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The molecular basis of antimalarial drug resistance in *Plasmodium vivax*

Lucas E. Buyon [1], Brendan Elsworth [1], Manoj T. Duraisingh [*]

Department of Immunology and Infectious Diseases, Harvard T. H. Chan School of Public Health, Boston, 02115, MA, USA

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ABSTRACT

Plasmodium vivax is the most geographically widespread cause of human malaria and is responsible for the majority of cases outside of the African continent. While great progress has been made towards eliminating human malaria, drug resistant parasite strains pose a threat towards continued progress. Resistance has arisen to multiple antimalarials in *P. vivax,* including to chloroquine, which is currently the first line therapy for *P. vivax* in most regions. Despite its importance, an understanding of the molecular mechanisms of drug resistance in this species remains elusive, in large part due to the complex biology of *P. vivax* and the lack of *in vitro* culture. In this review, we will cover the extent and challenges of measuring clinical and *in vitro* drug resistance in *P. vivax*. We will consider the roles of candidate drug resistance genes. We will highlight the development of molecular approaches for studying *P. vivax* biology that provide the opportunity to validate the role of putative drug resistance mutations as well as identify novel mechanisms of drug resistance in this understudied parasite. Validated molecular determinants and markers of drug resistance are essential for the rapid and cost-effective monitoring of drug resistance in *P. vivax,* and will be useful for optimizing drug regimens and for informing drug policy in control and elimination settings.

1. Introduction

Plasmodium vivax and *Plasmodium falciparum* are responsible for the vast majority of malaria cases globally, with *P. vivax* causing most malaria cases outside of the African continent (Price et al., 2014; WHO, 2019b). The significant progress that has been made towards reducing malaria transmission is threatened by the emergence of drug resistance in *P. falciparum* to all clinically used antimalarials (WHO, 2019a). Several recent reviews have summed up the state of knowledge around *P. falciparum* drug resistance (Haldar et al., 2018; Wicht et al., 2020). *P. vivax* resistance to antimalarial drugs, including chloroquine (CQ), mefloquine (MQ), sulfadoxine, and pyrimethamine (SP), has also been reported in many regions of the world (Baird et al., 1997; Fryauff et al., 1998; Hastings et al., 2005; Hastings and Sibley, 2002; Price et al., 2014). High-grade CQ-resistance (CQR) in *P. vivax* has emerged in Indonesia, Malaysia, and Papua New Guinea, and has been associated with severe and fatal malaria due to treatment failure (Asih et al., 2011; Grigg et al., 2016). However, the extent and public health consequences of drug resistance in *P. vivax* remains poorly defined in most regions of the world.

The standard treatment regimen for *P. vivax* in most regions of the world is a combination of CQ, which targets the blood stage, with either primaquine (PQ) or tafenoquine (TQ), which are used to clear dormant liver stage forms, known as hypnozoites, for radical cure. Hypnozoites can remain dormant for weeks to years before reactivating and initiating a new blood stage infection, known as a relapse (Fig. 1) (Hill et al., 2006; White, 2011). In several regions, the first-line treatment is now artemisinin combination therapies (ACTs) + PQ/TQ due to CQR or a high frequency of mixed infections with CQR *P. falciparum* (Douglas et al., 2010; Price et al., 2014; WHO, 2019a). The requirement to treat patients with PQ or TQ for radical cure is complicated by their propensity for causing hemolysis in patients with G6PD deficiency, preventing their use in a significant subset of patients (5% of global population according some estimates) (Luzzatto et al., 2020; Nkhoma et al., 2009). Another host genetic factor that can affect drug efficacy is polymorphisms in CYP2D6, that result in the reduced metabolism of PQ into its active metabolites for the clearance of hypnozoites (Baird et al., 2018; Bennett et al., 2013; Camarda et al., 2019).

In comparison to *P. falciparum*, CQR has spread relatively slowly in *P. vivax*. It has been hypothesized that there is reduced selection pressure in *P. vivax* compared to *P. falciparum* due to its lower parasite biomass, its ability to produce gametocytes early in infection, and to

* Corresponding author.
E-mail address: mduraisi@hsph.harvard.edu (M.T. Duraisingh).
[1] These authors contributed equally to this work.

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relapse from liver hypnozoites, which allows the parasite to transmit before drug treatment or after drug concentration has waned (Schneider and Escalante, 2013). The blood stage activity of PQ/TQ could also reduce the transmission of CQR parasites.

Despite more than two decades of research into drug-resistant *P. vivax*, and as molecular markers of drug resistance in *P. falciparum* have been identified, molecular markers of resistance in this species remain elusive (Price et al., 2014; WHO, 2019a). This is in large part due to the lack of *in vitro* culture and transgenic systems for *P. vivax,* which has been essential for studying *P. falciparum* drug resistance. The literature often refers to the presence of polymorphisms in putative resistance genes as evidence of drug resistance in that region, however, the current evidence for the role of many of these mutations in drug resistance is relatively weak, potentially leading to false conclusions that could impact drug policy. Validated drug resistance mutations could be used to rapidly and cost effectively survey drug resistance in an area over time. This information is essential for identifying the most effective treatment policy in a region and determining when it is appropriate to switch between first line antimalarials. Furthermore, an understanding of the molecular mechanisms of drug resistance, as well as the development of heterologous model systems to study them will be useful for the development of future antimalarials.

Despite substantial technical challenges towards interrogating the molecular mechanisms of drug resistance in *P. vivax*, significant progress has been made in recent years. In this review, we will summarize the current knowledge pertaining to the molecular basis of *P. vivax* drug resistance. We will consider the usefulness of polymorphisms in putative resistance genes as predictors of *in vivo* and *in vitro* drug resistance, and describe the identification of novel putative resistance markers. We will also highlight recent technological breakthroughs in studying *P. vivax*, and discuss the potential of these new techniques to identify and validate drug resistance markers.

2. Assessing *in vivo* and *in vitro* drug resistance in *P. vivax*: a lack of robust phenotypes

A large number of polymorphisms have been identified in putative drug resistance genes, including *multidrug resistance gene 1* (*pvmdr1*), *chloroquine resistance transporter* (*pvcrt*), *kelch 12* (*pvk12*), *dihydrofolate reductase-thymidylate synthase* (*pvdhfr-ts*), and *dihydropteroate synthase* (*pvdhps*). However, it is likely that many of these mutations do not affect drug sensitivity. Linking *P. vivax* mutations to a drug sensitivity phenotype, *in vivo* and *ex vivo*, is essential for narrowing the number of polymorphisms to validate as resistance markers. However, identification of resistance mutations in *P. vivax* remains difficult for some drugs, including CQ, in large part because defining drug-resistant *P. vivax* phenotypes is itself is difficult. We will discuss the strengths and limitations of currently available methods to measure drug sensitivity in *P. vivax* to the different antimalarials used clinically, and the current evidence of drug resistance globally.

2.1. Measuring in vivo drug resistance

Identifying resistance to CQ and ACTs has remained difficult in *P. vivax* for multiple reasons. The most clear definition of CQR in *P. vivax* is the ability to grow in CQ concentrations that would normally kill (CQ concentration >100 ng/ml), although it should be noted that this cutoff for resistance is somewhat arbitrary and may not identify low-grade resistance (Baird et al., 1997; Price et al., 2014). A high rate of treatment failure by day 28 is often used as a measure of resistance in the populations, however, the majority of recurrences that occur after day 21, and as early as day 14, are associated with low blood concentrations of CQ (<100 ng/ml) at the time of treatment failure (Añez et al., 2012, 2015; Baird et al., 1995; Congpuon et al., 2011; Getachew et al., 2015; Guthmann et al., 2008; Hwang et al., 2013; Phong et al., 2019; Phyo

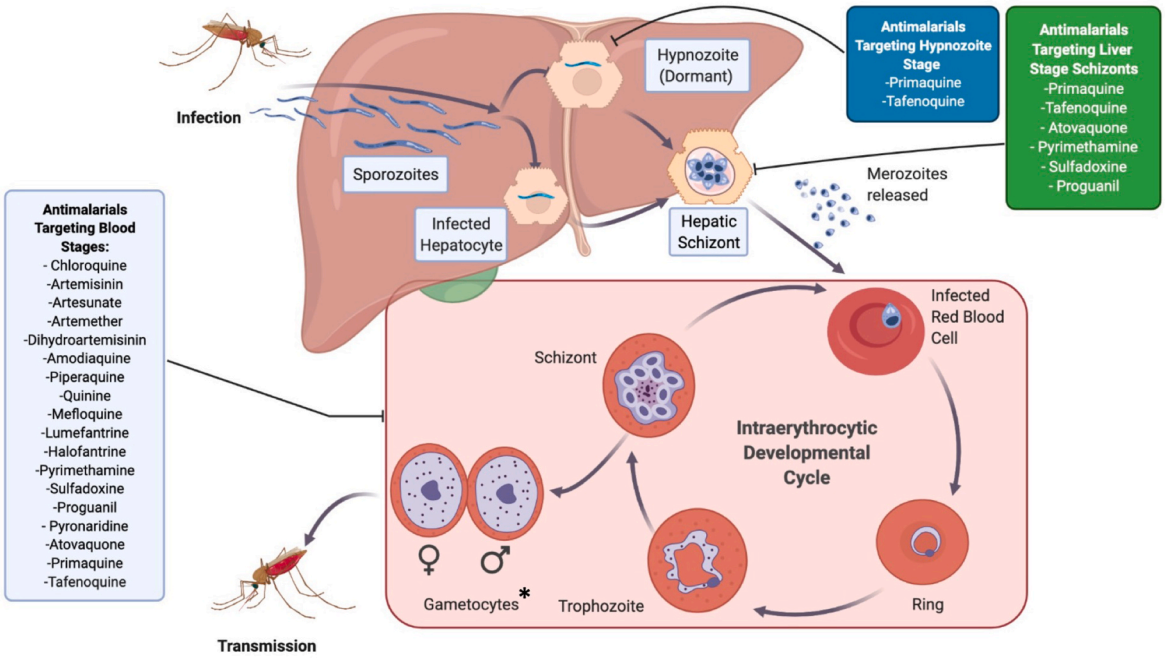

Fig. 1. Phases of the *P. vivax* life cycle targeted by various antimalarial compounds. Infected mosquitoes take up a human blood meal and inject sporozoites into the bloodstream. Sporozoites then migrate to the liver, where they either become hepatic schizonts which release merozoites into the bloodstream, or become dormant hypnozoites. Merozoites in the blood infect RBCs, where they develop from rings, to trophozoites and finally to mature schizonts. The majority of antimalarials target this intraerythrocytic stage. *Antimalarial compounds are generally thought to be more active against *P. vivax* gametocytes compared to *P. falciparum*, however, this may in part be an indirect effect through targeting asexual stage parasites and requires further study. A subset of intraerythrocytic parasites will develop into sexual stage gametocytes which are then taken up by the mosquito during a blood meal where they eventually develop into sporozoites to begin the cycle anew. Most antimalarials target the blood stage, while primaquine (PQ) and tafenoquine (TQ) also target the liver stage, including hypnozoites. PQ and TQ are required to achieve "radical cure" to completely eliminate the parasite. Adapted from "*P. vivax* Replication Cycle", by BioRender.com (2020).

et al., 2011; Yohannes et al., 2011). Use of different definitions of clinical resistance can lead to vastly different conclusions about the degree, or even the presence, of CQR in an area (Ferreira et al., 2021). For these reasons, clinical studies that include measurement of blood CQ at the day of treatment failure, or with a high frequency of early treatment failures (≤14 days), provide a clearer picture of resistance, which has been demonstrated in Myanmar, Thailand, Ethiopia, Bolivia, and Brazil, ranging from ~0.5% to 10% treatment failure (Añez et al., 2012; Congpuon et al., 2011; Guthmann et al., 2008; Hwang et al., 2013; Ladeia-Andrade et al., 2019; Yohannes et al., 2011). In contrast, regions of Indonesia and Papua New Guinea have significantly higher rates of CQR in *P. vivax* populations, where treatment failure occurs in 20–97% of patients, with blood CQ levels >100 ng/ml, often including a high frequency of early treatment failures (Asih et al., 2011; Baird et al., 1995; Fryauff et al., 1998, 2002; Ratcliff et al., 2007; Sumawinata et al., 2003; Sutanto et al., 2010). Treatment failures in such regions has made it necessary to switch to ACTs as the first line treatment.

Several other factors can complicate the association of clinical failure with drug resistance. *P. vivax* treatment with CQ is usually combined with PQ, or more recently TQ, in order to clear hypnozoites. While many clinical trials delay the addition of PQ/TQ until day 28, those that do not will mask CQR due to the blood-stage activity of these drugs (Baird et al., 1995; Fukuda et al., 2017; Ladeia-Andrade et al., 2019; Wilairatana et al., 1999). High levels of immunity can also suppress parasitemia following treatment, therefore reducing the rate of recrudescence, which can mistakenly be classified as having lower rates of treatment failure, and drug resistance, compared to regions with lower immunity (McIntosh and Olliaro, 2000; Stepniewska et al., 2010; WWARN, 2015; WWARN et al., 2015). Genotyping *P. vivax* at the time of treatment and at recrudescence has been used to identify relapse, where parasite clones are observed at recrudescence that were not present in the initial population (Chiang et al., 2012; Imwong et al., 2007). However, the presence of a homologous clone before and after treatment can not differentiate between recrudescence and relapse, limiting its use to ruling out recrudescence but not relapse.

ACTs remain clinically active against CQR *P. vivax*, including artemisinin (ART) in combination with MQ, artemether in combination with lumefantrine (LM) (combination denoted as AL) or dihydroartemisinin (DHA) in combination with piperaquine (PPQ) (Abdallah et al., 2012; Abreha et al., 2017; Commons et al., 2019; Hwang et al., 2013; Karunajeewa et al., 2008; Ratcliff et al., 2007; Senn et al., 2013; Yohannes et al., 2011). A recent meta-analysis of AL compared to DHA-PPQ, demonstrated a higher recrudescence rate in patients treated with AL in the first 42 days (Commons et al., 2019). This is likely due to the longer half-life of PPQ compared to LM, which provides a longer lasting prophylactic effect against relapse from hypnozoites, which is supported by the more similar rates of recrudescence by day 63 between drugs, as well as significant effect of PQ in both treatments (Commons et al., 2019).

SP has not been used to treat *P. vivax* due to the concept that it was naturally resistant in earlier literature, which was most likely an artifact of hypnozoite relapse (Hawkins et al., 2007). SP has historically been used to treat *P. falciparum* and is still used for intermittent preventive treatment in pregnancy (IPTp), which is indiscriminate of species (WHO, 2019b). Interestingly, *P. vivax* SP resistance is relatively common even though it was never intentionally treated with SP, demonstrating strong pressure from inadvertent treatment of *P. falciparum* (Alam et al., 2007; Kaur et al., 2006).

2.2. Measuring ex vivo drug susceptibility

Given the limitations, cost and difficulty involved with *in vivo* studies for drug resistance, studies in *P. falciparum* heavily rely on *in vitro* drug sensitivity assays. *In vitro* assays are not affected by some of the confounding factors associated with *in vivo* studies, including differing levels of immunity, drug absorption and metabolism, allowing for more comparable data between isolates, regions and patient age. However, the lack of a continuous *in vitro* culture system for *P. vivax* has significantly hampered the ability to accurately measure drug susceptibility. Instead, measurement of *P. vivax* drug sensitivity has relied on short-term *ex vivo* assays that measure schizont maturation over a single replication cycle (ring to schizont). These assays have been useful in identifying drug resistance in *P. vivax*, however, require patient isolates with appropriate parasitemias, skilled microscopists, and are labor intensive, together limiting the number of studies that have used *ex vivo* drug sensitivity assays to measure resistance (Chaorattanakawee et al., 2017; Li et al., 2020; Suwanarusk et al., 2007, 2008). Using *ex vivo* assays has identified *P. vivax* trophozoites as being less sensitive to most clinically used drugs (Russell et al., 2008; Sharrock et al., 2008; Suwanarusk et al., 2007), complicating the determination of precise IC_{50} values between parasite isolates that vary in stage composition between individuals. Furthermore, the length of the assay, independent of the initial parasite age, also impacts the IC_{50} calculated for both *P. vivax* and *P. falciparum* in single cycle assays and exaggerates stage dependent differences if it is not carefully controlled for (Russell et al., 2008; Sharrock et al., 2008).

Significant improvements have been made in recent years to improve the quality and ease of *ex vivo P. vivax* drug assays. *P. vivax* patient samples typically contain low parasitemia (<0.1%). Several methods have been used to enrich *P. vivax* infected red blood cells (RBCs), including Percoll or Nycodenz density gradients, however, these methods typically purify mature schizonts, which are less suitable for drug sensitivity assays (Mons et al., 1988; Ribaut et al., 2008; Russell et al., 2011).

Novel methods using a potassium chloride Percoll gradient can enrich reticulocytes and therefore *P. vivax* parasites that are restricted to these cells (Rangel et al., 2018). Culture conditions have also been improved, with IMDM culture media supporting schizont maturation more than previously used media (Rangel et al., 2018). Optimized growth conditions and enrichment methods increase the ease and accuracy of *ex vivo* assays and makes the use of higher throughput assays achievable, including flow-cytometry and radiolabeled hypoxanthine uptake growth assays (Rangel et al., 2018; Wirjanata et al., 2015). However, these technologies require equipment that is not always present in resource-poor settings. Therefore, the use of cryopreserved isolates is advantageous as they can be transported to better equipped laboratories and used in large batches, which also improves consistency between samples as well as making it possible to perform multiple repeated biological assays with the same isolate. Cryopreservation also has the advantage of being selective for younger parasites, favoring synchronicity at ring stage parasites and thereby reducing the stage dependent differences between isolates (Rangel et al., 2018). However, freeze-thawing of isolates results in a loss of some parasites that may inadvertently select for a subset of the population with altered drug susceptibility. *Ex vivo* assays have been used to determine the activity of novel antimalarial compounds against *P. vivax,* which in some cases has highlighted significant differences between *P. falciparum* and *P. vivax* that could affect their clinical use for *P. vivax* (Kuhen et al., 2014; Leimanis et al., 2010; Lek-Uthai et al., 2008; Llanos-Cuentas et al., 2018; Marfurt et al., 2011; McNamara et al., 2013; Phillips et al., 2015; 2016; Price et al., 2010; Pukrittayakamee et al., 1994; Rottmann et al., 2010). For example, DSM265 was found to be less active against recombinant PvDHODH and subsequently demonstrated reduced *in vivo* effectiveness against *P. vivax* compared to *P. falciparum*, however, the newer DHODH inhibitor, DSM421, was found to have equal activity in *ex vivo* isolates between *P. falciparum* and *P. vivax* (Llanos-Cuentas et al., 2018; Phillips et al., 2015, 2016; Phillips et al., 2015). The use of high-throughput screening methods and cryopreserved isolates could ease the barrier to *ex vivo* drug screening and encourage testing lead compounds against *P. vivax* at an earlier stage of drug development. An increased number of studies that accurately measure *in vivo* and/or standardized *ex vivo* drug sensitivity assays, combined with genome sequencing will set the

foundation for genotype-phenotype association studies to identify putative resistance mutations.

3. Candidate *P. vivax* drug resistance genes

Candidate *P. vivax* drug resistance genes have been identified based primarily on orthology from known *P. falciparum* drug resistance genes. Here we discuss what is known about each of the candidate *P. vivax* drug resistance genes, and current evidence for their role in mediating drug resistance (summarized in Supplementary Table 1).

3.1. *Plasmodium vivax multidrug resistance gene 1 (PvMDR1)*

pvmdr1 is the ortholog of the *P. falciparum pfmdr1* gene, which mediates drug sensitivity to MQ, LM, and CQ in this species (Price et al., 1999). In both parasites, *mdr1* encodes a transmembrane protein localized to the digestive vacuole (DV), where the parasite digests host cell proteins, and converts heme from hemoglobin into non-toxic hemozoin (Reiling and Rohrbach, 2015; Rohrbach et al., 2006; Sanchez et al., 2008). *pvmdr1* was identified in 2005, and due to its strong sequence conservation with *pfmdr1*, has subsequently become one of the primary candidate genes assessed in *P. vivax* drug sensitivity studies (Barnadas et al., 2008; Brega et al., 2005; Orjuela-Sánchez et al., 2009). PvMDR1 localizes to the digestive vacuole when overexpressed in *P. knowlesi*, which is more closely related to *P. vivax* than *P. falciparum* is, suggesting that it plays a similar role to PfMDR1 in *P. vivax* (Verzier et al., 2019).

PfMDR1 is thought to transport drugs, including CQ and MQ into the DV (Reiling and Rohrbach, 2015; Rohrbach et al., 2006; Veiga et al., 2016). Several molecular epidemiological and functional studies have associated amino acid changes at positions 86, 184, 1034, 1042, and 1246 in PfMDR1 with drug resistance (Veiga et al., 2016; Wurtz et al., 2014). CQ primarily acts to inhibit hemozoin formation in the DV, which results in a toxic build up of heme, thus reduced CQ in the DV leads to CQR (Olafson et al., 2015; Veiga et al., 2016). Indeed, mutations in *pfmdr1* are associated with CQR and with increased sensitivity to MQ and LM (Reed et al., 2000; Sidhu et al., 2005). MQ and LM are thought to primarily act on cytoplasmic proteins, although their mechanisms of action are less clear than CQ, and therefore reduced transport by PfMDR1 (and likely by PvMDR1 as well) into the DV, which sequesters it from its target, would lead to increased sensitivity to these compounds (Reiling and Rohrbach, 2015; Rohrbach et al., 2006; Sanchez et al., 2008).

Sequencing of *pvmdr1* across several regions of the world has revealed more than fifty polymorphisms in this gene, as well as copy number variants (CNVs). These single nucleotide polymorphisms (SNPs) correspond to amino acid changes throughout the protein sequence, including in the ATP binding domains and multiple transmembrane regions (Sá et al., 2005) (Fig. 2A). No single SNP, or set of SNPs, have emerged as definitive drug resistance markers. However, six SNPs have been reported at high frequency in multiple studies in regions with reported drug resistance; (relative to the SAL-1 reference) S513R, G698S, M908L, T958M, Y976F and F1076L (Brega et al., 2005; Huang et al., 2014; Joy et al., 2018; Orjuela-Sánchez et al., 2009; Vargas-Rodríguez et al., 2012). Amino acid changes at Y976F and F1076L in particular

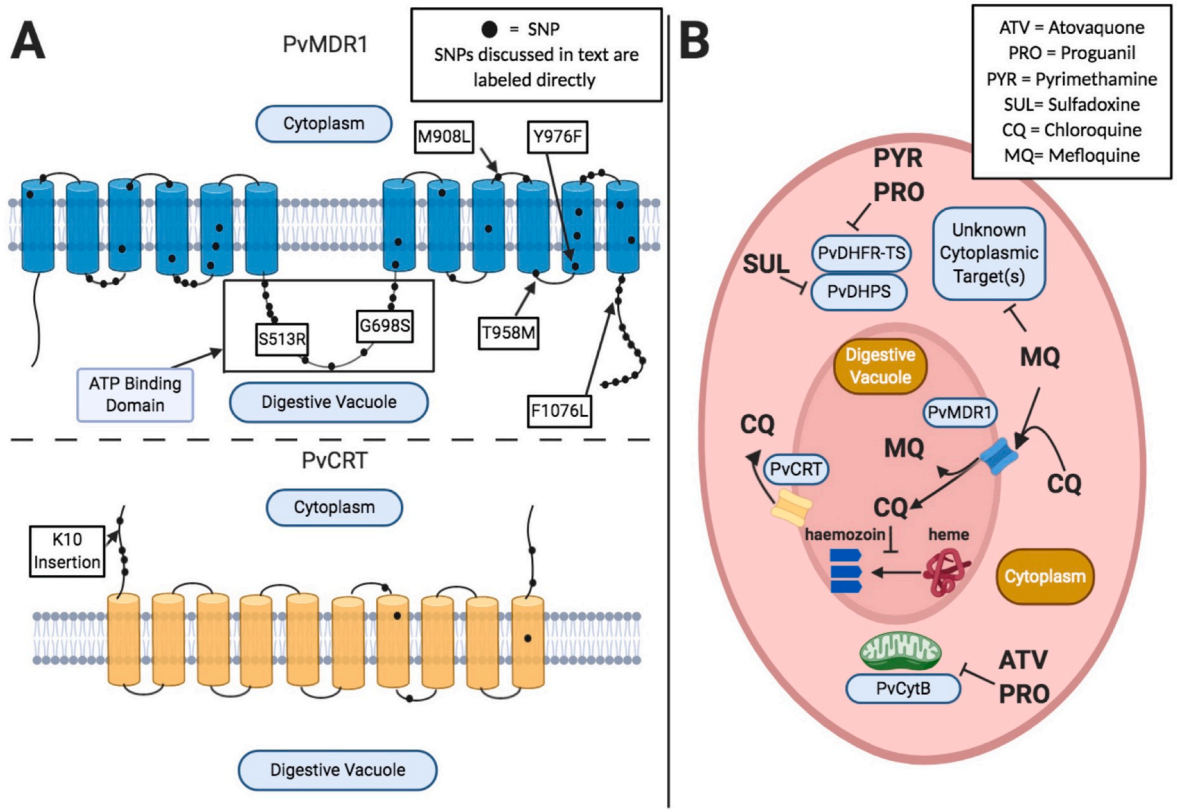

Fig. 2. A) Depicts the putative structures of PvMDR1 and PvCRT including their predicted transmembrane domains and where observed polymorphisms are positioned within the proteins. Specific SNPs discussed in text are labeled, while other observed mutations are shown as a black dot. Transmembrane domain locations are adapted from Sá et al., 2005 for PvMDR1, and Nomura et al., 2001 for PvCRT. SNP locations are approximate and inferred based on location in protein sequence. **B)** Depicts putative mechanisms of antimalarial compound action and suspected molecular resistance mechanisms. PvMDR1 and PvCRT are digestive vacuole (DV) transmembrane proteins. Based on orthology to *P. falciparum,* it is thought that PvMDR1 mutants confer CQ resistance by reducing transport of CQ into the digestive vacuole. PvCRT is thought to act as an efflux pump; actively transporting CQ out of the DV and away from its target (the process of converting heme into hemozoin). PvMDR1 is thought to confer resistance to MQ and possibly other compounds, by transporting them into the DV, thereby sequestering them away from their putative cytoplasmic targets.

have been cited as possible markers of drug resistance (Brega et al., 2005; Spotin et al., 2020; Suwanarusk et al., 2008). However, these SNPs are also found in regions without reported CQR, making their association with drug resistance uncertain (Spotin et al., 2020). The T958M, Y976F, F1076L variants (and possibly others) in PvMDR1, have been shown to arise independently, even within the same population (Schousboe et al., 2015). This finding suggests that *pvmdr1* mutations can arise on different genetic backgrounds and that they have not been subject to a selective sweep, possibly due to a lack of direct pressure from drug (Schousboe et al., 2015). If, and how, these SNPs play a role in mediating *P. vivax* drug resistance remains to be directly demonstrated in functional studies.

Studies exploring the relationship between these SNPs and drug sensitivity paint a mixed picture. A survey of isolates from China that associated sequencing of *pvmdr1* with *ex vivo* measurements of drug susceptibility found an association between M908L and reduced susceptibility to CQ, MQ, pyronaridine, PPQ, quinine, ART, and DHA (Li et al., 2020). A study in Cambodia found a correlation between Y976F and F1076L mutations and resistance to MQ and PPQ, but not CQ (Chaorattanakawee et al., 2017). Similarly Suwanarusk et al. found an association between Y976F and reduced susceptibility to CQ (Suwanarusk et al., 2008). Yet multiple other studies have found no relationship between polymorphisms in *pvmdr1* and drug resistance (Faway et al., 2016; Li et al., 2020; Nyunt et al., 2017; Orjuela-Sánchez et al., 2009). This in part could stem from methodological differences in *ex vivo* phenotyping, and the fact that several studies are conducted in regions with low rates of CQR. Differences between studies may simply reflect real geographical differences. It will be critical to evaluate these polymorphisms in an *in vitro* culture system to assess their impact on drug resistance. Two *pvmdr1* haplotypes were episomally overexpressed in a *P. knowlesi* model system, both of which encoded the 698S, 908L and 958T mutations, but did not alter sensitivity to either CQ or MQ (Verzier et al., 2019), suggesting that at least in this genetic background and expression system, these mutations did not confer resistance (discussed further in section 4.1). Another limitation is that many studies sequence only part of the gene, thus limiting and biasing our understanding of mutations to these regions of the gene.

Copy number variation (CNV) of *pvmdr1* is distributed globally at variable frequencies (7–31.6%), including Thailand, Cambodia, Laos, India, Brazil, French Guinea, and other countries (Costa et al., 2017; Faway et al., 2016; Imwong et al., 2008; Joy et al., 2018; Khim et al., 2014; Suwanarusk et al., 2008; Vargas-Rodríguez et al., 2012). CNVs in *pvmdr1* have more clearly been implicated as a potential cause of *P. vivax* drug resistance (Costa et al., 2017; Imwong et al., 2008; Suwanarusk et al., 2008). Sequencing of isolates in Thailand and Indonesia found a correlation between increases in IC_{50} of amodiaquine (AQ), ART, and MQ, measured by *ex vivo* drug susceptibility assays, and *pvmdr1* amplification (Auburn et al., 2016; Suwanarusk et al., 2008). Notably this mirrors the similar effect of *pfmdr1* amplification on MQ resistance in *P. falciparum* (Preechapornkul et al., 2009).

3.2. *Plasmodium vivax chloroquine resistance transporter (PvCRT)*

The *pvcrt* gene (also referred to as *pvcrt-o*) emerged as a candidate drug resistance gene due to its orthology with the *pfcrt* gene that mediates CQR in *P. falciparum* (Fig. 2A). The *pfcrt* gene was first identified as a determinant of CQR in a genetic cross between a CQ-sensitive parasites line and CQR parasites (Su et al., 1997). Numerous *pfcrt* polymorphisms have been implicated in causing CQ drug resistance (Ecker et al., 2012; Fidock et al., 2000,; Johnson et al., 2004; Pulcini et al., 2015; Summers et al., 2014). Additionally, polymorphisms in *pfcrt* are associated with PPQ resistance, and conversely, increased sensitivity to MQ and ART (Johnson et al., 2004; Phyo et al., 2011; Pulcini et al., 2015; van der Pluijm et al., 2019).

Unlike *pfcrt*, and in contrast to *pvmdr*1, very few SNPs (~10) in *pvcrt* have been reported, however, most occur at very low frequency (Barnadas et al., 2008; Joy et al., 2018; Noisang et al., 2020). The most common PvCRT polymorphism is a lysine insertion at position 10 (K10) (Barnadas et al., 2008; Joy et al., 2018; Noisang et al., 2020; Silva et al., 2018a). The K10 insertion has been observed in both Southeast Asian and South American parasites (Noisang et al., 2020; Silva et al., 2018b.; Zhao et al., 2020). However, no association between the K10 insertion and *in vitro P. vivax* drug resistance has been found (Silva et al., 2018a; Suwanarusk et al., 2007). It is currently unknown if *pvcrt* variants that may be associated with CQR have a deleterious impact on parasite fitness, which is the case for *P. falciparum* and could explain the low frequency of *pvcrt* mutations (Ord et al., 2007; Petersen et al., 2015).

There is evidence that increased expression of *pvcrt* potentially mediates CQR (Melo et al., 2014; Sá et al., 2019; Silva et al., 2018b). Sa and colleagues recently performed a *P. vivax* genetic cross between the CQR *P.* vivax NIH-1993-R line and the CQS NIH-1993-S line. Bulk segregant analysis of blood stage progeny implicated a 76 KB region on chromosome one, that includes *pvcrt*, as having a role in CQR. The study found no SNPs in *pvcrt,* but did identify a TGAAGH motif with an increased number of repeats both upstream of the 5' UTR of *pvcrt,* as well as a deletion within intron nine of the gene, and that CQR progeny had increased expression of *pvcrt*.

Evidence that overexpression of *pvcrt*, resulting from either increased gene copy numbers, or enhanced transcription levels, is linked to drug resistance has been reported in several studies of patient isolates from Brazil (Costa et al., 2017; Melo et al., 2014; Silva et al., 2018a). However, a study of *pvcrt* expression in parasites from Indonesia, where there is high-grade drug resistance, found no relationship between *pvcrt* expression levels and *ex vivo* susceptibility to CQ, PPQ, MQ and Artesunate (AS) (Pava et al., 2015). As with *pvmdr1*, whether or not these discrepancies are due to differences in the genetic background, such as *pvmdr1* polymorphisms and CNVs, or whether they are due to technical differences will require further investigation.

3.3. *P. vivax dihydrofolate reductase-thymidylate synthase (DHFR-TS) and dihydropteroate synthase (DHPS)*

The essential enzymes dihydrofolate reductase-thymidylate synthase (PvDHFR-TS*)* and dihydropteroate synthase (PvDHPS) are involved in folate synthesis, and are the targets of pyrimethamine and sulfadoxine, respectively (Asih et al., 2015; Auliff et al., 2006; 2010; Barnadas et al., 2008; Hastings et al., 2005; Hastings and Sibley, 2002). SP has long been used to treat *P. falciparum*, but is now primarily only used for IPTp due to widespread resistance (WHO, 2007). PfDHPS and PfDHFR-TS are well understood in *P. falciparum* and highly conserved in *P. vivax* (Hawkins et al., 2007). While SP has not been intentionally used for *P. vivax* treatment, mutations in PvDHPS and PvDHFR-TS, that are conserved with mutations known to confer resistance in *P.* falciparum, are widespread in *P. vivax* populations around the world, resulting in treatment failure in more than 50% of patients (Maguire et al., 2006; Pukrittayakamee et al., 1994; Tjitra et al., 2002). It has been suggested that genomic surveillance of *pvdhfr-ts* and *pvdhps* could provide an understanding of which regions these drugs could be used to cheaply and safely treat *P. vivax* infection, although to date SP has not be recommended for *P. vivax* (Hawkins et al., 2007). *pvdhfr-ts* and *pvdhps* also represent a key example of how molecular studies could be performed to characterize other resistance alleles and monitor the spread of resistance.

Amino acid changes in PvDHFR-TS at positions N50I, S58R, S117N and I173L exist as double, triple, and quadruple mutants at frequencies ranging from as 20–90% in Malaysia, Thailand, Papua New Guinea, and Indonesia (Auburn et al., 2019; Imwong et al., 2001; Marfurt et al., 2008). These mutations align with mutations N51I, C59R, S108N and I164L in PfDHFR-TS, which are associated with pyrimethamine resistance (Briolant et al., 2012; Hastings and Sibley, 2002). An additional F57L mutation in PvDHFR-TS, which has no *P. falciparum* equivalent, has also been reported at high frequency in combination as a double

mutant with 58R or 117N, and a quadruple mutant with 58R/61M/117N (Asih et al., 2015; Auliff et al., 2006; Hastings et al., 2005; Hastings and Sibley, 2002).

Expression of mutant PvDHFR-TS in a yeast system demonstrated that these mutations confer high levels of pyrimethamine resistance. Single mutations at positions 57L and 117N, and double or triple mutants, 58R/117N and 117N/173L, 58R/117N/173L resulted in a 50-, 87-, 460-, 700- and 500-fold increase in resistance to pyrimethamine, respectively (Hastings and Sibley, 2002). Using an episomally expressed copy of PvDHFR-TS in *P. falciparum*, Auliff et al. found that a single mutation, 117N, resulted in 46-, 6-, 2- and 6-fold increases in resistance, relative to wild-type, to pyrimethamine, clociguanil, WR99210, and cycloguanil, respectively. Additionally, double PvDHFR-TS mutant haplotypes of 57L/117T and 58R/117T, resulted in 67- and 114-fold increases in pyrimethamine resistance, 6- and 4-fold increase in cycloguanil resistance, 10- and 26-fold increase in clociguanil resistance, and 22- and 10-fold increases in WR99210 resistance for each haplotype, respectively. They also found that a PvDHFR-TS quadruple mutant (57L/58R/61M/117T) resulted in high resistance to pyrimethamine, cycloguanil, chlorcycloguanil, and WR99210, with resistance increases of 8497-, 746-, 2565-, and 44-fold, respectively. Interestingly, expression of a triple PvDHFR-TS mutant (58R/61M/117T) resulted in susceptibilities similar to wild-type for all drugs with the exception of pyrimethamine, for which this haplotype conferred a 58-fold increase in resistance. The authors hypothesized that 61M may be a compensatory mutation to offset possible fitness costs with carrying other resistance mutations. Studies assessing PvDHFR-TS mutants and SP treatment outcomes have confirmed that these results correlate with *in vivo* efficacy, with treatment failure 23 times more likely to occur when infected with *P. vivax* parasites containing the 57L/58R/61M/117T haplotype (Auliff et al., 2006; Hastings et al., 2004). A structure analysis of PvDHFR-TS showed that a mutation at position 117 leads to steric conflict with pyrimethamine binding, resulting in resistance, similar to the equivalent mutation at position 108 in PfDHFR-TS (Kongsaeree et al., 2005). These data suggest that *P. vivax* resistance to antifolates arises from molecular changes and is highly conserved with *P. falciparum*.

Mutations at amino acids 383 and 553 in PvDHPS, correspond with known drug resistance mutations 437 and 581 In PfDHPS (Auliff et al., 2006; Imwong et al., 2005). Similar to SP resistance in *P. falciparum*, mutations in PvDHPS alone is thought to not be sufficient to provide resistance to SP (Imwong et al., 2005). Furthermore, the wild-type PvDHPS allele at position 585 is a Valine, which aligns with position 613 in PfDHPS, and is thought to reduce the binding to sulfadoxine (Korsinczky et al., 2004). This V585 allele is found commonly in isolates, suggesting possible innate resistance to sulfadoxine in this species (Auliff et al., 2006; Korsinczky et al., 2004). Structure analysis of PvDHPS mutants support the role of these mutations in providing resistance to sulfadoxine by reducing binding affinity to the mutant enzyme (Yogavel et al., 2018). Particularly, strains containing both PvDHPS 383G and 553G mutations and mutant PvDHFR-TS alleles, have been implicated in SP treatment failure (Imwong et al., 2005).

Studies looking at the distribution of *pvdhfr-ts* alleles in natural populations have found a high prevalence (80–90%) of double, triple, and quadruple mutations in Malaysia, Thailand, India, Indonesia, Madagascar, and China, which all have high SP treatment failure rates (Alam et al., 2007; Auburn et al., 2019; Barnadas et al., 2011; Hastings et al., 2005; Huang et al., 2014; Imwong et al., 2001). Global and regional population genetic studies of *P. vivax* have found *pvdhfr-ts* and *pvdhps* in regions of the genome with strong evidence of selection by linkage disequilibrium and integrated haplotype score metrics (Auburn et al., 2018; Hupalo et al., 2016). Strong signals of genetic differentiation were found between Ethiopian populations, which have a low prevalence of *pvdhfr-ts* mutants, compared to Indonesian or Thai populations, which have high prevalence of *pvdhfr-ts* mutants, providing evidence of region-specific drug pressure (Auburn et al., 2019). Furthermore, a study comparing *P. vivax* isolates in Indonesia, Malaysia, and Thailand, found signals of genetic differentiation of *pvdhfr-ts* and *pvdhps* between regions, which all have a high prevalence of *pvdhfr-ts* mutants, suggesting that mutant haplotypes can arise on different genetic backgrounds (Auburn et al., 2018).

Two studies in India found a higher prevalence of *pvdhfr-ts* mutations in regions where *P. falciparum* and *P. vivax* are co-endemic, than regions where *P. vivax* is the dominant parasite (Alam et al., 2007; Kaur et al., 2006). These results suggest that pressure of SP treatment of *P. falciparum* can lead to co-selection of *pvdhfr-ts* mutant alleles, which could also occur for other drugs, including ACT partner drugs. Hence, the authors suggest that future studies should consider not only the drugs used to treat *P. vivax*, but also those for used *P. falciparum* in co-endemic regions.

3.4. *P. vivax kelch 12 (PvK12): ortholog of the P. falciparum kelch 13 (PfK13) determinant of reduced ART susceptibility*

ACTs are the recommended frontline treatment for *P. falciparum*, due to widespread resistance to older antimalarials, as well as for *P. vivax* in areas of high CQR and in the case of mixed infections. ACTs combines ART, which rapidly kills parasites but has a short elimination half-life, with a longer-lasting partner drug, usually MQ, LM, SP, PPQ or AQ (Commons et al., 2019). *P. falciparum* ART resistance, which first arose in the Mekong Subregion (GMS) in 2008, is mediated by the *pfkelch13* gene (Ariey et al., 2014; Ménard et al., 2016; Miotto et al., 2015; Takala-Harrison et al., 2015; Tun et al., 2015). *P. vivax* treatment with ACTs is recommended by the WHO mainly in Africa (with the exception of Ethiopia), The Western Pacific, and Eastern Mediterranean regions (WHO, 2019a).

To date, no cases of *P. vivax* resistance to ACTs have been reported. However, several studies have monitored the *P. vivax* ortholog of *pfkelch13*, *pvkelch12*, for polymorphisms that may lead to ART resistance (Brazeau et al., 2016; Deng et al., 2016; Gresty et al., 2019). While several polymorphisms have been found in *pvkelch12*, they do not align to the respective mutations in *pfkelch13* that mediate artemisinin resistance (Brazeau et al., 2016; Deng et al., 2016; Gresty et al., 2019). These results suggest a lack or reduced selection pressure from ART on *pvkelch12* to date. Notably there is a lack of persistence of *pvkech12* SNPs over time in the GMS, and no polymorphism in *pvkelch12* in Papua New Guinea, where one might expect selection pressure from ART indirectly from *P. falciparum* treatment, or directly as the first-line therapy in this region (Brazeau et al., 2016; Gresty et al., 2019). These results suggest a lack of strong selection pressure from ART on *pvkelch12* at this time (Brazeau et al., 2016; Gresty et al., 2019). Recently, an alternative gene, *pfcoronin*, has been identified to confer resistance to ART in *P. falciparum* but the ortholog has not been studied in *P. vivax* (Demas et al., 2018). Should ART resistance in *P. vivax* arise, it could be mediated by one of these genes or through other *P. vivax*-specific mechanisms. Regardless, continued monitoring of both clinical ART resistance and possible mutations in *pvkelch12* and *pvcoronin* orthologs is warranted.

3.5. *Plasmodium vivax multidrug resistance protein 1 (PvMRP1)*

P. vivax multidrug resistance protein 1 (*pvmrp1*) has emerged as a candidate drug resistance gene because of signals of selection in population genetic studies (Dharia et al., 2010; Flannery et al., 2015). *pvmrp1* is an ortholog of the *pfmrp1* gene, which transports glutathione adducts out of the parasite, a process that is thought to help the parasite regulate oxidative stress (Müller, 2015; Raj et al., 2009). *pfmrp1* knockouts in *P. falciparum* have increased susceptibility to quinine, CQ, ART, and PQ, as well a reduced parasite growth rate *in vitro* (Raj et al., 2009). SNPs in *pfmrp1* have also been associated with AL resistance, where the amino acid change, I876V, was found in recurrent infections after treatment with AL (Dahlström et al., 2009).

Genetic sequencing of *P. vivax* samples in South America has found

strong signals of molecular evolution in this gene, with a high ratio of nonsynonymous substitutions per synonymous site relative to the number of synonymous substitutions per synonymous sites (d_N/d_S) (Dharia et al., 2010; Flannery et al., 2015). Sequencing of a Peruvian isolate also demonstrated evidence of *pvmrp1* gene amplification (Flannery et al., 2015). Studies in Sudan, Thailand, Indonesia, and Cambodia found *pvmrp1* is situated in a long region of homozygosity, which indicates recent selection (Auburn et al., 2018, 2019; Bright et al., 2013,; Parobek et al., 2016).

Sequencing of a *P. vivax* isolate from a patient, with no mutations in CYP genes, that experienced PQ failure found several SNPs in *pvmrp1* (Bright et al., 2013). PQ and TQ are the only approved drugs that can eliminate hypnozoites, which is required to achieve radical cure of *P. vivax* (Chiang et al., 2012; Hill et al., 2006; Llanos-Cuentas et al., 2019; Taylor et al., 2019). PQ is oxidized by CYPD26 into hydroxyl-metabolites whose oxidation, in turn, generates quinoneimine and subsequently generates hydrogen peroxide (H_2O_2) (Camarda et al., 2019). Qinoneimine is also a substrate for CPR, CYPD26's redox partner, which leads to accumulation of H_2O_2 and subsequent antimalarial activity through oxidative stress in the parasite. PQ tolerance, leading to relapse of infection is rare but has been reported in cases following the correct treatment regimen (Chiang et al., 2012; Townell et al., 2012). Knockouts of *pfmrp1* have increased sensitivity to PQ in *P. falciparum*, further supporting that this protein plays a role in mediating tolerance to PQ, although the active metabolites were not tested (Raj et al., 2009). The experimental evidence of *pfmrp1* mediating PQ tolerance and evidence of selection pressure on *pvmrp1* have led to this gene as a candidate for mediating PQ tolerance in *P. vivax* (Bright et al., 2013, 2014; Dharia et al., 2010; Flannery et al., 2015).

Dharia and colleagues proposed a model of *pvmrp1* mediating PQ tolerance, where *pvmrp1* mutations improve the transport of glutathione adducts resulting from PQ treatment and thus allow for PQ tolerance by mitigating oxidative damage (Dharia et al., 2010). In light of this evidence, *pvmrp1* is a compelling research target as a putative *P. vivax* drug resistance gene, and elucidating if and how it contributes to *P. vivax* drug resistance warrants further investigation.

3.6. Other putative resistance genes

Population genetic studies looking for evidence of positive selection via increased polymorphism or selective sweeps of drug resistance-associated loci have identified several putative *P. vivax* drug resistance genes (Auburn et al., 2018, 2019; Brazeau et al., 2016; Dharia et al., 2010; Flannery et al., 2015; Parobek et al., 2016). These include the candidate genes described above (*pvmdr1, pvcrt, pvdhfr-ts, pvdhps,* and *pvmrp1*), as well as several additional genes of interest.

Several studies of parasites in the GMS found evidence of selective sweeps via extended haplotype homozygosity (XP-EHH) and iHS tests around the *pvmdr2* and *pvmrp2* genes in Cambodia (Auburn et al., 2018; Brashear et al., 2020; Parobek et al., 2016). Another study in the Peruvian Amazon found a higher number of SNPs in *pvmrp2*, though this study did not evaluate any measures of selection on this gene (Cowell et al., 2018). The *P. falciparum* ortholog of *pvmrp2* has been implicated in conferring resistance to CQ, MQ and PPQ (Mok et al., 2014; Nogueira et al., 2008; Veiga et al., 2014). Similarly, *pfmdr2*, the *P. falciparum* ortholog of *pvmdr2,* has been implicated in pyrimethamine resistance (Briolant et al., 2012).

A study of *P. vivax* parasites in the Peruvian Amazon uncovered possible evidence of increased copy number of the PVX_000585 gene. PVX_000585 is predicted to encode a homologue of the *E. coli emrE* gene, a multidrug transporter, but has not been evaluated for a role in drug resistance in any *Plasmodium* spp. (Flannery et al., 2015). Additionally, a study of *P. vivax* found evidence of selection and significant genetic differentiation of the I165V variant in the *P. vivax plasmepsin IV* gene, an ortholog of the *P. falciparum plasmepsin II*, between Malaysian parasite populations, where there is a higher reported prevalence of CQR, and Thailand, where there is reportedly a lower prevalence of CQR (Auburn et al., 2018). *P. falciparum plasmepsin II* has been implicated in mediating PPQ resistance in this species (Bopp et al., 2018). The study also found evidence of significant genetic differentiation of variants in the putative drug/metabolite transporters *pvdmt1*, and a CG2-related protein (PVP01_1450700) between Thailand and Malaysia (Auburn et al., 2018). While these genes have not previously been the subject of intense research focus, the evidence of recent selection or increased copy number variation at loci encompassing them suggests that further research effort is merited.

4. Experimental approaches for assessing drug resistance and drug resistance determinants

4.1. Culturable heterologous model systems and reverse genetics

Significant improvements have been made to *P. vivax ex vivo* drug susceptibility assays, however, they remain limited to short single cycle assays with relatively poor growth compared to *P. falciparum* as well as requiring patient isolates that can not be well controlled for parasite stage and parasitemia, limiting their accuracy. Improved growth conditions and the use of multiple-cycle assays would reduce the differences observed from starting parasite age and parasitemia, as well as making it possible to study slower-acting drugs, including TQ (Russell et al., 2003). Improved *ex vivo* assays will significantly improve the ability to determine drug susceptibility in natural isolates and link drug phenotypes to specific resistance loci. However, validation of putative resistance alleles will remain unobtainable in *P. vivax* until robust *ex vivo* short-term culture, or more ideal methods for a continuous culture system are developed. In lieu of a stable culture system, heterologous systems will be invaluable for validation studies in *P. vivax*.

P. falciparum has been used to episomally express *P. vivax dhfr* alleles, which demonstrated the role of polymorphisms observed in the field in mediating pyrimethamine resistance (Auliff et al., 2010). The role and protein sequence of DHFR-TS, and its resistance mutations, are relatively well conserved between *Plasmodium* spp., as well as to higher eukaryotes (Hastings and Sibley, 2002). In contrast, the sequence of other putative resistance genes, as well as mutations found within them, are more divergent between *Plasmodium* spp., which may impact their accuracy as model systems. For these reasons, the field has moved towards using *Plasmodium* spp. that are more closely related to *P. vivax*. *P. knowlesi* primarily infects primates, but can also cause outbreaks in humans, and is more closely related to *P. vivax* than *P. falciparum* is. *P. knowlesi* has long been used for research, but only recently has been adapted to grow in human RBCs, which opens up its use to the wider community (Lim et al., 2013; Moon et al., 2013). *P. knowlesi* is also genetically tractable and a CRISPR/Cas9 system has been developed, which was successfully used to replace *pkdbp* with its *P. vivax* ortholog for vaccine studies (Mohring et al., 2019). *P. knowlesi* has also been used to overexpress two PvMDR1 variants, which correctly localized to the digestive vacuole (Verzier et al., 2019). Expression of these variants did not alter sensitivity to CQ or MQ (Verzier et al., 2019). Whether or not the lack of an effect is due to PvMDR1, or the variants tested, not being involved in drug resistance or due to technical reasons, such as poor expression levels or the presence of the wild-type PkMDR1, will require further study. Allelic replacement using CRISPR/Cas9 could replace the native *pkmdr1* with *pvmdr1* variants, which would ensure it is expressed from the native promoter in the absence of *pkmdr1*. The same methods could also be used to study other putative resistance genes.

More recently, *P. cynomolgi*, another primate malaria which is even more closely related to *P. vivax,* has been adapted to *in vitro* culture in rhesus macaque RBCs (Chua et al., 2019). The closer evolutionary relationship between *P. cynomolgi* and *P. vivax* may make it a better model for drug resistance mechanisms. Transgenic methods have been developed for *P. cynomolgi in vivo* using a primate model, which was used to produce fluorescent or luminescent reporter lines for high-throughput

screening of compounds active against hypnozoites (Voorberg-van der Wel et al., 2013; Voorberg-van der Wel et al., 2020). Development of hypnozoites is a significant advantage of using *P. cynomolgi* over *P. knowlesi,* and could be used to investigate putative resistance markers of PQ or TQ. The development of transfection for *in vitro* cultured *P. cynomolgi,* as well as a CRISPR/Cas9 system, would significantly advance the *P. cynomolgi* model for understanding *P. vivax* biology. Advances in these model systems will make it possible to definitively define the role of polymorphisms in *P. vivax* genes on drug susceptibility.

4.2. Genetic crosses for mapping resistance determinants

Genetic crosses are powerful tools to help map genetic loci to phenotypic characteristics (Sá et al., 2019; Wellems et al., 1991). Genetic crosses of *P. falciparum* lines have been used successfully to map loci involved in CQR and cell invasion ligands (Hayton et al., 2008; Wellems et al., 1991). *P. falciparum* genetic crosses are labor-intensive, as gametocytes from two different strains need to be cultured, mixed together, and fed to mosquitos as part of a blood meal. Once inside the mosquito, parasites undergo recombination (the only diploid stage of the life cycle). Furthermore, parent lines with desired phenotypes to be investigated may not yet exist, thereby limiting the number of phenotypes for which crosses will be informative. The infected mosquitos are used to infect splenectomized chimpanzees, and blood-stage parasites are isolated and grown in culture for further analysis (Walliker et al., 1987).

The lack of an *in vitro* culture system for *P. vivax* has complicated development of a genetic cross, however as described previously, Sa and colleagues used a bulk segregant approach on a cross of a CQS line and a CQR line to identify a region on *P. vivax* chromosome one, which includes *pvcrt*, that is linked to a CQR phenotype (Sá et al., 2019). In place of the *in vitro* culture steps used for *P. falciparum*, *in vivo* infection of Aotus and Saimiri monkeys was required to propagate *P. vivax*. It should be noted that in the United States, the NIH has stopped supporting research using chimpanzees, though research with other non-human primates (NHP) is still allowed (NIH, 2015).

More recently, *P. falciparum* crosses have been performed in human hepatocyte-liver chimeric mice, significantly reducing the cost and labor of generating crosses (Minkah et al., 2018; Vaughan et al., 2015). Another advantage of the mouse model is that multiple mice can be used for a cross, and subsequently more progeny from a cross can be collected, allowing for more powerful and fine-scale analysis linking genetic variation to phenotypes (Vendrely et al., 2020). Development of a *P. vivax* humanized mouse model would also open up new avenues to identify *P. vivax* loci involved in cell invasion, drug resistance, and other biological processes using genetic crosses. Furthermore, pairing genetic crosses with single-cell sequencing can provide powerful approaches towards understanding within host dynamics of genetic variation (Nair et al., 2014). With regards to drug resistance, single-cell analysis of genetic crosses could shed light on different genetic backgrounds upon which resistance can arise, within-host competition of different drug resistance haplotypes, and help understand the full range of genetic diversity at drug resistance loci (Nair et al., 2014). Ultimately, innovations in propagating *P. vivax in vitro* would ease the process of performing *P. vivax* genetic crosses.

4.3. Beyond candidate SNPs/CNVs: advances in genomics and transcriptomics

Advances in genomics and transcriptomics over the past decade have opened a range of possibilities for conducting population genetics and molecular epidemiological studies of *P. vivax*. Whole Genome Sequencing (WGS) of *P. vivax* directly from patient samples can be difficult because of the typically low parasitemia, and the subsequent difficulty of separating the *P. vivax* DNA from the background human genome (Cowell et al., 2017; Melnikov et al., 2011). Leukocyte depletion, which removes the background human DNA, and hybrid selection to enrich parasite DNA, has been used to successfully sequence *P. vivax* genomes from clinical samples (Hupalo et al., 2016; Melnikov et al., 2011; Pearson et al., 2016). Similarly, selective whole genome amplification approaches, which use a highly processive polymerase to amplify the parasite genome from the background human genome, for WGS is a low-cost and easily scalable method for conducting WGS on *P. vivax* clinical samples (Cowell et al., 2017).

These technologies have opened up the possibility of conducting large scale population molecular epidemiological studies in *P. vivax* and hold promise to help conduct genetic association studies highlighting selected loci that are under different selective pressures, (i.e. from drugs) between regions. Furthermore, whole-genome data can help illuminate how drug resistance alleles spread in a population, and be used to conduct molecular surveillance of drug resistance alleles to inform control efforts (Brazeau et al., 2016; Hupalo et al., 2016; Pearson et al., 2016).

However, whole genome approaches can miss rare variants due to lower sequence coverage, which limits the ability to uncover rare variants that could cause drug resistance (Gruenberg et al., 2019; Rao et al., 2016). Additionally, low sequencing coverage can affect the ability to differentiate between parasite lineages in a patient, which could be used to identify mixed infections and determine if persistent malaria is the result of recrudescence, relapse, or reinfection (Lin et al., 2015). Targeted sequencing approaches such as amplicon sequencing, where genes are amplified in a PCR reaction in parallel and then sequenced, allows for deep sequencing of select loci to uncover low frequency variants and uncover the full range of genetic variation in a population (Boyce et al., 2018; Gruenberg et al., 2019; Lin et al., 2015). *P. vivax* amplicon sequencing has been used to differentiate between recrudescence, reinfection, or relapse as a cause of recurrent infection, which could help determine if a patient has drug resistant *P. vivax* (Lin et al., 2015). Amplicon sequencing in *P. falciparum* has been used to identify low-frequency SNPs in the drug resistance genes *pfcrt, pfkelch13, pfmdr1, pfmrp1,* and *pfdhfr-ts* (Rao et al., 2016; Talundzic et al., 2017). Development of *P. vivax* amplicon panels for putative resistance genes could help identify SNPs associated with drug resistance (Lin et al., 2015; Rao et al., 2016; Talundzic et al., 2017).

The low parasitemia and lack of culture system in *P. vivax* has also hindered the use of transcriptomics to study gene expression. Recent advances in the ability to conduct transcriptomics from clinical samples and single-cell transcriptomics from monkey adapted lines demonstrate the feasibility to conduct transcriptomic analysis on *P. vivax* (Kim et al., 2019; Rangel et al., 2020; Sà et al., 2020). *P. vivax* transcriptomes from Cambodian patients treated with or without CQ, showed no differential expression of the candidate CQR genes *pvmdr1* and *pvcrt* between the two treatment groups (Kim et al., 2019). The lack of an association could be due to a lack of drug resistance alleles in this sample set, or a lack of a change in gene expression in response to drug pressure (Kim et al., 2019). Comparative transcriptomics between CQR and CQS patient isolates could identify putative resistance loci that have elevated expression in CQR parasites, since increased expression is known to mediate *P. falciparum* drug resistance and there is evidence that the same is true in *P. vivax* (Kim et al., 2019; Sà et al., 2020; Sá et al., 2019).

5. Looking forward

The complex biology of *P. vivax*, that includes dormant liver stage hypnozoites, the lack of *in vitro* culture, and the difficulty in performing *ex vivo* assays has made it hard to readily determine clinical resistance to CQ and other drugs in this species. Improved techniques for *ex vivo* drug assays will allow researchers to more readily screen larger numbers of *P. vivax* isolates and determine the frequency and level of drug resistance within different populations. If paired with WGS and transcriptomics, putative molecular markers of drug resistance could be determined with greater detail and accuracy (Fig. 3). Many of the resistance mutations in

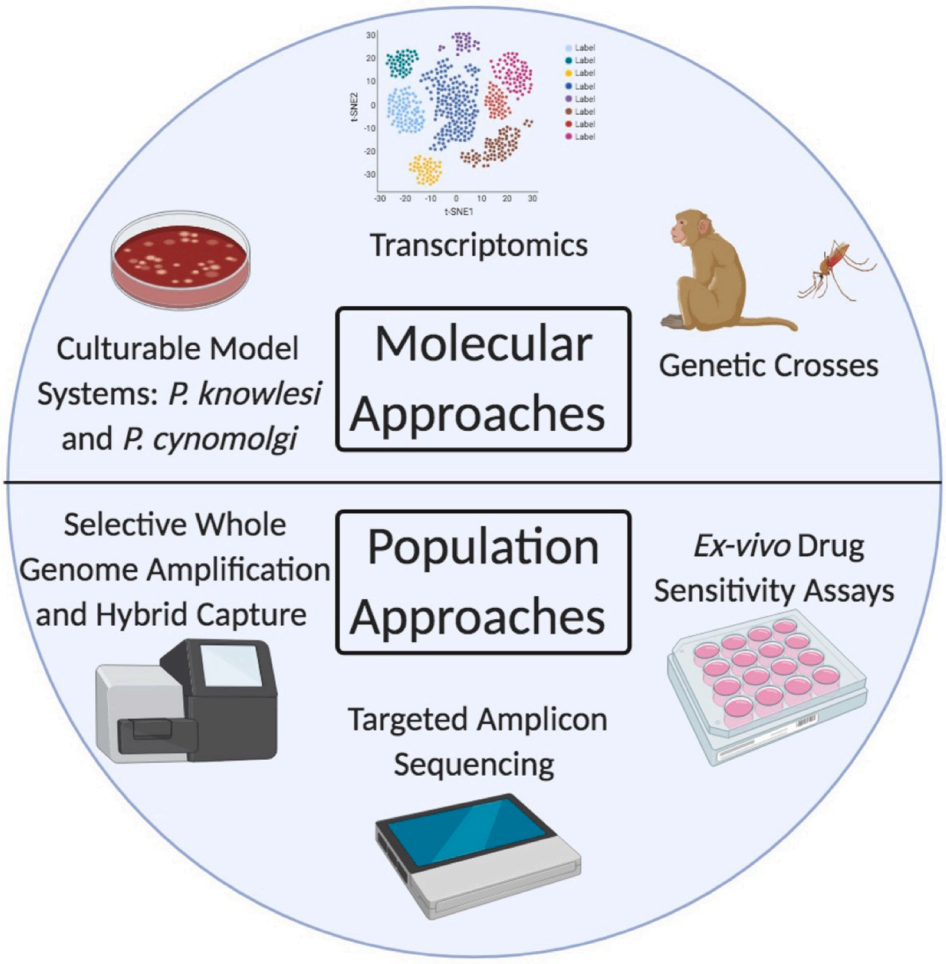

Fig. 3. Summary of current and emerging techniques used to interrogate *P. vivax* drug resistance.

P. falciparum are not found in *P. vivax*, limiting the value of using orthology to infer resistance mutations in *P. vivax*. This could be due to biological differences at the molecular level, including a strong divergence of putative resistance alleles between species, where mutations occurring in similar regions of the gene, but different positions, could confer the same resistance, or the divergent genes may also have significantly different abilities to transport antimalarial compounds. Furthermore, biological differences in *P. vivax* likely significantly alter the selection pressure it faces compared to *P. falciparum*. For example, compared to *P. falciparum*, *P. vivax* has a reduced biomass, a different host cell preference (restriction to reticulocytes), produces transmissible gametocytes early in infection and can relapse from dormant hypnozoites. Using genomic tools that are not biased towards suspected resistance genes based on orthology to *P. falciparum*, may reveal novel *P. vivax* specific mechanisms of drug resistance that have remained largely unexplored. Putative resistance markers will need to be validated in a heterologous system, such as using transgenic systems in *P. knowlesi* or *P. cynomolgi*.

In the literature, putative resistance mutations are frequently referred to as markers of drug resistance in *P. vivax*. Given the relatively limited evidence supporting these putative resistance markers, care should be taken when using sequencing data to inform the levels of CQR or drug policy in a given geographical region. Instead, *ex vivo* assays and clinical treatment failure in the presence of adequate drug concentrations in the blood are the only reliable methods currently available for determining the level of drug resistance in a population. Verified genetic markers of drug resistance could be used to replace these laborious and costly approaches, allowing high-throughput and frequent sampling of parasite populations that can be used to inform drug policy in that region. Molecular surveillance for resistance markers would allow for treatment regimens to be changed upon the emergence of significant drug resistance in a population (Ehrlich et al., 2020; Nkhoma et al., 2007). Understanding of the evolutionary and population dynamics of drug resistance will be critical for molecular surveillance to both identify when these alleles arise and understand how they move throughout and between populations. Investigation of candidate *P. vivax* drug resistance genes using advances in *in vitro* culture, *ex vivo* assays, and sequencing will help make this possible and improve *P. vivax* control and elimination efforts.

Decaration of competing interest

The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.04.002.

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