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Monitoring *Plasmodium vivax* resistance to antimalarials: Persisting challenges and future directions

Marcelo U. Ferreira [a,b,*], Tais Nobrega de Sousa [c], Gabriel W. Rangel [d], Igor C. Johansen [a], Rodrigo M. Corder [a], Simone Ladeia-Andrade [e], José Pedro Gil [f]

[a] Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[b] Global Health and Tropical Medicine, Institute of Hygiene and Tropical Medicine, Nova University of Lisbon, Lisbon, Portugal
[c] Molecular Biology and Malaria Immunology Research Group, René Rachou Institute, Fiocruz, Belo Horizonte, Brazil
[d] Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA, USA
[e] Laboratory of Parasitic Diseases, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Brazil
[f] Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Solna, Sweden

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ABSTRACT

Emerging antimalarial drug resistance may undermine current efforts to control and eliminate *Plasmodium vivax*, the most geographically widespread yet neglected human malaria parasite. Endemic countries are expected to assess regularly the therapeutic efficacy of antimalarial drugs in use in order to adjust their malaria treatment policies, but proper funding and trained human resources are often lacking to execute relatively complex and expensive clinical studies, ideally complemented by *ex vivo* assays of drug resistance. Here we review the challenges for assessing *in vivo P. vivax* responses to commonly used antimalarials, especially chloroquine and primaquine, in the presence of confounding factors such as variable drug absorption, metabolism and interaction, and the risk of new infections following successful radical cure. We introduce a simple modeling approach to quantify the relative contribution of relapses and new infections to recurring parasitemias in clinical studies of hypnozoitocides. Finally, we examine recent methodological advances that may render *ex vivo* assays more practical and widely used to confirm *P. vivax* drug resistance phenotypes in endemic settings and review current approaches to the development of robust genetic markers for monitoring chloroquine resistance in *P. vivax* populations.

1. Global distribution of antimalarial resistance in *Plasmodium vivax*

Plasmodium vivax, the most geographically widespread yet relatively neglected human malaria parasite, causes significant morbidity in Central and South America, the Middle East, parts of Africa, Central, South, and Southeast Asia, and the Western Pacific. Nearly 3.3 billion people are at risk of infection with this species worldwide and 14.3 million clinical vivax malaria cases estimated to occur each year (Battle et al., 2019).

The emergence of antimalarial drug resistance complicates current global efforts to eliminate malaria (Menard and Dondorp, 2017) and national malaria control programs are expected to assess regularly the therapeutic efficacy of antimalarial drugs in use (World Health Organization, 2015). Drug resistance has been classically defined as "the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the tolerance of the subject" (World Health Organization, 1967). Moreover, "the form of the drug active against the parasite must be able to gain access to the parasite or

Abbreviations: ACT, antemisinin-based combination therapy; bp, base pairs; CQ, chloroquine; CYP2D6, cytochrome P450 2D6; cPQ, carboxyprimaquine; DCQ, desethylchloroquine; DEN, dextromethorphan; DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; EDTA, ethylenediamine tetraacetic acid; G6PD, glucose-6-phosphate dehydrogenase; IMDM, Iscove's modified Dulbecco's medium; MAO-A, Monoamine oxidase A; MQ, mefloquine; PQ, primaquine; *pvcrt-o*, *Plasmodium vivax chloroquine resistance transporter ortholog*; *pvmdr-1*, *Plasmodium vivax* multidrug resistance 1; QN, quinine; SNP, single-nucleotide polymorphism; SP, sulphadoxine-pyrimethamine; TBM, transmembrane domain; TQ, tafenoquine.

* Corresponding author. Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes 1374, São Paulo (SP), 05508-900 , Brazil.
E-mail address: muferrei@usp.br (M.U. Ferreira).

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the infected erythrocyte for the duration of the time necessary for its normal action" (Bruce-Chwatt, 1986). Due to the limited use of ex-vivo assays and the lack of robust molecular markers, treatment failure is often used as a proxy of emerging *P. vivax* resistance to antimalarials. Although therapeutic efficacy studies play a central role in monitoring drug resistance, treatment failure is not synonymous with drug resistance. Indeed, poor drug absorption and metabolism and other factors may reduce the therapeutic efficacy of antimalarials to which no true resistance has been characterized. Conversely, semi-immune patients may successfully clear parasitemias despite the use of a suboptimal treatment regimen.

Compared with the globally dominant human malaria parasite, *P. falciparum*, resistance to chloroquine (CQ) and other commonly used antimalarials has spread much more slowly in *P. vivax* populations (Price et al., 2014). Possible causes include the relatively small parasite biomass in *P. vivax* infections, compared to *P. falciparum*, rendering rare mutations much less likely to emerge within individuals undergoing antimalarial treatment, and the frequent use of drug combinations for the radical cure of vivax malaria – i.e., elimination of hypnozoites and asexual blood stages to prevent relapses and recrudescences, respectively. Other potential contributors to the delayed emergence of CQ-resistant *P. vivax* strains include early gametocytogenesis and transmission, before resistant clones are selected in the host under drug pressure, and the very high genetic diversity in natural *P. vivax* populations, which renders resistant phenotypes less likely to become fixed.

CQ, a safe, inexpensive, and fast-acting 4-aminoquinoline derivative that has been extensively used since 1950, offers an example of delayed resistance in *P. vivax*. CQ resistance emerged independently, in the early 1960s, in *P. falciparum* populations from Southeast Asia and South America and spread to Africa in the late 1970s (Payne, 1987; D'Alessandro and Buttiëns, 2001). In contrast, *P. vivax* resistance to CQ was first documented nearly three decades later, in two Australian soldiers returning from Papua New Guinea (Rieckmann et al., 1989). CQ-resistant strains subsequently spread to the Indonesian part of the island of Papua, where high-grade resistance is now very common in local *P. vivax* populations. In most of the remaining *P. vivax* endemic countries, CQ remains efficacious as a blood schizontocide but resistance is increasing and has been detected in Southeast Asia, South Asia, the Middle East, East Africa, and the Americas (Price et al., 2014; Gonçalves et al., 2014; Chu and White, 2016). On the Indian subcontinent, which accounts for most of the global burden of vivax malaria (Battle et al., 2019), CQ-resistant *P. vivax* strains remain uncommon (Price et al., 2014; Chu and White, 2016).

Fig. 1 shows the global distribution of CQ-resistant *P. vivax* infections inferred from therapeutic efficacy studies and clinical trials. We note that the limited available data from ex-vivo sensitivity assays do not provide a geographically representative picture of phenotypic *P. vivax* resistance to this antimalarial drug. CQ remains the first-line drug for vivax malaria in most endemic countries, except for Indonesia, Papua New Guinea, Solomon Islands, Vanuatu, Cambodia, Eritrea, and South Sudan (Chu and White, 2016; Price et al., 2020). The remaining malaria-endemic countries are recommended to monitor CQ resistance regularly and change for an artemisinin-based combination therapy (ACT) when the treatment failure rate of CQ exceeds 10% at day 28 (World Health Organization, 2015).

Mefloquine (MQ) monotherapy has never been recommended as a first-line treatment for single-species *P. vivax* infections, despite its proven efficacy against CQ-resistant strains in Indonesia (Maguire et al., 2006). However, *P. vivax* has been extensively exposed to MQ used to treat *P. falciparum* or mixed-species malaria in areas where *P. falciparum*

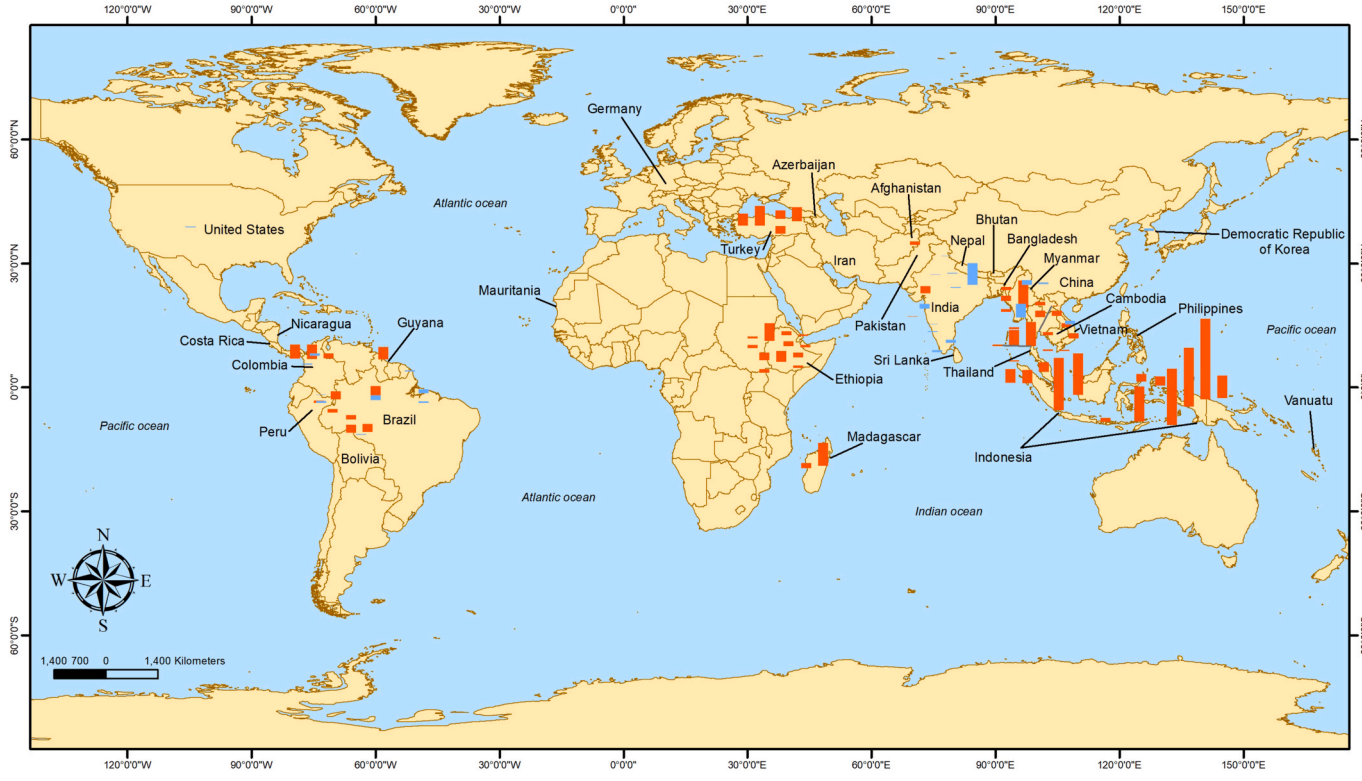

Fig. 1. Global distribution of chloroquine (CQ)-resistant *P. vivax* infections documented in therapeutic efficacy studies and clinical trials. Bar heights are directly proportional to failure rates. Orange bars represent patients treated with CQ alone who presented parasite recurrences until day 28 (n = 116 CQ treatment arms; treatment failure rate ranging between 0 and 100% among studies). Blue bars represent patients treated with CQ and primaquine who presented recurrences (n = 75 CQ-PQ treatment arms; treatment failure rate ranging between 0 and 22.7% among studies). Source: WorldWide Antimalarial Resistance Network (WWARN), available at: http://www.wwarn.org/vivax/surveyor/#0. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and *P. vivax* co-exist (Khim et al., 2014). Moreover, MQ is administered as a partner drug in a widely used ACT, artesunate-MQ, which is currently recommended in several countries to treat suspected or confirmed cases of CQ-resistant vivax malaria (Chu and White, 2016). Artemisinin resistance remains undescribed in *P. vivax,* but MQ resistance may be a matter of concern when using this ACT. Amplification of the *multidrug resistance 1* (*pvmdr-1*) gene, which associates with decreased susceptibility to amodiaquine, artesunate and MQ *in vitro* (Suwanarusk et al., 2008), has emerged in Southeast Asia and South America, where MQ monotherapy was widely deployed in the late 1980s and 1990s (Vargas-Rodríguez et al., 2012; Khim et al., 2014; Costa et al., 2017). Fig. 2 shows the current geographic distribution of *pvmdr-1* copy number variation. It remains unclear whether the current prevalence of *pvmdr-1* gene amplification represents an impediment for artesunate-MQ use for vivax malaria treatment in Southeast Asia and South America.

Dihydropteroate synthase (DHPS) inhibitors, such as sulphadoxine, and dihydrofolate reductase (DHFR) inhibitors, such as proguanil and pyrimethamine, disrupt parasite's folate synthesis. However, mutations at the *pvdhps* and *pvdhfr* loci leading to antifolate resistance are commonly found in *P. vivax* isolates from across the globe (Imwong et al., 2001; Hastings et al., 2004). *P. vivax* therapeutic responses to the sulphadoxine-pyrimethamine (SP) combination are generally poor (e.g., Pukrittayakamee et al., 2000). Interestingly, this parasite appears to have become SP-resistant faster than *P. falciparum*. Antifolates are not currently recommended as a first-line treatment of vivax malaria (World Health Organization, 2015).

One distinctive feature of *P. vivax* is its ability to stay in the liver as a dormant stage, the hypnozoite, following a primary infection. As a result, repeated episodes of blood-stage infection, known as relapses, may originate over the next weeks or months from hypnozoites reactivating at different time points following a single mosquito inoculation (White, 2011). CQ and other blood schizontocides, such as MQ and artemisinin derivatives, can eliminate *P. vivax* asexual blood stages but not its hypnozoites. Relapses are estimated to account for 50–96% of all post-treatment *P. vivax* recurrences in Papua New Guinea, Thailand, and Ethiopia (Betuela et al., 2012; Adekunle et al., 2015; Robinson et al., 2015; Abreha et al., 2017), suggesting that a large fraction of the vivax malaria burden in endemic settings is preventable by effective anti-relapse therapy.

Only 8-aminoquinoline derivatives have been found to eradicate both tissue schizonts and hypnozoites. The 8-aminoquinolines primaquine (PQ) or tafenoquine (TQ) are combined with blood schizontocides as a radical cure to prevent relapses of vivax and ovale malaria. Importantly, PQ also has some activity against *P. vivax* asexual blood stages (Pukrittayakamee et al., 1994) and can eliminate partially CQ-resistant parasites (Price et al., 2014). Some *P. vivax* strains appear to be tolerant or partially resistant to PQ, as relapses have been repeatedly observed in natural or experimental infections treated with standard CQ-PQ regimens in malaria-free settings (Fernando et al., 2011; Thomas et al., 2016). Two examples are the Brazil-1 strain (original infection acquired in the Brazilian Amazon in 1994; Nayar et al., 1997) and the Chesson strain (original infection acquired in Papua New Guinea in 1944; Alving et al., 1960). High-dose PQ regiments are currently recommended for radical cure of vivax malaria in Southeast Asia and Oceania (Hill et al., 2006; World Health Organization, 2015; Chu and White, 2016), but the global distribution of putative PQ tolerance remains unknown (Goller et al., 2007; Thomas et al., 2016). Indeed, the recommendation of high-dose PQ appears to be supported by surprisingly limited clinical evidence (John et al., 2012; Milligan et al., 2020). Clinical studies of PQ efficacy in malaria-endemic regions are often confounded by the reduced compliance with lengthy therapeutic regimens, poor metabolization of PQ into active metabolites, and frequent *P. vivax* reinfections (and sometimes late recrudescences) that

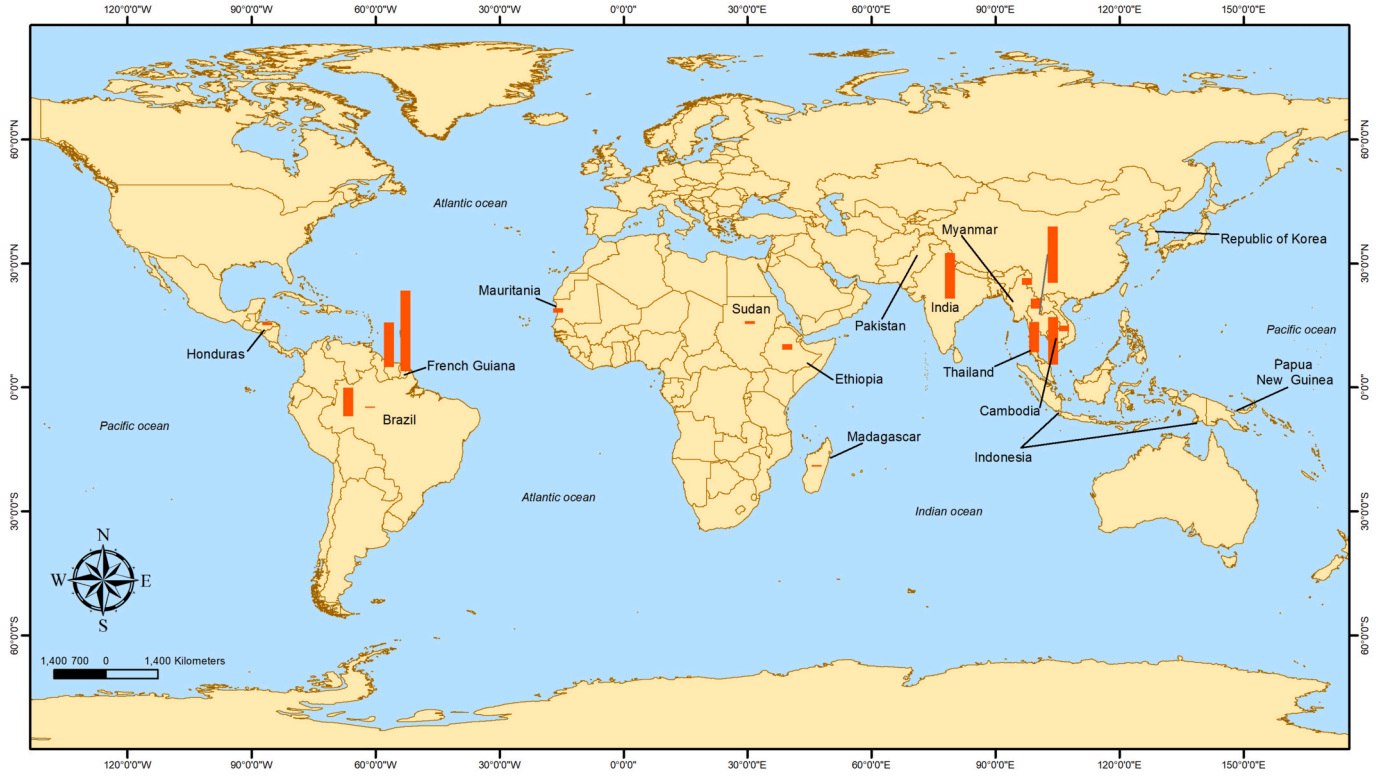

Fig. 2. Global distribution of amplification at the *multidrug resistance 1* (*mdr-1*) locus of *Plasmodium vivax* that is associated with resistance to mefloquine. Bar heights are directly proportional to the percentage of parasite samples from each location with two or more *pvmdr-1* gene copies (n = 14 studies), ranging between 0 and 59%. Data compiled from: Auburn et al. (2016); Chaorattanakawee et al. (2017); Costa et al. (2017); Htun et al. (2017); Imwong et al. (2008); Joy et al. (2018); Li et al. (2020); Lo et al. (2017); Musset et al. (2019); Roesch et al. (2020); Silva et al. (2018); Suwanarusk et al. (2007), and Vargas-Rodríguez et al. (2012).

cannot be readily distinguished from relapses (Fernando et al., 2011).

2. Therapeutic efficacy trials of blood schizontocides

A total of 1221 therapeutic efficacy studies of antimalarials were published between 1946 and 2018, but only 177 (17%) of them enrolled exclusively patients with single-species *P. vivax* infection (Takata et al., 2020). Table 1 summarizes key aspects of clinical trial protocols to assess the therapeutic efficacy of commonly used blood schizontocides in vivax malaria. Topics for debate include the use of molecular diagnostic methods for early recurrence detection and quantification, the timing of hypnozoitocide administration, and the optimal duration of patient follow-up.

Investigators typically recruit patients aged >6 months who present fever or history of fever and a single-species, uncomplicated *P. vivax* infection diagnosed by microscopy (World Health Organization, 2015; WorldWide Antimalarial Resistance Network (WWARN), 2015). Although standard microscopy remains the method of choice for monitoring parasite clearance in clinical trials (Baird et al., 2016), more sensitive (and species-specific) molecular diagnostic techniques may be used to: (a) rule out mixed-species infections missed by microscopy at enrolment (Siqueira et al., 2017; Ladeia-Andrade et al., 2019), (b) detect and quantify low-level recrudescences that may be missed by microscopy (Lo et al., 2016; Ladeia-Andrade et al., 2019), and (c) monitor the clearance of gametocyte-specific RNA transcripts following treatment (Ladeia-Andrade et al., 2019). Rapid diagnostic tests for parasite antigen detection should not be used in therapeutic efficacy studies to detect recrudescences due to the prolonged antigenemia observed after successful treatment (Phuong et al., 2015).

Patients with parasitemias above a certain threshold – typically 250 asexual blood stages/μl (World Health Organization, 2015) – are recruited to increase the likelihood that the associated fever is due to the current infection (Baird et al., 2016). However, the threshold is often ignored in areas where early malaria diagnosis and prompt treatment are readily available, as relatively high asexual parasite counts are seldom observed, and low-transmission settings where even low parasite densities are commonly associated with malaria-related symptoms.

Table 1
Challenges and suggestions for designing therapeutic efficacy studies of blood schizontocides.

Question	Rationale for the suggested approach
Parasitemia at enrolment	A minimum asexual parasite count of 250/μl is often suggested as an enrolment criterion, but may restrict patient recruitment in countries where parasitemias are low due to prompt diagnosis and treatment.
Molecular diagnosis	Molecular confirmation of single-species *P. vivax* infection is desirable. Molecular techniques may detect low-level parasite recrudescences earlier than microscopy as well as gametocyte-specific RNA transcripts.
Co-administration of PQ/TQ	PQ and TQ display blood schizontocidal activity and may mask partial resistance to CQ. Withholding CQ or TQ until day 28 is required to study CQ efficacy. Whether the late administration of hypnozoitocides affects their antirelapse efficacy remains unclear.
Supervised treatment	Multiple-dose regimens must be fully supervised and measurement of drug blood levels helps to confirm whether antimalarials were properly absorbed.
Follow-up duration	Follow-up must capture early and late parasite recrudescences, either symptomatic or not. At least 28 days are required for CQ, with longer follow-up to study more slowly eliminated blood schizontocides.
Blood CQ measurement	CQ levels must be measured at the day of parasite recurrence; drug levels at day 7 help to confirm adequate CQ intake and absorption.
Parasite genotyping	Parasite recrudescences, early relapses and new infections may occur during the study follow-up. Genetically identical parasites at day 0 and the day or recrudescence indicate a recrudescence or early relapse.

Abbreviations: CQ: chloroquine; PQ: primaquine; TQ: tafenoquine.

P. vivax infections in many areas of endemicity, especially in the Americas (Gonçalves et al., 2014), are routinely treated with a combination therapy: PQ is co-administered with CQ or another schizontocide for the radical cure (Price et al., 2014; Baird et al., 2016). Because of the significant blood schizontocidal activity of PQ *in vivo*, its concomitant administration may obscure the emergence of *P. vivax* resistance to CQ and other antimalarials (Baird et al., 2016; Commons et al., 2018). Therefore, withholding PQ until day 28 is strongly recommended to monitor CQ efficacy *in vivo* (WorldWide Antimalarial Resistance Network (WWARN), 2015). Even later administration of 8-aminoquinolines (e.g., at day 42) may be required after use of blood schizontocides with longer half-lives, such as MQ or piperaquine. Since the co-administration of CQ or other blood schizontocides may be required for the hypnozoitocidal activity of PQ (Baird and Hoffman, 2004), study participants who receive PQ at day 28 or later, when therapeutic CQ blood levels are no longer present, must be carefully monitored for relapses.

Previous use of antimalarials and the proper absorption and metabolization of study drugs must be evaluated in efficacy trials. Blood levels of CQ and other locally available blood schizontocides should ideally be measured at patients' enrolment, to exclude study participants presenting partially suppressive drug concentrations prior to treatment, but surprisingly this is rarely done in clinical trials. Drug levels measured at day 7 can confirm whether study drugs were properly absorbed and metabolized (WorldWide Antimalarial Resistance Network (WWARN), 2015). They must be further monitored at the day of parasite recurrence to confirm that blood-stage parasites have survived therapeutic levels of study drugs. A whole-blood concentration above 100 ng/ml of CQ and its main metabolite, desethylchloroquine (DCQ), is widely accepted although not thoroughly validated as the therapeutic level of CQ in in vivo studies (Baird et al., 1997). Therapeutic levels of other blood schizontocides, including partner drugs in widely used ACTs such as MQ and lumefantrine, remain to be determined for *P. vivax*.

CQ resistance is typically evaluated over 28 days of follow-up (PAHO, 2003; World Health Organization, 2015), although an extended 42-day follow-up may be required to capture late recrudescences (WorldWide Antimalarial Resistance Network (WWARN), 2015; Commons et al., 2018). Indeed, most recurring parasitemias following CQ treatment in a trial in Brazil (Siqueira et al., 2017), although not in Myanmar (Yuan et al., 2015), were diagnosed by microscopy between days 29 and 42, arguing for an extended follow-up to properly characterize CQ resistance *in vivo*. We speculate that at least some of these treatment failures between days 29 and 42 might have been detected earlier, at day 28, by using sensitive molecular diagnostic methods (Ladeia-Andrade et al., 2019). Moreover, delayed parasite clearance (on or after day 3) appears to predict CQ treatment failure, being associated with an increased rate of *P. vivax* recurrence by day 28 in a large individual patient pooled meta-analysis (Commons et al., 2018). Whether monitoring CQ responses *in vivo* requires more sensitive diagnostic methods and extended follow-up remains open to debate. Further data on CQ blood levels beyond day 28 of standard CQ regimens are urgently needed to inform clinicians and policy makers. Increased trial cost and the risk of cohort attrition due to the extended study duration and extra blood sampling must be considered. However, trials of slowly eliminated blood schizontocides such as MQ will surely require a longer follow-up.

There are no standard protocols for comparing day 0 and recurrence parasite genotypes in order to classify *P. vivax* recurrences as recrudescences, early relapses, or new infections. Indeed, parasites recovered from relapses following vivax malaria treatment can be either identical to or genetically different from those that caused the primary infection (de Araujo et al., 2012; Imwong et al., 2007; Lin et al., 2015; Van den Eede et al., 2010; Imwong et al., 2012). Furthermore, *P. vivax* infections often comprise multiple different (but sometimes closely

related) parasite genotypes, which further complicates the molecular characterization of recurrences (de Araujo et al., 2012; Lin et al., 2015). The reactivation of heterologous latent hypnozoites and the consecutive (during separate mosquito bites) or simultaneous inoculation of genetically different sporozoites (during a single mosquito bite; Bright et al., 2014; Nkhoma et al., 2012) all can originate multiple-clone infections. Moreover, putative molecular markers of drug resistance should ideally be characterized in recrudescent parasites that survive therapeutic drug levels, as currently done for *P. falciparum* (Malmberg et al., 2013).

Microsatellites and other length-polymorphic sequences are widely used as genotyping markers in clinical trials, but studies vary in the number of markers used and how differences between genotypes are interpreted (e.g., Koepfli et al., 2009; Negreiros et al., 2016; Siqueira et al., 2017; Ladeia-Andrade et al., 2019). A limited number of markers may lack discriminatory power when closely related parasites circulate in the study site (Popovici et al., 2018; Cowell et al., 2018). In addition, the use of different genotyping strategies reduces the comparability among studies (de Souza et al., 2015; Koepfli et al., 2009; Lin et al., 2015). Finally, amplification bias may impair the detectability of particular alleles in multiple-clone infections (de Souza et al., 2015; Havryliuk et al., 2008; Messerli et al., 2017) – e.g., template competition often favours the PCR amplification of shorter fragments. Importantly, although genotype-adjusted estimates of failure rates are often provided in clinical trials to characterize true recrudescences, any parasite recurrence in a patient that currently has therapeutic blood levels of a blood schizontocide may indicate drug resistance, regardless of its origin (whether a relapse, recrudescence or new infection).

P. vivax drug susceptibility may also be assessed *ex vivo* to allow for a more objective assessment of resistance patterns, with no influence of confounding factors such as patients' acquired immunity and antimalarial drug absorption and metabolism (Price et al., 2012). However, few studies have combined *in vivo* and *ex vivo* monitoring of antimalarial resistance in the same patients (e.g., Phyo et al., 2011; Ladeia-Andrade et al., 2019). Schizont maturation assays have gradually become more practical (Russell et al., 2012; Rangel et al., 2018) and are expected to be more often used in *P. vivax* drug resistance monitoring.

3. Natural variation in cytochrome P450 2D6 activity and metabolization of 8-aminoquinolines

PQ has been used as an anti-relapse drug for over 70 years, but only recently its hypnozoitocidal efficacy was attributed to active metabolites generated by the drug-metabolizing isoenzyme cytochrome P450 (CYP) 2D6 (Bennett et al., 2013; Pybus et al., 2012, 2013). Importantly, relapses despite PQ use can only be taken as an indirect evidence of PQ tolerance or resistance in patients displaying normal CYP2D6 activity.

The exact mechanism of action of PQ remains unknown, but there is evidence that the same metabolites that cause hemolytic toxicity in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency also have anti-relapse effect. Reactive oxygen species such as hydrogen peroxide (H_2O_2) were the first molecules implied in PQ-mediated oxidative damage and hemotoxic effects (Vasquez-Vivar and Augusto, 1992; Vásquez-Vivar and Augusto, 1994; reviewed in Marcsisin et al., 2016). These compounds are generated by oxidation and redox cycling of hydroxylated PQ metabolites, mainly 5-hydroxyprimaquine, produced by CYP2D6. The monoamine oxidase A (MAO-A), another key liver enzyme, is involved in the formation of carboxyprimaquine (cPQ) (Constantino et al., 1999; Pybus et al., 2012), which appears to lack pharmacological or toxic effects (Constantino et al., 1999; Mihaly et al., 1985). The rapid metabolism by the MAO-A pathway might be responsible for the short half-life of PQ, estimated at 4 h (Avula et al., 2018).

CYP2D6 is primarily expressed in the liver, where the majority of drugs are biotransformed prior to elimination (Zanger et al., 2008). CYP2D6 accounts for only 1–4% of the total hepatic CYPs, but metabolizes 20–25% of all drugs in clinical use – mostly antidepressants, antipsychotics, analgesics, beta-blockers, and antiarrhythmics (Ingelman-Sundberg, 2005; Zanger et al., 2004; Zanger and Schwab, 2013). Extensive genetic diversity at the *cyp2d6* locus, with over 130 alleles (https://www.pharmvar.org/gene/CYP2D6), causes significant variation in individual CYP2D6 activity. Alleles are associated with loss of activity, decreased, normal, or increased function.

CYP2D6 activity in therapeutic efficacy trials of PQ-containing regimens is typically inferred from genotyping results, but translating CYP2D6 genotypes into phenotypes remains a major challenge. Four pharmacogenetic phenotypes are traditionally inferred from the number of functional alleles: (a) carriers of two nonfunctional alleles are termed poor metabolisers; (b) intermediate metaboliser refers to carriers of one normal or functionally deficient allele, resulting in partially deficient metabolic capacity; (c) normal metaboliser refers to the expression of two functional alleles; and (d) ultrarapid metabolisers carry at least one increased function allele (i.e., multiple copies of a functional allele on one chromosome) in addition to a functional allele. Alternatively, activity scores (AS) for CYP2D6 alleles have been inferred from urinary metabolic ratios of a probe drug and its respective metabolite to quantify enzyme activity levels (see below; Gaedigk et al., 2017; Gaedigk et al., 2008). The AS system assigns a value ranging between 0 and 1 to each allele based on its expected enzyme activity. The sum of the values for both alleles define AS groups, which range from complete dysfunction ($AS = 0$) to ultra-rapid metabolism ($AS > 2$) (Gaedigk et al., 2008; Hicks et al., 2014).

Although the AS system has improved CYP2D6 phenotype prediction, important limitations persist (Gaedigk et al., 2018; Llerena et al., 2012; Ning et al., 2018). Multiple genetic factors may regulate CYP2D6 expression and activity, such as gene duplication/multiplication, gene deletion, defective splicing and single-nucleotide polymorphism (SNP) that regulate enzyme stability and its affinity for the substrate. For instance, Ray et al. (2019) showed that enhancer SNPs (e.g. rs5758550, 115 kb downstream of the CYP2D6 promoter region) substantially affect CYP2D6 activity (Ray et al., 2019; Wang et al., 2015). Furthermore, Ning et al. (2018) showed that CYP2D6 protein levels in human liver tissue samples are the major determinant of enzyme activity, while the AS system remains a good predictor only of poor metaboliser phenotypes.

The link between therapeutic efficacy of PQ and CYP2D6 activity has been established in observational and experimental studies using knockout mouse models (Potter et al., 2015; Pybus et al., 2013). The first clinical evidence came from a clinical trial of a *P. vivax* vaccine. Higher risk of malaria relapse and increased number of relapses were found in sporozoite-challenged volunteers with greatly reduced or absent CYP2D6 activity (Bennett et al., 2013). Since then, a large body of evidence for an association between clinical failure of PQ and impaired CYP2D6 metabolism has accumulated (Baird et al., 2018a,b; Brasil et al., 2018; Ingram et al., 2014; Silvino et al., 2016, 2020).

Reduced CYP2D6 activity may represent a major confounder in therapeutic efficacy studies across malaria-endemic settings. Two cohort studies in the Amazon Basin of Brazil found 20–25% of individuals with impaired CYP2D6 enzyme ($AS \leq 1$) (Silvino et al., 2020), who had increased risk of *P. vivax* recurrence following CQ-PQ treatment after adjusting for confounding variables (Silvino et al., 2016, 2020). Nonimmune individuals with impaired enzyme activity were significantly more susceptible to *P. vivax* recurrence (Silvino et al., 2020). A third study in the Amazon found 35% of subjects with reduced CYP2D6 activity predicted by genotyping, but no association between impaired enzyme activity and risk of *P. vivax* recurrence after standard CQ-PQ treatment was found; no adjustment for malaria immunity was made (Ladeia-Andrade et al., 2019).

Alternatively, the opioid dextromethorphan (DEM), which is metabolized by CYP2D6, has been used as a probe to directly measure enzyme activity in malaria patients. This is assessed by the log of the metabolic ratio of DEM to its metabolite dextrorphan in urine (Baird et al., 2018b; Bennett et al., 2013). Other probe drugs can be used, such

as sparteine, debrisoquine, and metoprolol, whose metabolites of interest are 2- and 5-dehydrosparteine, 4-hydroxydebrisoquine, and α-hydroxymetoprolol, respectively (Ito et al., 2010). Indeed, low CYP2D6 activity has been associated with elevated risk of *P. vivax* malaria recurrence in PQ-treated patients (Baird et al., 2018a,b). However, CYP2D6 activity measured with DEM may not be necessarily extrapolated to other CYP2D6 substrates, such as PQ (Hicks et al., 2014). The recent demonstration that CYP2D6 polymorphisms affect the production of active phenolic metabolites of PQ represents a major advance in the field (Spring et al., 2019). Reduced urinary levels of 5,6-ortho-quinone, a surrogate marker of the presumed active metabolite 5-hydroxyprimaquine, were measured in intermediate and poor metabolizers, consistent with the notion that CYP2D6-mediated biotransformation is essential for the therapeutic efficacy of PQ (Spring et al., 2019).

TQ is a slowly eliminated 8-aminoquinoline derivative with anti-relapse activity (Lacerda et al., 2019; Llanos-Cuentas et al., 2014; Llanos-Cuentas et al., 2019; reviewed in Baird, 2019). It remains to be determined how CYP2D6 polymorphisms impact TQ efficacy. Experimental studies with CYP2D knock-out mice (poor metabolisers) showed that TQ biotransformation by CYP2D is required for its activity as a hepatic schizontocide against *P. berghei* (Marcsisin et al., 2014). Importantly, changes in pharmacokinetic parameters of TQ and lower levels of its 5,6-ortho-quinone metabolite in knock-out mice indicate that these animals were less efficient at metabolizing TQ (Vuong et al., 2015). Noticeably, TQ pharmacokinetics was less affected than that of PQ in the same knock-out mouse model (Milner et al., 2016; Potter et al., 2015; Vuong et al., 2015) and TQ retained its tissue schizontocidal effect against *P. berghei* (Milner et al., 2016). Conflicting results between these experimental studies may be due to differences in the TQ doses used, approximately 8 times higher in Milner et al. (2016) compared with Marcsisin et al. (2014).

Data on the impact of CYP2D6 polymorphisms on TQ anti-relapse activity in humans remain limited (Lacerda et al., 2019; St Jean et al., 2016). Few poor metabolisers were enrolled in available studies and TQ efficacy did not appear to be reduced among intermediate metabolisers of CYP2D6. Further investigations are required to determine the impact of more extreme CYP2D6 phenotypes (i.e. AS < 1) on clinical efficacy of TQ.

4. Therapeutic efficacy trials of hypnozoitocides

Relapses are the main origin of recurrent *P. vivax* malaria infections in PQ-untreated individuals across endemic settings (Betuela et al., 2012; Adekunle et al., 2015; Robinson et al., 2015; Abreha et al., 2017; Commons et al., 2020). To prevent relapses, the World Health Organization recommends the co-administration of CQ and standard- (total, 3.5 mg/kg) or high-dose PQ (total, 7.0 mg/kg) distributed over 14 days to patients aged >6 months with normal G6PD activity who are neither pregnant nor breastfeeding (World Health Organization, 2015). Between 9% and 100% of *P. vivax* infections in different endemic settings are estimated to eventually relapse within 12 months if PQ is omitted (Baird and Hoffman, 2004). However, even PQ-treated individuals often experience one or more *P. vivax* relapses, highlighting the importance of more effective hypnozoitocidal therapies (Thomas et al., 2016). For example, 38% of the *P. vivax* recurrences observed over 12 months in a cohort of Brazilian patients treated with unsupervised CQ and standard PQ dose were attributable to relapses or late recrudescences; 12% of PQ-treated individuals were estimated to relapse during the follow-up (Corder et al., 2020a). Table 2 summarizes some key challenges for the design of clinical protocols to monitor the efficacy of anti-relapse regimens and the emergence of PQ-resistant *P. vivax* strains.

Because hepatocyte culture models that sustain hypnozoite development remain limited to very few research laboratories, therapeutic efficacy studies are the only practical way of monitoring the efficacy of hypnozoitocidal drugs. True PQ resistance has not been convincingly characterized *in vivo* (Fernando et al., 2011; Chu and White, 2016; Thomas et al., 2016; Milligan et al., 2020), but therapeutic efficacy studies may have overlooked emerging PQ resistance due to a wide range of design constraints. The optimal dose of PQ (and possibly of TQ) for radical cure of *P. vivax* is likely to differ across endemic settings (Goller et al., 2007; John et al., 2012; Price et al., 2020). Weight-based dosing is crucial, as subtherapeutic PQ doses are often prescribed for adult patients (Duarte et al., 2001). Ideally, PQ failure would be defined as a *P. vivax* recurrence after day 28 of treatment with PQ and an efficacious blood schizontocide (to minimize the risk of recrudescences) in a patient unexposed to new infections who is followed-up for at least 3–12 months to capture nearly all first relapses. However, large numbers of malaria patients are rarely available for enrolment and long-term follow-up in non-endemic sites. Not surprisingly, the vast majority of clinical trials of hypnozoitocides have been carried out in endemic settings, where relapses cannot be reliably distinguished from late recrudescences and new infections despite the widespread use of molecular genotyping in recent years (White and Imwong, 2012; Chu and White, 2016).

The concomitant use of PQ and blood schizontocides poses additional challenges to clinical trials. On the one hand, clinical trials in the 1950s showed that volunteers treated with PQ plus quinine (QN) or CQ and unexposed to reinfection experienced much less relapses than those given 3.5 mg/kg of PQ alone (Edgcomb et al., 1950; Alving et al., 1955), consistent with a potentiation of the hypnozoitocidal effect of PQ by CQ and QN (Alving et al., 1955; Baird and Hoffman, 2004). Importantly,

Table 2
Challenges and suggestions for designing therapeutic efficacy studies of hypnozoitocides.

Question	Rationale for the suggested approach
Type of infection at enrollment	Treatment-time-to-infection studies assume that the primary infection is sporozoite-induced and prone to relapse, but patients may be experiencing a relapse at the time of enrollment. Enrolling patients with no laboratory-confirmed *P. vivax* malaria episodes within the previous 6–12 months minimizes this risk.
G6PD deficiency screening	Patients with ≥30% of the normal G6PD activity are eligible for PQ use; only those above the 70% threshold are eligible for TQ. Quantitative tests are recommended to detect moderate G6PD deficiency.
CYP2D6 activity screening	Reduced CYP2D6 activity impairs PQ metabolization into active compounds and the simultaneous use of schizontocides may further inhibit CYP2D6 activity. Genetic variation in CYP2D6 activity and the timing of drug administration must be considered in study design and data analysis.
Supervised PQ administration	Compliance with multiple-dose PQ regimens is poor unless treatment is fully supervised.
Choice of partner schizonticide	Local patterns of CQ resistance must be considered and ACTs may be required to minimize the risk of recrudescence. Pharmacokinetic interactions between partner schizontocides and 8-aminoquinolines must be considered.
Inclusion of a control arm	Local relapse patterns should be ideally characterized by including a PQ- and TQ-untreated control arm; otherwise, PQ- and TQ-ineligible individuals may serve as historical controls.
Follow-up duration	Six months of follow-up capture nearly all relapses in tropical settings, but at least 12 months may be required in temperate regions.
Monitoring haemolysis	Hemoglobin levels must be monitored to quantify the combined effect of malaria-induced and PQ- or TQ-induced hemolysis.
Parasite genotyping	Hypnozoites from previous inoculations may be activated by the current infection and cause relapses of unrelated parasite genotypes, but repeated infections with identical parasites are very likely to be relapses, suggesting a role for molecular genotyping.

Modified from John et al. (2012). Abbreviations: ACT: artemisinin-based combination therapy; CQ: chloroquine; CYP2D6: cytochrome P450 2D6; G6PD: glucose-6-phosphate dehydrogenase; PQ: primaquine; QN: quinine; TQ: tafenoquine.

these studies showed a very limited anti-relapse action of PQ when administered alone (Baird and Hoffman, 2004), contrasting with recent results from Indonesia showing a high (>90%) anti-relapse efficacy of high-dose PQ even when not co-administered with a blood schizontocide (Nelwan et al., 2015). Interestingly, co-administration of CQ does not appear to enhance the hepatic schizontocidal activity of PQ that is required for primary prophylaxis of *P. vivax* malaria (Soto et al., 1999).

On the other hand, CQ can partially inhibit CYP2D6 activity, as evaluated by the phenotypic probes debrisoquine (Adedoyin et al., 1998) and metoprolol (Lancaster et al., 1990; Almeida et al., 2020), thereby decreasing the generation of pharmacologically active PQ metabolites. A decrease in CYP2D6-mediated biotransformation might explain the increased plasma levels of PQ and cPQ measured in healthy individuals co-administered CQ and PQ, compared with those given PQ alone (Pukrittayakamee et al., 2014). Other blood schizontocides, such as QN and artemisinin derivatives, appear to have similar CYP2D6-inhibitory effects (Marcsisin et al., 2016). The decrease in CYP2D6-mediated biotransformation of PQ following CQ administration is modest (Adedoyin et al., 1998), but might favor relapses in patients already carrying low-activity CYP2D6 variants who concomitantly receive standard doses of PQ. A delicate balance between the putative CQ-mediated potentiation of PQ activity and CQ-dependent inhibition of CYP2D6-mediated generation of active PQ metabolites will determine the efficacy of CQ-PQ regimens for the radical cure of vivax malaria (Ladeia-Andrade et al., 2019). Interactions between TQ and blood schizontocides remain largely unexplored, but there are recent concerns over the potential inhibitory effect of co-administered artemisinin derivatives on the anti-relapse activity of TQ. We suggest that the timing of PQ (and possibly TQ) administration relative to CQ and other blood schizontocides that inhibit CYP2D6 activity may require further optimization in order to maximize radical cure (Nelwan et al., 2015; Ladeia-Andrade et al., 2019).

Therapeutic efficacy studies and clinical trials of 8-aminoquinolines must exclude subjects with moderate to severe forms of G6PD deficiency, as they may develop life-threatening hemolysis following PQ or TQ treatment (Baird et al., 2018a). Therefore, G6PD deficiency screening is a key component of *in vivo* monitoring of PQ and TQ efficacy. G6PD plays an essential role in maintaining reduction–oxidation (redox) equilibrium of the cytoplasm and, ultimately, red blood cell integrity (Beutler, 1991). G6PD catalyses the rate-limiting step in the pentose phosphate pathway that reduces NADP while oxidizing glucose-6-phosphate. Since this pathway is the only source of NADPH for red blood cells, G6PD deficiency renders them specifically vulnerable to oxidative damage.

G6PD deficiency is inherited as an X-linked recessive disorder. It affects mainly men; hemizygous males who carry one deficient allele have residual enzyme activity typically below 30%. The phenotype is more variable in females, due to alternate X-chromosome inactivation resulting in red cell mosaicism (Beutler et al., 1962): heterozygous female displays varying degrees of G6PD activity, usually between 30% and 80% of normal G6PD activity (Domingo et al., 2019). Approximately 8% of individuals exposed to endemic *P. vivax* malaria carry one (or, rarely, two) of the numerous alleles causing G6PD deficiency worldwide (Baird et al., 2018a) – 60% are male hemizygotes and 36% female heterozygotes (Howes et al., 2012). G6PD deficiency is very prevalent in sub-Saharan Africa (where the mild A-variant predominates), but the greatest risk of severe hemolysis is found in Asian countries where variants conferring severe G6PD deficiency predominate. Over 35% of the population at risk of *P. vivax* malaria may be unable to receive safe and effective PQ therapy mainly due to G6PD deficiency and impaired CYP2D6 activity (Baird et al., 2018a).

Patients with 30–70% of the "normal" G6PD activity are not eligible for TQ use but may still receive PQ; those with <30% activity cannot be safely given PQ or TQ. A single 300 mg dose of TQ is equivalent to a total dose of 210 mg of PQ over 14 days in the magnitude of hemolysis it causes in G6PD deficient heterozygous females with 40–60% of normal activity (Rueangweerayut et al., 2017). Qualitative point-of-care tests are currently able to identify individuals with 30–40% of normal G6PD activity, including male hemizygotes and female homozygotes, but they may overlook female heterozygotes with a mixture of G6PD deficient and normal erythrocytes. A recent meta-analysis identified substantial inter-laboratory variation in measurements around the 70% threshold of G6PD activity using ultraviolet spectrophotometry, the most popular quantitative diagnostic method for G6PD deficiency. Measurements appear to be more robust at the 30% threshold (Pfeffer et al., 2020). Hand-held biosensors for point-of-care quantification of G6PD activity, which are currently under development, are expected to become available in the near future in malaria clinics worldwide.

Finally, the efficacy of supervised PQ treatment, measured in clinical trials with a PQ-untreated control arm, which is typically above 85% (John et al., 2012), does not directly translate into effectiveness estimates in real-life settings where the multiple-dose PQ treatment is unsupervised and adherence is likely to be poor (Grietens et al., 2010; Thriemer et al., 2018). Indeed, unsupervised PQ was shown to reduce by only 12% the risk of vivax malaria recurrence over one year of post-treatment follow-up in Papua, Indonesia (Douglas et al., 2017).

5. The mathematics of *Plasmodium vivax* relapses

Different mathematical models have been designed to quantify the proportion of *P. vivax* malaria recurrences that is caused by relapses. The simplest models assume that PQ eradicates all liver hypnozoites and estimate the proportion of relapses by comparing the time to first recurrence of PQ-treated and PQ-untreated patients using non-parametric survival analysis (Commons et al., 2020). Importantly, these analyses may be confounded by competing risk events, since late recrudescences and new infections often preclude the detection of relapses during the post-treatment follow-up (WorldWide Antimalarial Resistance Network (WWARN), 2019). Conversely, the most complex models account for competing risks and use a mixture of up to four parametric distributions to estimate the relative contribution of relapses in time-to-recurrence survival analysis (Taylor et al., 2019). Here we revisit a simple two-component mixture modeling approach (Corder et al., 2020a) and briefly discuss some key issues to consider in quantitative analyses of *P. vivax* relapses. We argue that exponential models can capture the dynamics of *P. vivax* relapses provided that clinical studies are properly designed (Corder et al., 2020a).

The simplest parametric survival distribution is given by assuming a constant risk over time, so the hazard is

$$\lambda(t) = \lambda \tag{1}$$

for all t. The corresponding survival function $S(\cdot)$ is defined by,

$$S(t) = e^{-\lambda t}. \tag{2}$$

Under the key assumption that hypnozoites have a constant activation rate over time, as expected for sympatric parasites, relapses have been parametrically modelled by exponential survival distributions (Equation (2); Adekunle et al., 2015; Corder et al., 2020a). This assumption does not hold for heterogeneous populations, where more complex mixture distributions may be necessary to properly model time-to-recurrence datasets (Lover et al., 2014; Taylor et al., 2019). For example, the combined analysis of times to relapse of experimental infections with *P. vivax* strains of disparate geographic origin yielded a bimodal distribution, with clearly separated early and late relapse patterns, which was best fitted with a mixture of Gompertz distributions to account for early and late relapse patterns (Lover et al., 2014). Relapses observed in multicentric clinical trials (e.g., Lacerda et al., 2019; Llanos-Cuentas et al., 2019) are also expected to violate the assumption of constant hypnozoite activation rate over time, with clear (often overlooked) implications for data analysis. Conversely, unimodal distributions in the times to *P. vivax* relapse tend to be observed in

geographically homogeneous samples (e.g., Simões et al., 2014), but more geographically representative data are needed to draw definitive conclusions.

The assumption of constant new infection rates over time may also not hold for new infections. This is due to the substantial risk heterogeneity in endemic populations, which affects the dynamics of new infections in time-to-event analysis. Typically, 20% of the exposed individuals contribute over 80% of the overall *P. vivax* malaria burden (e.g., Corder et al., 2020b). Because high-risk individuals tend to be infected earlier during the post-treatment follow-up, they are selectively removed from the susceptible population and those who remain uninfected are at low risk of infection, due to limited exposure or acquired immunity. As a consequence, the incidence of new cases gradually decreases over time during the follow-up, violating the constant rate assumption (Corder et al., 2020b).

We can, however, enroll study participants who are at a roughly similar risk of new infections. To this end, we suggest to exclude patients with one or more recent malaria episodes (e.g., within the past 6 months) before the current infection, as these subjects are more likely to be at higher-than-average risk of repeated infections (Corder et al., 2020a). Importantly, this approach also minimizes the risk that the current infection in treatment-time-to-reinfection studies is actually a relapse from a recent infection (either PQ-treated or not), rather than a sporozoite-induced, relapse-prone new infection. Finally, excluding patients with recent malaria episodes reduces the probability that patients have, at the time of enrolment, a pre-existing heterogeneous pool of hypnozoites from previous infections that can reactivate at different times during the post-treatment follow-up.

The survival function described by Equation (2) assumes that all patients will have recurrences as time goes to infinity. This assumption does not hold when only relapses are considered – only patients with hypnozoites are susceptible to this event. A large proportion of PQ-untreated patients is expected to harbor hypnozoites and eventually relapse, compared with a small proportion of those treated with PQ, who can still relapse due to poor adherence to PQ treatment, reduced PQ biotransformation, or partial PQ resistance or tolerance. Assuming (a) a constant hypnozoite activation rate λ and (b) that a fraction p of the population is susceptible to relapses, the parametric survival function can be written as

$$S(t) = (1-p) + pe^{-\lambda t}. \tag{3}$$

P. vivax infection following treatment in endemic settings may originate from recrudescences of blood-stage parasites that resisted schizontocidal treatment, new sporozoite-induced infections with either *P. vivax* or another malaria parasite, or relapses. Moreover, relapses may originate from hypnozoites from the primary sporozoite-induced infection or from previous infections, especially in countries where PQ is not widely used. These typical competing risk events (Lau et al., 2009) can bias survival analysis in high-risk individuals because new infections preclude the detection of relapses, the event of interest, and, conversely, frequent relapses due to a large pre-existing hypnozoite reservoir preclude the detection of new infections. These recurrent events cannot be distinguished on clinical grounds and molecular genotyping may be only partially helpful. Finding genetically identical parasites during the primary infection and the recurrence in a genetically diverse population strongly suggests a relapse, rather than a new infection, but recrudescences are also possible in areas where resistance to commonly used schizontocides is present (e.g., Taylor et al., 2019). Moreover, primary infections often comprise multiple clones, many of them meiotic siblings and half-siblings co-inoculated during a single mosquito bloodmeal. Not surprisingly, relapses can be caused by siblings that remained undetected during the primary infection (e.g., Bright et al., 2014; Cowell et al., 2018). However, recurrences caused by meiotic siblings are not synonymous with relapses, as siblings can occasionally be found in new infections, especially in areas with low to moderate malaria transmission where highly inbred parasite lineages co-circulate over extended periods of time (de Oliveira et al., 2020).

If individuals at very high risk are not enrolled, we can readily distinguish the fast dynamics dominated by PQ-preventable relapses/late recrudescences and the slow dynamics dominated by new infections. Most relapses and perhaps a few late recrudescences – if some degree of resistance to the blood schizontocide in use is present – occur within weeks or few months after a primary sporozoite-induced infection and are described by the hypnozoite activation rate λ. New infections are described by the constant infection rate β, with $\beta \ll \lambda$. Assuming that only a fraction p of the population is susceptible to relapses and late recrudescences and all enrolled patients are susceptible to new infections, the parametric survival function can be written as

$$S(t) = (1-p)e^{-(\beta)t} + pe^{-(\lambda+\beta)t}. \tag{4}$$

The fraction p of the population that is susceptible to relapses accounts for those who actually relapsed and those who would eventually relapse but had a new infection (competing event) first.

To fit competing risk survival models to empirical data (Fig. 3), let Y be the time to event (malaria episode – either relapse/late recrudescence or new infection) with survival distribution function $S(\cdot, \boldsymbol{\theta})$ and density function $f(\cdot, \boldsymbol{\theta})$, where $\boldsymbol{\theta}$ is a parameter vector (in this case, $\boldsymbol{\theta} = (p, \lambda, \beta)$). Let Z be a censoring event (e.g., a *P. falciparum* infection or the end of follow-up) such that the observable time variable (X) is the minimum between Y and Z, and δ, an indicator variable denoting whether a time of malaria episode or a time of censoring was observed. The actual data to be observed for each subject may then be represented by

$$X = \min(Y, Z) \quad \text{and} \quad \delta = \mathrm{I}(X = Y).$$

Assuming independent and non-informative censoring, the likelihood function for the underlined survival model for inference on $\boldsymbol{\theta}$, based on data from N individuals, is defined as follows,

$$l(\boldsymbol{\theta}|(\boldsymbol{X}, \boldsymbol{\delta})) = \prod_{i=1}^{N} [f(X_i, \boldsymbol{\theta})]^{\delta_i} \cdot [S(X_i, \boldsymbol{\theta})]^{1-\delta_i} \tag{5}$$

with $\boldsymbol{X} = (X_1, \ldots, X_n)$ and $\boldsymbol{\delta} = (\delta_1, \ldots, \delta_n)$. If study selected subjects are randomly allocated to the PQ or no-PQ arms, rates λ and β should be the same for both groups. The only expected difference between treatment arms is in the proportion p of individuals who are susceptible to relapses, i.e., $p_{PQ} \neq p_{nPQ}$. In this case, the product $l_{PQ} \cdot l_{nPQ}$ of the likelihood functions of PQ-treated and -untreated individuals, respectively, should be maximized over the parameters $(p_{PQ}, p_{nPQ}, \lambda, \beta)$ in order to improve parameter identifiability. However, due to the low number of parameters and that $\beta \ll \lambda$, parameter identifiability may not be an issue in this modeling approach. The maximum likelihood parameter and their respective credible intervals can be estimated using Bayesian approaches (e.g., Corder et al., 2020a).

The simple modeling approach outlined here can be readily applied to different treatment scenarios, such as clinical trials (Llanos-Cuentas et al., 2014, 2019; Lacerda et al., 2019) and prolonged antimalarial stock outages (Douglas et al., 2017), to estimate how many relapses and late recrudescences can be prevented by PQ or TQ in the presence of competing risks, provided that the 8-aminoquinoline treatment is omitted in the comparator group. No parasite genotyping data are required for inference. Importantly, the proposed analysis does not assume complete relapse suppression by treatment (Commons et al., 2020), but rather estimates the proportion of infections described by the fast and slow dynamics (Fig. 3). This approach is consistent with real-life scenarios with variable absorption and biotransformation of drugs and relatively poor adherence to treatment regimens.

6. *Ex vivo* monitoring of resistance to blood schizontocides

Ex vivo assays of *P. vivax* drug susceptibility are carried out over 42–46 h with patient-derived parasite samples cultured in the presence

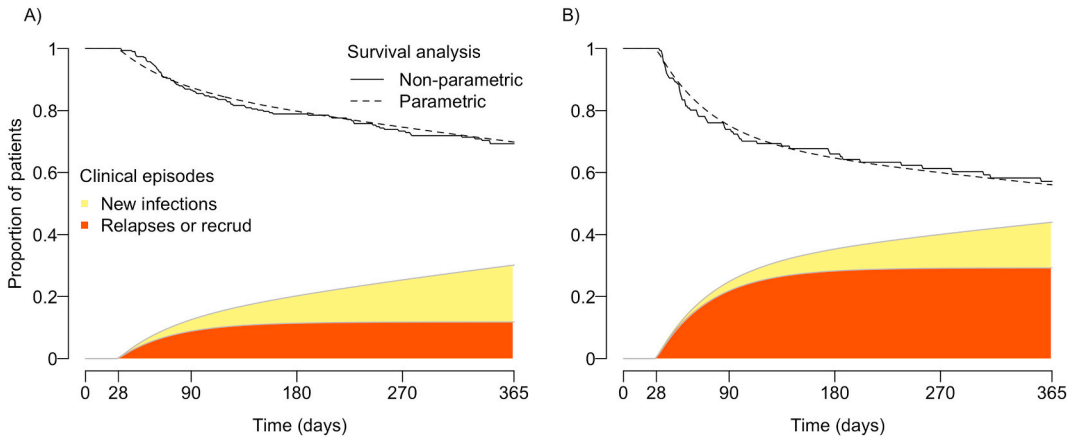

Fig. 3. Competing risk survival model applied to real-life data from vivax malaria patients who received standard chloroquine regimens with (A) or without (B) concomitant primaquine. Continuous lines represent the non-parametric Kaplan-Meier survival function and dashed lines represent the fitting of a competing risk survival model (Equation (3)) to empirical data. The red area represents the cumulative proportion of patients experiencing PQ-preventable relapses/late recrudescences following treatment (fast dynamics) and the yellow area represents the cumulative proportion of patients experiencing new infections (slow dynamics). Redrawn from Corder et al. (2020a). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of increasing drug concentrations, but the low parasitemias that are characteristic of clinical isolates (often <0.1% and are rarely >0.5%) severely limit their use. Moreover, test results are affected by factors such as previous patient use of antimalarials, the synchronicity and blood stage composition of clinical infections, time delays in processing clinical samples, variations in methods used for sample cryopreservation and thawing and differences in assay protocols, including the choice of culture media and methods for quantifying parasite growth (Russell et al., 2008; Russell et al., 2012; Lim et al., 2016b; Rangel et al., 2018; Thomson-Luque et al., 2019).

Due to the lack of a robust *in vitro* continuous culture system for *P. vivax* (Thomson-;Luque et al., 2019), primary isolates, fresh or cryopreserved, can typically only be tested for antimalarial resistance at a single time point; assay reproducibility within and between laboratories remains largely unaddressed (Baird et al., 2016). Importantly, short-term assays are not well suited to evaluate slow-acting antimalarials such as antibiotics and 8-aminoquinolines (Russell et al., 2012). Given all these limitations, *ex vivo* assays with *P. vivax* remain mostly restricted to research settings. However, recent technical advances can lead to a more frequent use of *P. vivax* schizont maturation tests in clinical and public health settings. Significant progress has been made over the past few years to improve access to and use of clinical isolates (such as the use of cryopreserved isolates and development of better protocols for parasite enrichment) and to process samples prior to drug assays (simple and efficient leukocyte depletion).

Fig. 4 summarizes the steps of sample processing, cryopreservation and thawing (if deemed necessary), and enrichment for *P. vivax* blood stages. Choosing an appropriate anticoagulant for sample collection (heparin or citrate) is essential, since the often-used ethylenediamine tetraacetic acid (EDTA) may reduce parasite viability (Russell et al., 2012). Leukocyte depletion prior to sample cryopreservation is another key step, which prevents parasite phagocytosis leading to a decrease in parasitemia during *ex vivo* culture that can be mistakenly interpreted as an effect of the antimalarial drug being evaluated (Russell et al., 2012).

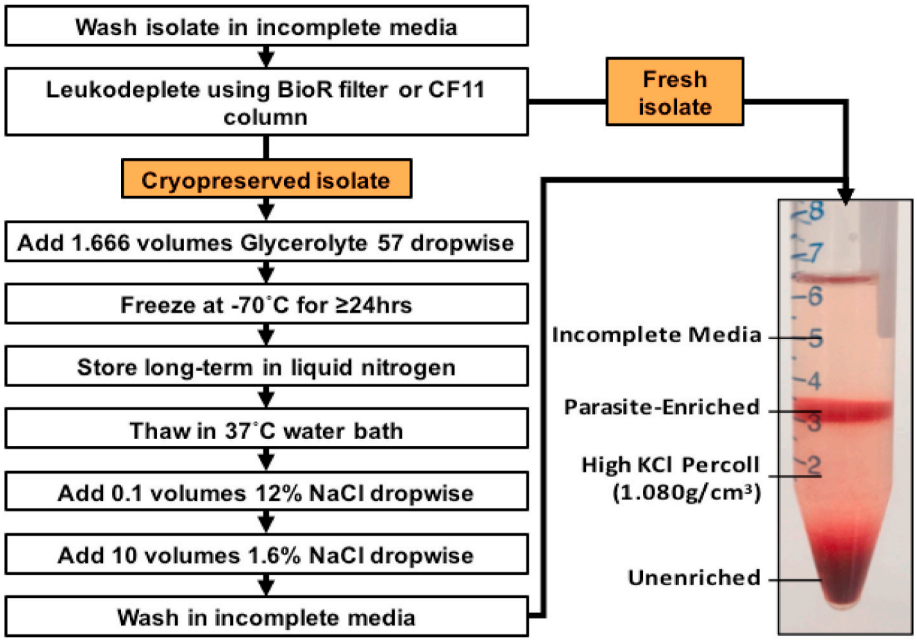

Fig. 4. Workflow for blood sample processing in the field, cryopreservation and thawing (if deemed necessary) and *Plasmodium vivax* enrichment prior to *ex vivo* schizont maturation assays.

The most popular method for leukocyte removal is cellulose filtration using CF11 (Sriprawat et al., 2009 Venkatesan et al., 2012) or another commercially available medium-sized cellulose fibre powder (Russell et al., 2012). Leukocyte depletion filters used in transfusion medicine (e.g., BioR 01 Plus; Fresenius Kabi, Germany) are more practical for field use than cellulose columns, and likely more efficient for complete leukocyte removal (de Oliveira et al., 2017). Alternatively, leukocyte depletion may be achieved with commercially available Plasmodipur filters (Janse et al., 1994) and non-woven fabric filters (Tao et al., 2011).

The use of fresh primary isolates of *P. vivax* is always preferable for drug resistance monitoring, since the freeze-thaw cycle may selectively kill minor parasite clones and surviving parasites may not be entirely representative of the original sample. However, using fresh parasites requires a laboratory infrastructure that is often lacking in resource-poor endemic settings. Better protocols are now available for sample cryopreservation and thawing of viable parasites (Shaw-Saliba et al., 2016) that may be combined with a 10- to 100-fold parasite enrichment, by KCl Percoll gradient centrifugation, prior to *ex vivo* testing (Fig. 4; Rangel et al., 2018). If a basic laboratory structure for leukocyte filtering, sample aliquoting, and freezing in liquid nitrogen is available in the field, cryopreserved *P. vivax* blood stages can be shipped to well-equipped reference national or international laboratories for use in a wide range of experiments, including *ex vivo* maturation assays.

Because only early-stage parasites typically survive cryopreservation and thawing (Rangel et al., 2018), cryopreserved samples are highly synchronous whereas fresh isolates often comprise mixtures of several parasite development stages (Lim et al., 2016a). Parasite synchronization is key for evaluating the sensitivity to antimalarials with striking stage-specific differences in efficacy. CQ, for example, is active against early ring stages but late trophozoites and schizonts are inherently CQ-resistant (Russell et al., 2008). Parasite synchronization also facilitates DNA-based quantification methods, such as ^{3}H-hypoxanthine incorporation (see below), as the assays can be timed for readout during schizogony, where DNA content is maximized, increasing sensitivity (Rangel et al., 2018).

Short-term culture conditions have been further optimized for *P. vivax* in recent years. Compared with the commonly used McCoy's 5 A culture medium, the alternative Iscove's modified Dulbecco's medium (IMDM) has recently been shown to double the survival rate of *P. vivax* through the initial round of *ex vivo* maturation (Rangel et al., 2018). It remains unclear whether IMDM provides additional nutrients required for survival of *P. vivax* blood stages that other media (including McCoy's 5 A) fail to provide or, alternatively, whether IMDM has less of a particular component present in other media that may be detrimental to the parasite (Rangel, 2019).

Finally, more practical methods are now available for accurately and rapidly quantifying low parasitemias that are characteristic of *P. vivax*, either via optical microscopy (Lim et al., 2016b) or flow cytometry (Wirjanata et al., 2015). Isotopic metabolic labelling with ^{3}H-hypoxanthine incorporation, little used in the past decade (Kosaisavee et al., 2006), was recently improved and became a valuable option to scale up assay reading (Rangel et al., 2018). The currently available robust protocols for sample processing, *ex vivo* parasite maturation and assay readout now allow parallel experimentation with a large number of cryopreserved *P. vivax* samples, thereby greatly reducing inter-assay variation (Rangel, 2019).

7. Molecular monitoring of chloroquine resistance

A genetic marker of drug resistance would ideally be a single mutation event with a clearly measurable phenotypic consequence; its presence would accurately predict a change in phenotype. The K76T substitution at the *P. falciparum chloroquine resistance transporter* (*pfcrt*) locus stands out as an example, being firmly (although not absolutely) associated with *ex vivo* resistance assay outputs and treatment failure *in vivo*. A genetic marker of CQ resistance remains unavailable for *P. vivax*, perhaps because the phenotype depends on several alternative molecular mechanisms converging for the same outcome; no optimal mutation leading to the CQ-resistant phenotype appears to have been selected. Markers may alternatively be quantitative – e.g., the increased expression of the *pfcrt* orthologous gene of *P. vivax*, *pvcrt-o*, has been tentatively associated with CQ treatment failure (see below). However, the requirement for RNA transcript quantification limits their use unless transcription changes are associated with gene amplification and other structural changes that can be readily identified on genomic DNA samples.

The search for CQ resistance markers in *P. vivax* has focused on orthologs of genes with known role in *P. falciparum* resistance to this drug — namely, *pvcrt-o* and *pvmdr1*. The discovery of the *pfcrt* gene (Fidock et al., 2000) and of the K76T single mutation required for CQ resistance and treatment failure in *P. falciparum* (Djimdé et al., 2001) raised the possibility that a similar CQ resistance mechanism would exist in *P. vivax*. Nevertheless, presently available *in vivo* and *ex vivo* data does not support the use of nucleotide substitutions and copy number variation at the *pvmdr1* and *pvcrt-o* loci as robust markers of *P. vivax* response to CQ.

The *P. vivax* ortholog of *pfcrt*, initially referred to as *pvcg10* but currently known as *pvcrt-o*, was characterized nearly two decades ago (Nomura et al., 2001). Similar to its *P. falciparum* ortholog, *pvcrt-o* displays an intron-rich gene structure with 14 exons (size range, 45–266 base pairs [bp]) that encodes a protein with 451 amino acids and 10 membrane domains. Sequence polymorphism is relatively limited at the *pvcrt-o* locus (Fig. 5), with most minor alleles present at frequencies $<$ 5%. One of them stands out: a lysine (AAG) insertion at amino acid position 10 (K10), which was initially discovered in Southeast Asian strains and suggested to be associated with CQ resistance (Lu et al., 2011). Copy number variation also occurs in *pvcrt-o* and has similarly been associated with the CQ resistant phenotype in Brazil (Silva et al., 2018) but not in Cambodia (Roesch et al., 2020). However, no *pvcrt-o* polymorphism has so far been unequivocally shown to predict CQ treatment failure *in vivo* or CQ resistance *ex vivo* in different laboratories across the World (Suwanarusk et al., 2007, 2008; Barnadas et al., 2008; Orjuela-Sánchez et al., 2009; Lu et al., 2011, 2012; Ganguly et al., 2013; Melo et al., 2014; Marques et al., 2014; Li et al., 2020; Roesch et al., 2020). Interestingly, a recent analysis of a genetic cross progeny appears to support upregulated *pvcrt-o* gene expression as a mechanism of CQ resistance in *P. vivax* (Sá et al., 2019). The association between increased *pvcrt-o* gene expression and CQ treatment failure originally described in a clinical trial in Brazil (Melo et al., 2014) has not been reproduced in Indonesia (Pava et al., 2015), Additionally, *pfcrt*-driven CQ resistance in *P. falciparum* is reversed by the calcium-channel blocker verapamil, but such an effect has not been observed with up to 0.9 μM of verapamil added to *ex vivo* short-term cultures of CQ-resistant *P. vivax* from Thailand (Suwanarusk et al., 2007). These negative findings have recently been confirmed in Papua, Indonesia (Wirjanata et al., 2017). Taken together, these findings point to different mechanisms of CQ resistance in these two species.

The gene coding for the *P. vivax* ortholog of *pfmdr-1*, a secondary CQ resistance factor in *P. falciparum*, was characterized in 2005 (Brega et al., 2005; Sá et al., 2005). It encodes a protein with 12 transmembrane domains and 1464 amino acids, with an apparent molecular mass of 165 kDa (Fig. 6). Two common nonsynonymous nucleotide substitutions were soon identified at the *pvmdr-1* locus, Y976F and F1076L, but no causal association could be established with CQ resistance. However, IC_{50} values for CQ were found to be 4 to 6-fold higher among *P. vivax* samples from Papua, Indonesia, that harbor the Y976F change, compared with the wild type samples from Thailand (Suwanarusk et al., 2007). The near fixation of the Y976F change in Indonesia, where CQ resistance is extremely common, provided some indirect support for the association between *pvmdr-1* polymorphism and CQ resistance (Suwanarusk et al., 2007).

Nevertheless, this clear-cut association between Y976F change and

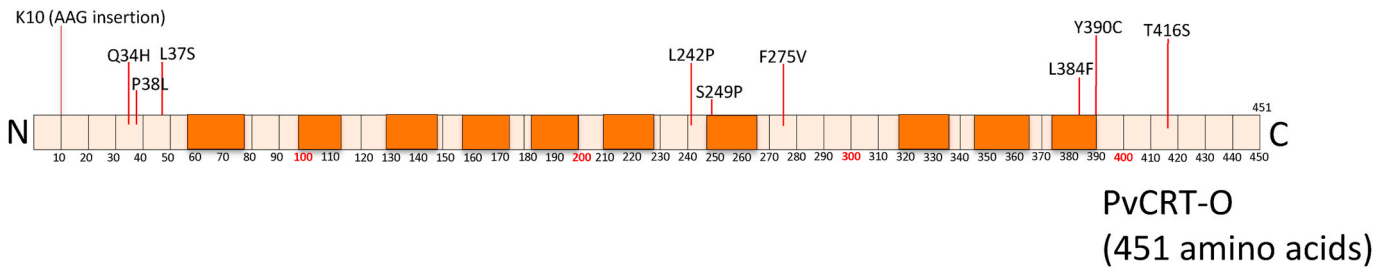

Fig. 5. Amino acid substitutions found in the *Plasmodium vivax* chloroquine resistance transporter protein (PvCRT-O). The 10 transmembrane domains (TMDs) are represented by orange boxes: amino acid positions 58–78 (TMD1), 95–112 (TMD2), 129–148 (TMD3), 157–174 (TMD4), 183–200 (TMD5), 209–228 (TMD6), 247–265 (TMD7), 317–336 (TMD8), 345–365 (TMD9), and 374–390 (TMD10). TMD position and sizes as per Nomura et al. (2001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

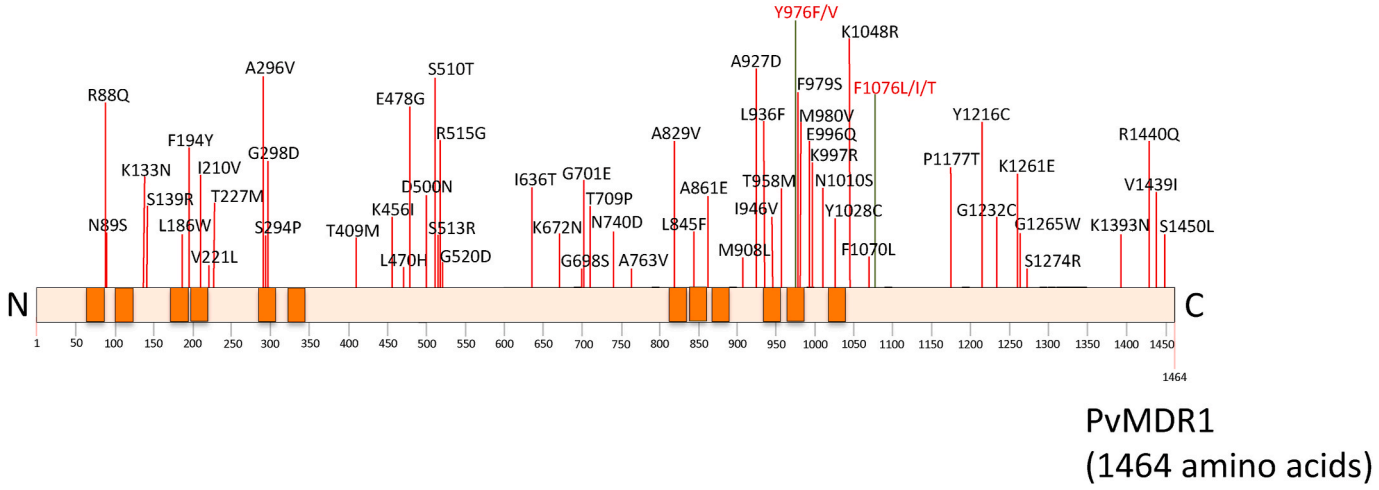

Fig. 6. Amino acid substitutions found in the *Plasmodium vivax* multidrug resistance 1 protein (PvMDR-1). The 12 transmembrane domains (TMDs) are represented by orange boxes: amino acid positions 63–85 (TMD1), 100–122 (TMD2), 171–193 (TMD3), 197–218 (TMD4), 285–307 (TMD5), 323–345 (TMD6), 813–835 (TMD7), 839–860 (TMD8), 868–890 (TMD9), 935–957 (TMD10), 965–986 (TMD11), and 1018–1040 (TMD12). TMD position and sizes as per Sá et al. (2005). Two common nonsynonymous substitutions found in PvMDR-1, Y976F and F1076L, are highlighted in red. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CQ response *ex vivo* has not been confirmed in subsequent studies in Southeast Asia (Shalini et al., 2014; Rungsihirunrat et al., 2015; Roesch et al., 2020; Li et al., 2020), East Asia (Lu et al., 2011, 2012; Li et al., 2020), and South America (Chehuan et al., 2013; Aguiar et al., 2014). Additionally, *pvmdr1* gene polymorphism does not predict CQ treatment failure in clinical trials (Orjuela-Sánchez et al., 2009; Ganguly et al., 2013; Shalini et al., 2014; Melo et al., 2014; Marques et al., 2014). Finally, copy number variation at the *pvmdr-1* does not appear to modulate CQ sensitivity (Suwanarusk et al., 2008; Lu et al., 2011; Htun et al., 2017; Silva et al., 2018; Li et al., 2020).

8. Final remarks

Monitoring *P. vivax* drug resistance relies mostly in therapeutic efficacy studies. Recommendations for change first-line blood schizontocidal regimens in use are based on estimated treatment failure rates *in vivo*. However, clinical studies are laborious and expensive; they are often underpowered for comparing different treatment regimens and for revealing interpretable temporal trends in drug resistance patterns. Although relapses are widely recognized as a major contributor to the overall burden of vivax malaria, data on the anti-relapse efficacy of available hypnozoitocides remain scarce and the very existence of PQ resistance is disputed. Clinical studies to fill this knowledge gap are particularly challenging to design, execute, and analyze. *Ex vivo* monitoring of *P. vivax* response to blood schizontocides remains underutilized by national malaria control programs, despite the gradual advances towards more practical and reproducible assay protocols. Laboratory personnel should be able to carry out *ex vivo* assays routinely in endemic countries, provided that appropriate training is offered. Finally, robust molecular markers of *P. vivax* drug resistance are urgently needed. Nucleotide substitutions, copy number variation, and changes in expression levels have been extensively characterized in the *pvcrt-o* and *pvmdr-1* genes, whose orthologs in *P. falciparum* genes play a well-established role in CQ efficacy, but they have not been unequivocally associated with the CQ resistant phenotype in *P. vivax*. The only exception may be the increased expression of *pvcrt*-o, which appears to be associated with the CQ resistance phenotype in a recent genetic cross of *P. vivax* (Sá et al., 2019). Clinical and *ex vivo* studies with a global sample of CQ-resistant parasites are now required to confirm this important finding.

Declaration of competing interest

The authors of the manuscript “Monitoring *Plasmodium vivax* resistance to antimalarials: persisting challenges and novel approaches” declare that they have no conflicts of interest.

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