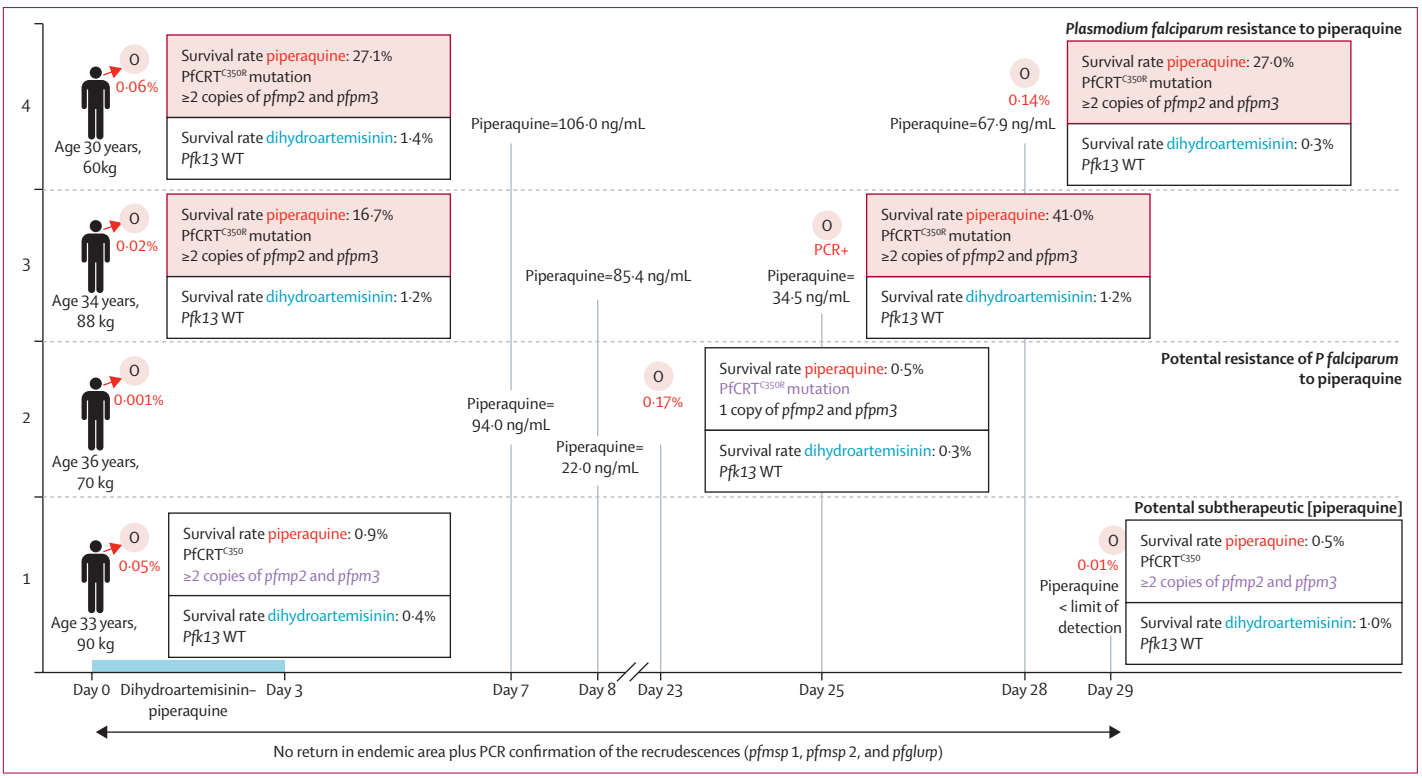

Figure 2: **Dihydroartemisinin–piperaquine therapeutics that did not work, reported in French Guiana, 2016–18**
Parasitaemia was calculated as: percent infected red blood cells=(number of infected red blood cells ÷ total number of red blood cells counted) × 100 or, positive PCR=positive results after real-time PCR *falciparum*-specific exhibiting a sensitivity of 1 parasite per µl of blood. Plasmatic drug dosages were evaluated by high-performance liquid chromatography with diode array; however dihydroartemisinin–piperaquine administration was supervised. The number of days elapsed from the beginning of the treatment is shown on the x-axis. Correct piperaquine intake was confirmed if plasma concentration was ≥30 ng/ml after day 7. No patients returned to endemic areas during or after treatment. Monoclonality and recrudescence of parasite was confirmed by PCR genotyping the *pfmsp1*, *pfmsp2*, and *pfglurp* alleles in samples at day 0 and the day the treatment was deemed to have not worked. Survival assays under dihydroartemisinin or piperaquine pressure (700 nM and 200 nM, respectively) defined the survival rate of the parasites in vitro. Red text boxes detail genetic markers associated with parasite resistance phenotypes and black text boxes for sensitive phenotypes. Piperaquine resistance markers are highlighted in purple. Following treatment that did not work, patients recovered after atovaquone–proguanil treatment. Pfk13 WT defines a genotype on the propeller region identical to the reference strain 3D7. *Pfpm2* and *pfpm3*=*plasmepsin 2* and the *plasmepsin 3*. *PfCRT*=*Plasmodium falciparum* chloroquine resistance transporter.

amplifications in these countries (figure 4). In contrast, in the Amazonas state of Brazil, *pfCRT*^{C350R} was observed at a low frequency. The wild-type *pfCRT*^{C350} allele was fixed, and no evidence of *pfpm2* and *pfpm3* amplifications were observed elsewhere. In addition, *pfCRT*^{C350R} mutations were not found in samples from Afghanistan (n=66), which also contain the *pfCRT* 72–76 SVMNT haplotype.

We further investigated the role of *plasmepsin* gene amplifications because of their association with piperaquine resistance in southeast Asia. *Pfpm2* and *pfpm3* gene amplifications were first detected in 2007, 5 years after the first observation of *pfCRT*^{C350R} (figure 5; appendix 3 pp 9, 12). Notably, we observed a positive correlation between *pfpm2* and *pfpm3* amplifications (Pearson's *r*=0·59, 95% CI 0·44–0·72; p<0·001), suggesting that both events either occur in a dependent manner or are present on the same genomic background (appendix 3 p 9). We therefore use the term *xpfpm2* and *pfpm3* to designate the amplification of *pfpm2* or *pfpm3* and *1pfpm2* and *pfpm3* to denote one copy of both genes. The prevalence of *pfpm2* and *pfpm3* amplification changed over time (Mann-Kendall test, τ=0·82; p=0·019). Initially, *pfpm2* and *pfpm3* amplification was highly associated with the *pfCRT*^{C350R} allele (*Z* test p=0·02) but, after 2011, the prevalence of *pfpm2* and *pfpm3* amplification in *pfCRT*^{C350} and *pfCRT*^{C350R} isolates was not significantly different (*Z* test p=0·4 for the period 2012–14 and p=0·9 for the period 2015–18; figure 5; appendix 3 p 9).

Although *pfpm2* and *pfpm3* amplification was significantly associated with piperaquine resistance in our in vitro assays (Welch's *t* test p<0·001; appendix 3 pp 9–10), we found that the phenotypic effect of this genetic marker was dependent on the *pfCRT* background. We showed that although *pfCRT*^{C350} isolates remained sensitive to piperaquine regardless of *pfpm2* and *pfpm3* gene status (*pfCRT*^{C350} 1*pfpm2* and *pfpm3* [median=0·0%; IQR=0–0·1%]; *pfCRT*^{C350} x*pfpm2* and *pfpm3* [0·4%; 0·1–0·9%]), *plasmepsin* gene amplification seemed to potentiate resistance to piperaquine in *pfCRT*^{C350R} mutant isolates [21·2%; 16·2–30·2%; p≤0·001]; figure 5). We similarly investigated *pfmdr1* copy number variation and observed 12 (14%) of 88 isolates with a co-amplification of

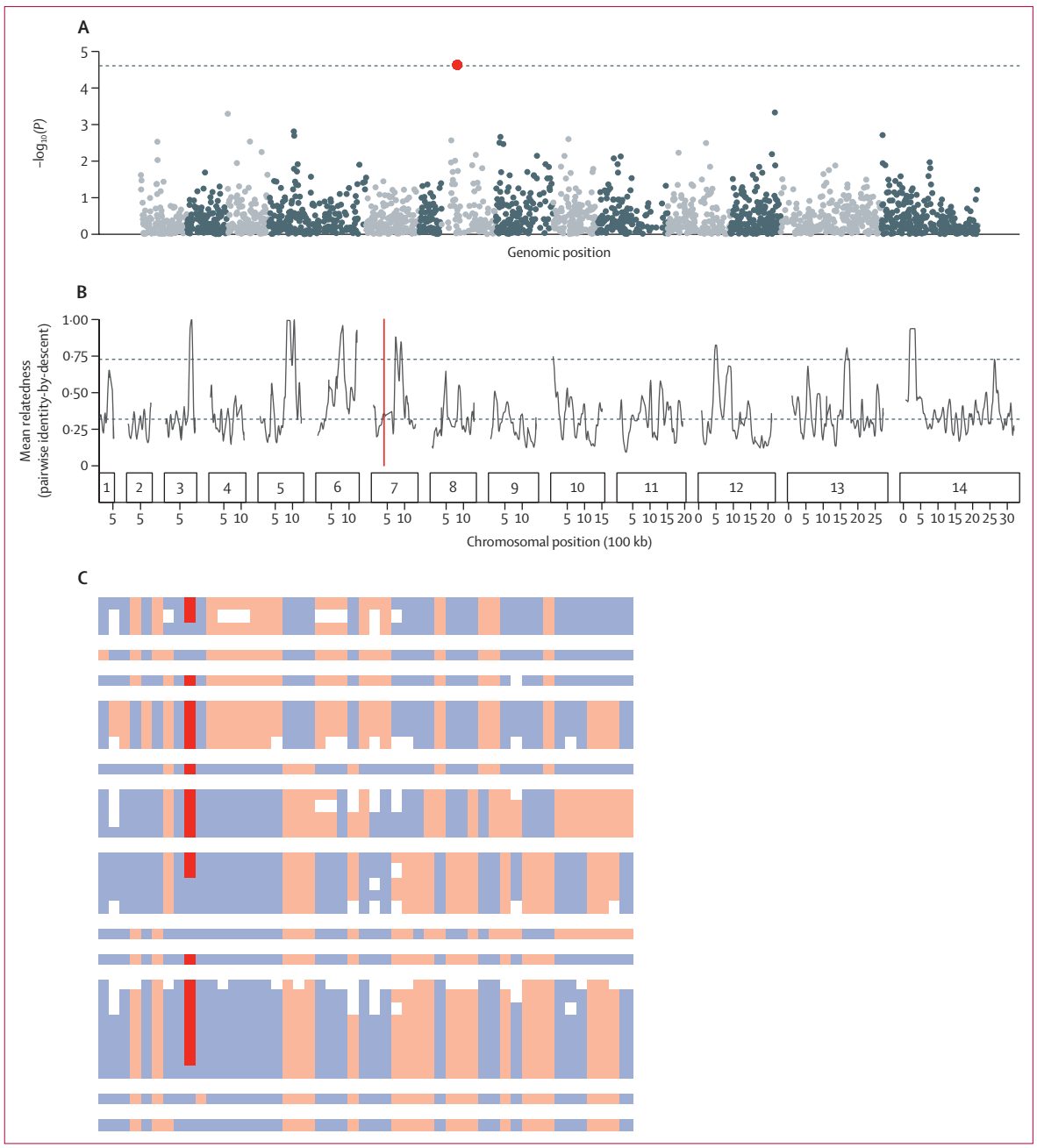

Figure 3: **Whole-genome sequencing of 50 phenotyped *Plasmodium falciparum* isolates**
(A) A genome-wide association analysis with 4600 single nucleotide polymorphisms shows a single outlier at the nucleotide position encoding the Cys350Arg substitution in the *pfCRT*^{350} locus (red). The dashed line shows the α=0·05 significance level after multiple test corrections. The mutation shows evidence of carrying a large phenotypic effect, as the effect size estimate (β in the generalised linear mixed model) was 9·8 (SE 1·7) with percent survival in the piperaquine survival assay ranging from 0% to 45% for parasites with the mutation. (B) Mean relatedness (identity-by-descent) between pairs of individuals calculated in 50 kb windows across the genome shows high variability genome wide. The genomic region surrounding the *pfCRT* locus (red line) does not show elevated relatedness, indicative of a soft selective sweep. Dashed grey lines depict the 50th and 95th percentiles. (C) The whole-genome sequenced samples of French Guiana origin (n=50) harbour 12 unique haplotypes in the 200 kb region surrounding the *pfCRT*^{350} locus (chromosome 7, positions 305 000 to 505 000). Each row corresponds to a single sample. Each column corresponds to a variant site. Blue denotes the *3D7* reference allele. Orange denotes the alternate allele. Red marks the mutation coding for *pfCRT*^{C350R}. Three of these haplotypes contain both wild-type and mutant *pfCRT*^{350} alleles. *PfCRT=plasmodium falciparum* chloroquine resistance transporter.

pfpm2 and *pfpm3* and *pfmdr1* (appendix 3 p 10). However, we found a correlation of 0 between *pfmdr1* and *pfpm2* and *pfpm3* amplification (Pearson's r=0·11, p=0·29), or with *pfCRT*^{C350R} allele (χ2 test, p=0·37), and with piperaquine resistance phenotype (Pearson's r=0·05, p=0·64; appendix 3 p 10).

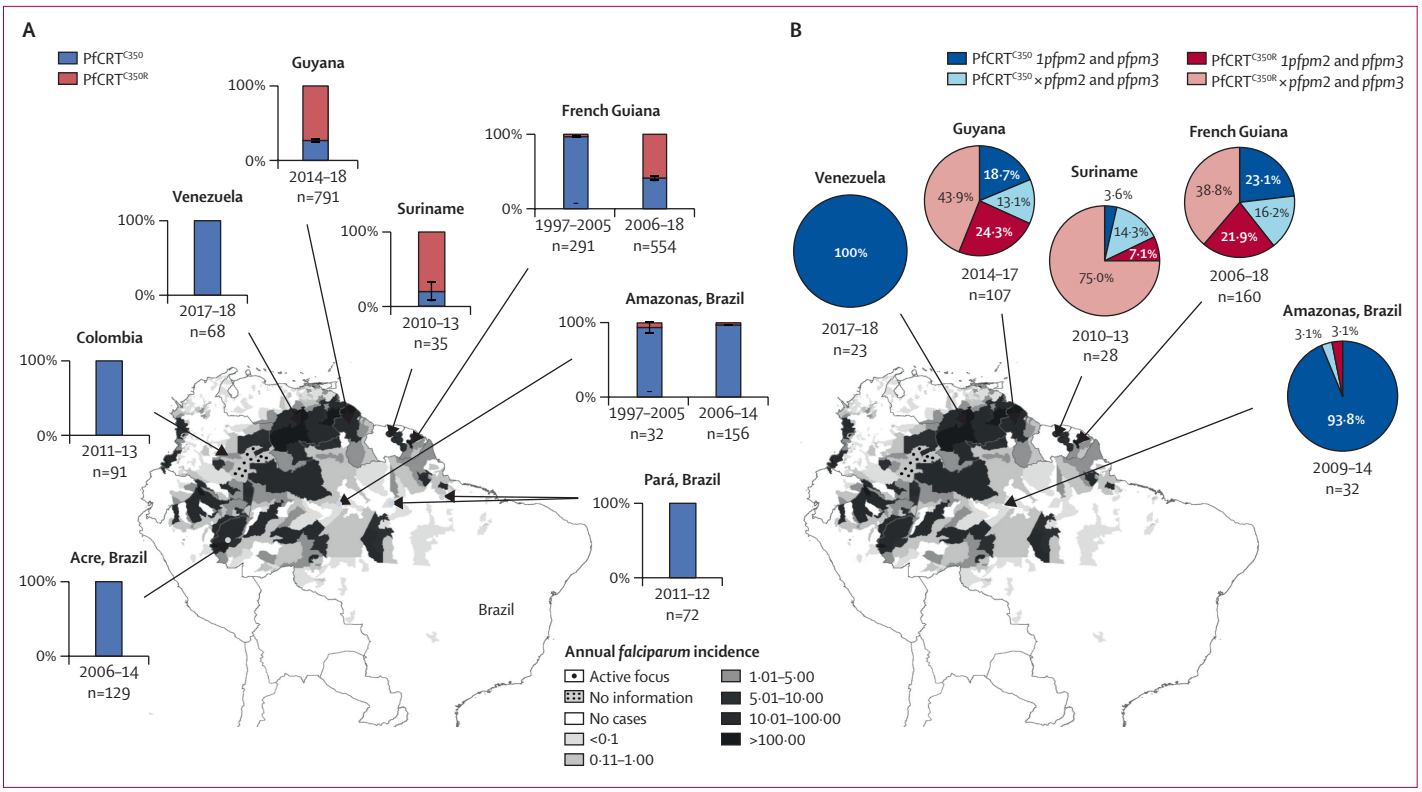

Figure 4: **Distribution of piperaquine resistance markers from isolates collected between 1997 and 2018 and spatial distribution of *Plasmodium falciparum* malaria infections in 2017**
(A) Frequencies of isolates with *pfCRT* muted (red bars) or not (blue bars) at position 350 showed disparities between the countries in the Amazon basin (isolate population=n). (B) Spatial distribution of piperaquine resistance markers (*pfCRT*^{C350R} [ie, Cys350Arg] and *pfpm2* and *pfpm3* amplification) in Guiana Shield countries from isolates collected between 2006 and 2018. Blue designated for *pfCRT*^{C350} carriers with a single copy of *pfpm2* and *pfpm3*, light blue for *pfCRT*^{C350} carriers with multiple copies of *pfpm2* and *pfpm3*, red for *pfCRT*^{C350R} mutants with a single copy of *pfpm2* and *pfpm3*, and pink for *pfCRT*^{C350R} mutants with multiple copies of *pfpm2 and pfpm3*. Adapted from annual country reports to the Pan American Health Organization, CDE/VT, and Malaria.[20] *Pfpm2* and *Pfpm3*=*plasmepsin 2* and *plasmepsin 3*. *PfCRT*=*Plasmodium falciparum* chloroquine resistance transporter.

Discussion

This study reports the de novo emergence of *P falciparum* piperaquine resistance in Guiana Shield countries, already exhibiting a high prevalence (40 [47%] of 86 isolates) in French Guiana between December, 2008, and April, 2018, based on in vitro phenotypes. Dihydroartemisinin–piperaquine therapeutic failure in patients has been associated with this resistant profile, in the absence of partial resistance to artemisinin and *pfk13* mutations. These observations, although on a small cohort, strongly support the importance of the partner drugs in ACTs, since mono-resistance to the partner drug has been shown to be sufficient to lead to therapeutic failures.[21] Such resistance could also lead to enhanced selection for artemisinin partial resistance. Piperaquine-resistant parasites are highly prevalent in the three countries of the Guiana Shield where miners in the deep forest widely self-medicate using Artecom. This drug pressure was most likely initiated shortly after the year 2000, during the most recent gold rush.[9,11] Therefore, we speculate that this unofficial underlying but strong piperaquine pressure was sufficient to select for and spread *pfCRT*^{C350R}.

Our results identify *pfCRT* as the key player involved in piperaquine resistance in the Guiana Shield. To date, *pfCRT*^{C350R} has solely been associated with *pfCRT* haplotype 7G8 (appendix 3, p 12) in South America and is predicted to localise on the lumen side of the transporter cavity where it affects the affinity of *pfCRT* to piperaquine.[12] Morphological observations revealed the characteristic presence of swollen translucent digestive vacuoles[14] in maturing trophozoites carrying the mutation (appendix 3 p 7).

This study further highlights the contrast in the piperaquine resistance evolutionary path between isolates from different regions as, in southeast Asia, piperaquine resistance is associated with the continuous emergence of several different but unique *pfCRT* mutations.[6,14] Furthermore, in southeast Asia, piperaquine resistance has arisen in close association with artemisinin partial resistance, whereas artemisinin partial resistance is not yet widespread in the Guiana Shield.[1] Historical patterns of drug selection might explain this disparate outcome. In southeast Asia, previous use of piperaquine and artemisinin as monotherapy against malaria had already led to emergences of piperaquine resistance in the 1980s[22] and artemisinin partial resistance in the mid-2000s.[23] The deployment of dihydroartemisinin–piperaquine later on

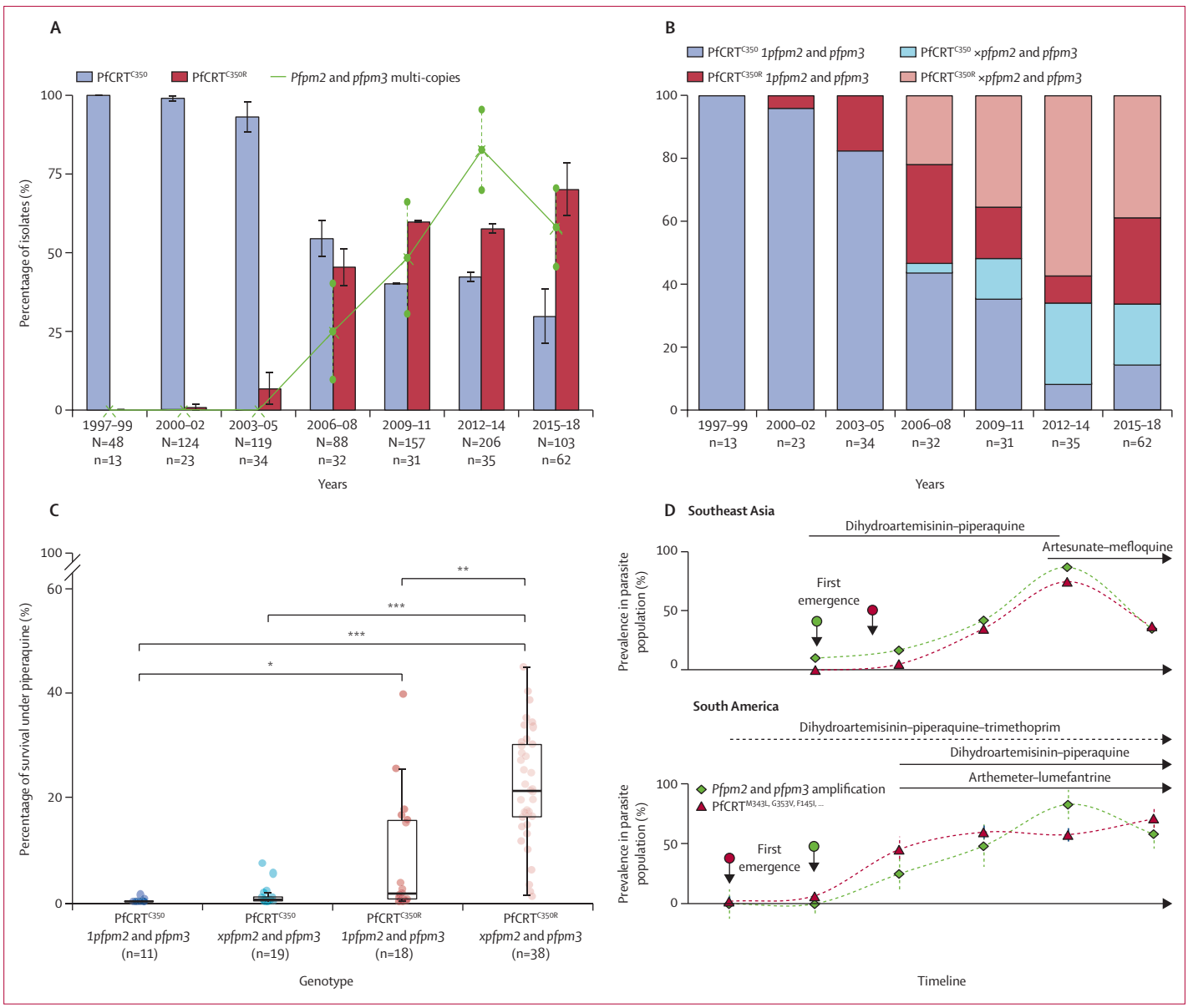

Figure 5: **Emergence and impact of plasmepsin gene amplifications (*pfpm2* and *pfpm3*) in French Guianese isolates between 1997 and 2018**
(A) Prevalence of *pfCRT*^{350} polymorphism (isolate population=N) and *pfpm2* and *pfpm3* multiple copies (isolate population=n). Like *pfCRT*^{350R} (ie, Cys350Arg), *pfpm2* and *pfpm3* amplifications rapidly rose in frequency after their initial emergence, culminating in 29 (83%) of 35 isolates (95% CI 70·4–95·3%) between 2012 and 2014. Blue bars represent *pfCRT*^{350} carriers and red bars, *pfCRT*^{350R} mutants; black error bars show the SD between the years. The green dashed line shows the percentage of isolates with multiple copies of *pfpm2* and *pfpm3*, green error bars display the 95% CI. (B) The *pfpm2* and *pfpm3* copy number status depending on the *pfCRT*^{350} polymorphism (isolate population=n). Blue bars represente *pfCRT*^{350} carriers with a single copy of *pfpm2* and *pfpm3*, light-blue bars for *pfCRT*^{350} carriers with multiple copies of *pfpm2* and *pfpm3*, red bars for *pfCRT*^{350R} mutants with a single copy of *pfpm2* and *pfpm3*, and pink bars for *pfCRT*^{350R} mutants with multiple copies of *pfpm2* and *pfpm3*. (C) Survival rate after piperaquine pressure depending on isolate genotype. Blue circles represent *pfCRT*^{350} carriers with a single copy of *pfpm2* and *pfpm3*, light-blue circles indicate *pfCRT*^{350} carriers with multiple copies of *pfpm2* and *pfpm3*, red circle indicate *pfCRT*^{350R} mutants with a single copy of *pfpm2* and *pfpm3*, and pink circles indicate *of pfCRT*^{350R} mutants with multiple copies of *pfpm2* and *pfpm3*. Multiple copies of genes is ≥2. Significance was determined by a Dunn test for multiple comparisons using R; *p<0·05, **p<0·01, and ***p<0·001. Sample size=n. (D) Temporal analysis of piperaquine resistance markers emergence and prevalence in southeast Asia and South America. *PfCRT* mutation is in red and *pfpm2* and *pfpm3* amplifications are in green. The timeline depicts periods from 2000 to 2020. Coloured arrows pinpoint the time of the first emergence for each piperaquine resistance markers. The different drug pressures are represented, with dashed arrows for non-conventional usage. *Pfpm2* and *pfpm3*=*plasmepsin 2* and the *plasmepsin 3*. *PfCRT*=*Plasmodium falciparum* chloroquine resistance transporter.

might have selected for a co-lineage resistant to both artemisinin and piperaquine.[4] In this case, the *pfpm2* amplification was the first piperaquine resistance marker selected[3] and, later on, *pfCRT* mutations[5] (figure 5). These amplifications were also associated with *pfmdr1* deamplification, due to the withdrawal of mefloquine.[6] In this context, given the sequence of emergence of the two piperaquine resistance markers,[3,6] *pfCRT* mutations and plasmepsin gene amplifications were suggested to have contributed additively rather than epistatically to

piperaquine resistance. Furthermore, the *pfpm2* amplification was acknowledged as sufficient to confer piperaquine resistance in the absence of *pfCRT* mutations[24] whereas, our results indicate that *pfpm2* and *pfpm3* amplification by itself could not lead to piperaquine resistance but led to the rapid selection of *pfCRT*^{C350R} in the *P falciparum* population and the potentiation of piperaquine resistance levels. This regionally distinctive evolutionary pattern has also been noted for strains from Africa versus southeast Asia, with *pfmdr1* mutations and amodiaquine resistance.[25,26]

For 16 (18%) of 86 French Guianese isolates, *pfCRT*^{C350R} does not lead to piperaquine resistance in vitro. This observation could be one of the reasons behind the presence of parasites carrying *pfCRT*^{C350R} in one of the adequate response cases under dihydroartemisinin–piperaquine treatment, the others being a possible low level of resistance in the absence of *pfpm2* and *pfpm3* amplification, or the patient-specific immune response. Hence, even though *pfCRT*^{C350R} is the only common feature identified among piperaquine resistance isolates, it appears that piperaquine resistance in South America could involve other factors. Our genome-wide association study supported the primacy of *pfCRT*^{C350R} in conferring resistance and did not identify additional genetic factors associated with it, but it was conducted with single nucleotide polymorphisms and not copy number variants (except *pfpm2* and *pfpm3*), which remain difficult to accurately genotype in *P falciparum* with Illumina data. *pfCRT*^{C350R} appears to have emerged via multiple recurrent mutational events within the past 20 years. This phenomenon, unique to this part of the world and occurring within a short timespan, suggests there are one or more unknown factors in the genetic background of the local parasite population conducive to the emergence of *pfCRT*^{C350R}. We attempted to test this hypothesis directly by generating gene-edited clones from several strains collected in French Guiana (*pfCRT*^{C350R} or not, *pfpm2* and *pfpm3* amplified or not, n=10 strains, 2 to 10 replicates each); however, we could not obtain viable clones. Further investigations are needed to thoroughly characterise other factors associated with *P falciparum* piperaquine resistance emergence in this region. Overall, we conclude that antimalarial drug resistance is not a deterministic process that will play out in the same manner in parasite populations around the world, but rather a phenomenon that could proceed along different mutational pathways as a function of local genomic diversity, historical selection pressure, and contemporary disease epidemiology. In this context, genetic surveillance needs to be tailored to specific parasite populations, and investigators and public health officers should recognise that genotypes might not map to phenotypes in a consistent manner across different geographical settings.

From a patient-care perspective, we recommend a halt to dihydroartemisinin–piperaquine treatment for *P falciparum* malaria in cases diagnosed in or imported from the Guiana Shield, given the high prevalence of piperaquine resistance, as well as its impact on dihydroartemisinin–piperaquine efficacy. Although French Guiana is the only country in the region to include dihydroartemisinin–piperaquine as a first-line therapy alongside artemether–lumefantrine, dihydroartemisinin–piperaquine is widely used for self-medication through the black market in the Guiana Shield. Curtailing its use will therefore be difficult but crucial if we want to preserve artemisinins and aim to eliminate *P falciparum* malaria in the region.

Based on the prevalence of antimalarial resistance markers in the Amazonian parasite population, the alternatives to dihydroartemisinin–piperaquine among ACTs are limited. For example, sulfadoxine–pyrimethamine should be avoided as an artemisinin partner drug, because triple resistance mutations in the *pfdhfr* and *pfdhps* loci have been fixed since the late 1980s. Amodiaquine resistance was also reported in the region in the 1970s, and exhibits high cross-resistance with chloroquine on this continent.[27] Although Pelleau and colleagues[11] showed that *pfCRT*^{C350R} mutant parasites reversed their resistance to amodiaquine in addition to chloroquine,[11] based on the actual prevalence of amodiaquine resistance in French Guiana (39 [51%] of 77, 2017–21, unpublished data), amodiaquine use is not a reliable option. Mefloquine was withdrawn decades ago because of *pfmdr1* amplification.[28] Since then, the prevalence of this amplification has decreased, but it has not disappeared (appendix 3 p 10). The recent deployment of artesunate–mefloquine in addition to artemether–lumefantrine in the Amazonas state of Brazil, requires tight monitoring.[29] Lumefantrine, currently in use as an antiretroviral therapy partner drug in most of the countries of the Amazon basin, is still highly efficient,[1] which could explain the failure of the *pfk13* C580Y artemisinin partial resistance mutation to spread following its emergence in Guyana in 2010, and repeated observation in 2016.[30] Finally, a WHO-recommended ACT that includes pyronaridine has not yet been deployed in South America, although recent data from southeast Asia showed its potency against piperaquine-resistance parasites.[31] Overall, artemether–lumefantrine is the better first-line treatment option to treat *P falciparum* in Amazonia and more generally in malaria endemic areas worldwide whereas dihydroartemisinin–piperaquine appears to be exposed to a rapid selection for resistant parasites particularly in low endemic countries.

Contributors

CF, FdL, AME, DEN, and LM contributed to the study design. DEN and LM oversaw the conduct of the study. CF, AME, SS, YL, SP, and MA performed the analysis. FdL, WMM, EM, FD, NT, FM, MM, MVGL, GMRV, SH, MRA, MUF, CJW, GRA, HC, M-PA, and LM contributed to the sample and data collection. LM and CF had full access to all the data in the study and LM had final responsibility for the decision to submit for publication. CF, AME, SS, and LM wrote the original draft. All

authors critically reviewed the manuscript during writing. CF, AME, DEN, PR, and LM wrote, reviewed, and edited the manuscript.

Declaration of interests

M-PA and PR are staff members of WHO. The authors alone are responsible for the views expressed in this publication, which do not necessarily represent the decisions, policies, or views of WHO. All other authors declare no competing interests.

Data sharing

The data supporting the findings of this study are available within the paper and appendix 3. Illumina sequence data have been deposited in the NCBI Sequence Read Archive in association with BioProjects PRJNA809398, PRJNA759191, and PRJNA809659.

Acknowledgments

We thank all the patients whose samples were analysed in this study. We also thank all the partners of the French National Reference Center for Malaria involved in malaria surveillance in French Guiana. This work was supported by the Global Malaria Program (WHO), the Communicable Diseases and Environmental Determinants of Health Department (Pan American Health Organization), the Guyamazon 3 programme, the French Ministry for Research, a European Commission Grant through the ELIMALAR project (Regional fund for development, Synergie GY0012082), Santé publique France as the National Reference Centre for Malaria, and an Investissement d'Avenir grant managed by Agence Nationale de la Recherche (Centre d'étude de la biodiversité amazonienne, reference ANR-10-LABX-25-01). The Manaus team was funded by Fundação de Amparo à Pesquisa do Estado do Amazonas-FAPEAM (PAPAC 005/2019, Posgrad and Pró-Estado). Samples from Cruzeiro do Sul, Brazil, were collected in the context of a therapeutic efficacy trial supported by the Amazonian Malaria Initiative and the Amazon Network for the Surveillance of Antimalarial Drug Resistance through the Pan American Health Organization (BR/LOA/0900212.001), the Ministry of Health of Brazil, and the Oswaldo Cruz Foundation (cooperation agreements 25380.4120/09-26 and 82/2012). MUF receives research scholarships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil, and Fundação Millennium BCP, Portugal. This project has been funded in whole or in part with federal funds from the National Institute of Allergy and Infectious Diseases, the National Institutes of Health, the Department of Health and Human Services, under the grant number U19AI110818 to the Broad Institute. Colombian sample collection and analysis were performed in the frame of the US National Institute of Allergy and Infectious Diseases International Center of Excellence for Malaria Research (ICEMR-5U19AI089702).

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