Ann Ist Super Sanità 2020 | Vol. 56, No. 4: 409-418
DOI: 10.4415/ANN_20_04_02

An insight on drug resistance in *Plasmodium vivax*, a still neglected human malaria parasite

Mariangela L'Episcopia[1], Edvige Perrotti[1], Francesco Severini[1], Stéphane Picot[2,3] and Carlo Severini[1]

[1]*Dipartimento di Malattie Infettive, Istituto Superiore di Sanità, Rome, Italy*
[2]*ICBMS CNRS 5246, SMITh, Malaria Research Unit, Campus Lyon-Tech La Doua, Lyon University, Lyon, France*
[3]*Groupement Hospitalier Nord, Institut de Parasitologie et Mycologie Médicale, Hospices Civils de Lyon, Lyon, France*

Abstract
Plasmodium vivax has been considered for years as responsible for a mild form of malaria, due to the absence in the majority of its infections of the severe form of the disease, typical instead of the deadly human parasite *P. falciparum*. In the last decade, studies on *vivax* malaria have had a partial step ahead especially after the completion of the whole genome project, but there is still a gap of knowledge in the biology of this parasite. Moreover, the emergence of *P. vivax* antimalarial resistance in 1980s and its subsequent spread in the Southeast Asia have indicated new concerns about the possibility to control this parasite. *P. vivax* drug resistance poses a major threat to endemic countries and without important international efforts, we could assist in a near future to the paradox of seeing different malaria co-endemic countries, that have successfully controlled/eliminated *P. falciparum*, still fighting against *P. vivax*.

Key words
- malaria
- *Plasmodium vivax*
- drug resistance

In memoriam of ***Professor Giancarlo Majori, 1943-2020***

INTRODUCTION

The biology of *Plasmodium vivax* is characterized by the existence of hypnozoites, which are characteristic dormant liver stage forms able to cause relapses weeks or years later from the first exposure and clinical manifestation *(Figure 1)*. The discovery of these dormant forms occurred in 1982 [1]. Some of the sporozoites inoculated by the bite of an *Anopheles* mosquitoes, on arriving in liver tissue do not turn into merozoites, which are responsible for the primary malaria attack, but instead become hypnozoites responsible for relapse of this disease. Relapses can occur after different time intervals, based on the *P. vivax* strain and climatic zone: infection by the tropical strain named "Chesson" can result in relapse after just two weeks, whereas strains originating from temperate zones may cause relapses even years after the infection. [2]. Some other biological features make this parasite peculiar and, in some way, more difficult to control than *P. falciparum.* From the epidemiological point of view, an important aspect for *vivax* transmission is the fact that the gametocytes, the parasite forms able to infect the mosquito vectors, appear in the human blood circulation very early, making the patient potentially infectious to the mosquitoes very soon, i.e. at the appearance of the clinical symptoms. Finally, the ability of this parasite to complete its life cycle in the vector at temperature below the 20 °C (the life cycle of *P. vivax* does not occur below the 15 °C) explains the presence of this parasite outside tropical areas and we can assume that in cases of resurgence of malaria in these regions, *P. vivax* will be the responsible plasmodial species [3, 4].

In Europe, in the early 20th century, before the industrial era and before the development of a malaria-control program initiated just after the Second World War, *P. vivax* was the most common species of the four human plasmodia then known. At that time, the annual number of cases of malaria caused by *P. vivax* was much greater than that caused by *P. falciparum*. *P. vivax* was the dominant parasite in temperate areas and most tropical areas except in Africa.

Nowadays, *P. vivax* represents the geographically most widespread *Plasmodium* species, with more than 2.5 billion people at risk of infection and an estimated 15.8 million of clinical cases per years. [5, 6]. According

Address for correspondence: Carlo Severini, Reparto di Malattie Trasmesse da Vettori, Dipartimento di Malattie Infettive, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: carlo.severini@iss.it.

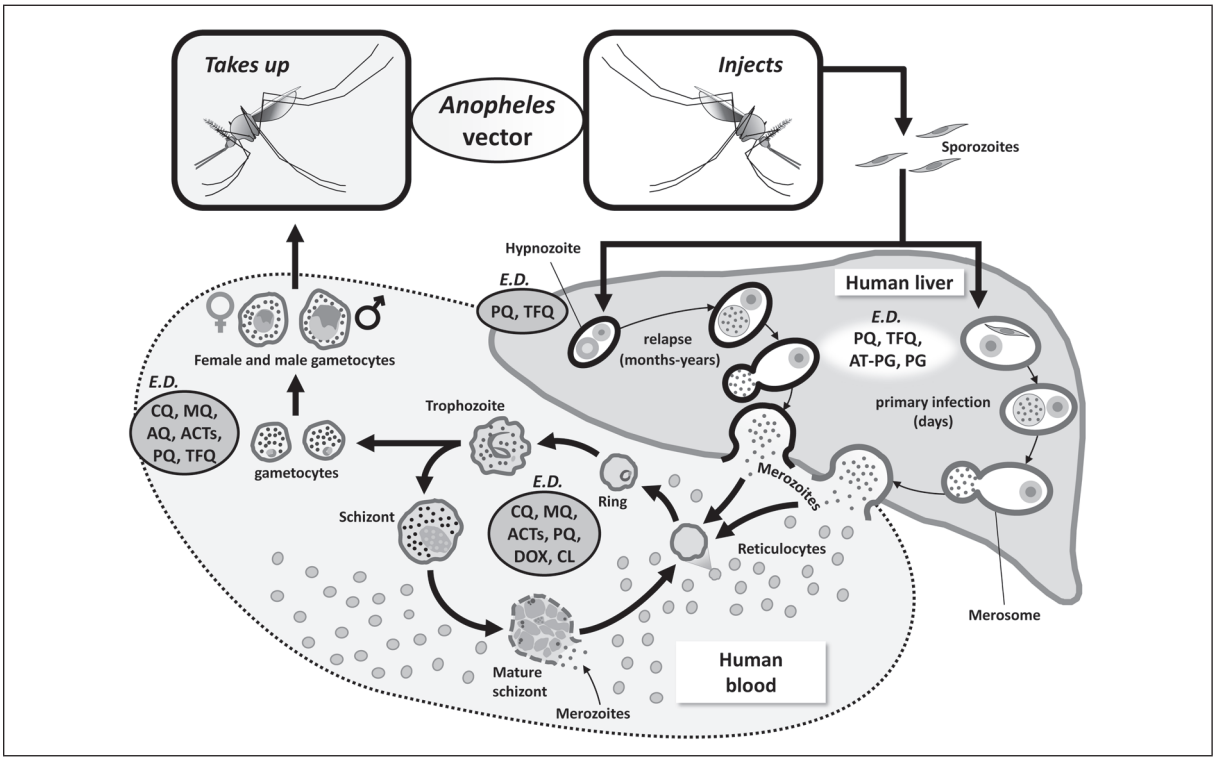

Figure 1

The complexity of the life cycle of *Plasmodium vivax* is illustrated by the fact that it is possible that three different parasitic populations can circulate in the blood of a person infected with *P. vivax*: a responsible population of primary attack, a resurgent population if the strain is resistant to drugs, and a population from a relapse of which hypnozoites are the origin. The diagnostic tools currently available are unable to distinguish these three populations. Specific antimalarial drugs for relevant parasite cell target are also indicated in this figure.

ED: effective drug; CQ: chloroquine; PQ: primaquine; AQ: amodiaquine; MQ: mefloquine; PG: proguanil; AT: atovaquone; TFQ: tafenoquine; DOX: doxycycline; CL: clindamycin; ACTs: artemisinin combination therapies.

to the latest Word Malaria Report, released by WHO in 2019, the highest incidence of *P. vivax* was reported in the region of America, with 75% of malaria cases in the 2018, and Southeast Asia; [7]. Until few years ago, *P. vivax* has been rarely studied in Africa, since the dominance of Duffy-negative blood group in blacks, as key determinant of natural resistance factor of *P. vivax* infection [8]. Nevertheless, several studies have recently shown evidence of *P. vivax* transmission across Africa in Duffy-negative populations [9-11].

In Europe malaria are currently reported as imported cases. Sporadic non-imported/indigenous cases of malaria caused by *P. vivax* were reported through the last years, as occurred in Italy in 1997 [12], in Corsica during the summer of 2006 [13] and in Spain in October 2010 [14]. Even if in 2016, the WHO European Region was declared malaria free [15], nevertheless an outbreak of *vivax* malaria occurred in Greece [16] in the district of Lakonia, Peloponnese between 2011 and 2012 and after an initial successful control of the situation in the two following years (0 local transmission in 2014) some indigenous cases have been still registered in the year 2015-2018 (10 cases in 2018). In France, Spain, and Italy, where malaria was endemic until the end of the sixties, some areas were monitored to assess the risk of reintroduction of malaria [17, 18]. These areas are the Camargue, the largest wetland in the south of France; the Ebro delta; and the Maremma, a great rural territory that stretches between Lazio and Tuscany, in central Italy. Particular eco-climatic conditions and a significant presence of mosquitoes potentially vectors of malaria (phenomenon known as "anophelism without malaria") make these zones prone to malaria reintroduction.

More recently, five events of local malaria transmission (non-imported cases) have been reported recently in the EU. Three of these events were associated with either mosquito-borne transmission from an imported case (introduced malaria) or an imported infected mosquito (airport malaria), in Greece and northern Cyprus (*P. vivax*), and in France (*P. falciparum*); and two of the cases were most likely associated with nosocomial mosquito-borne or iatrogenic transmission of *P. falciparum*, in Italy and Greece [19]. These cases demonstrate that the re-starting of malaria transmission in Europe, even if in the form of small epidemics, is a possibility and the magnitude of risk still under-evaluated.

For over 50 years, chloroquine has been used as the schizonticidal drug choice for the treatment of *vivax* malaria, administrated in combination with primaquine, a drug used to prevent relapses due to hypnozoites. Unfortunately, drug resistance to chloroquine started to emerge at the end of 1980s [20], and over the following years several reports described the extent of

Table 1
Therapeutic protocols currently in use for the treatment of uncomplicated *Plasmodium vivax* malaria

Drugs	Dose	Duration	Notes
Chloroquine (+ primaquine or tafenoquine as below)	600 mg initial 300 mg after 6-8 hrs 300 mg/single daily – day2 and day3	3 days	
Primaquine phosphate	30 mg/single daily *(0.75 mg/kg - single weekly × 8 weeks)*	14 days *(2 months)*	*Not to be used in pregnancy* *To be avoided in G6PD deficiency* *(Prevention of relapse in patients with mild-moderate G6PD deficiency)*
Tafenoquine	300 mg single dose (2 tablets of Krintafel®, 150 mg each)	1 day	*Not to be used in pregnancy* *To be avoided in G6PD deficiency*
Artemether-lumefantrine (20 mg/120 mg) (+ primaquine or tafenoquine as above)	4 tablets initial 4 tablets/single at 8, 24, 36, 48 and 60 hrs	3 days	
Dihydroartemisinin-piperaquine (320 mg/40 mg) (+ primaquine or tafenoquine as above)	3 tablets/single daily × 3 days (36-75 kg bw)	3 days	*>75 kg = 4 tablets/ single daily x 3 days*
Atovaquone-proguanil (+ primaquine or tafenoquine as above)	4 tablets/single daily × 3 days	3 days	

chloroquine resistance spread across most of *vivax*-endemic countries [21]. A summary of therapeutic protocols currently in use for the treatment of uncomplicated *Plasmodium vivax* malaria is reported in *Table 1*. In areas where chloroquine is high ineffective due to resistance, artemisinin combination therapy, co-administrated with primaquine, has been used as alternative drug strategy [22]. Recently, in July 2018, tafenoquine, commercially known as Krintafel® and Arakoda®, has been approved by FDA as a longer acting anti-hypnozoite drug for the radical cure of *vivax* malaria and as a possible substitute of primaquine. It is administrated in a single oral dose in combination along with a schizonticide, resulting in a better compliance linked to the dose regimen [23]. As for primaquine, this drug cannot be used in glucose-6-phosphate dehydrogenase (G6PD) deficiency and in pregnancy, due to the unclear risks of its use in these two circumstances.

Since years, scientific research on *P. vivax* has been fewer compared to *P. falciparum*. Moreover, in the recent decades, there have been relatively few programs and funds made available for targeted action against *P. vivax*, probably because malaria mortality is linked mainly to *P. falciparum* infections and occurs primarily in African children. Instead, *P. vivax* epidemiology shows several peculiarities that would suggest the importance of increasing international efforts to shed light on the biology of this parasite and to channel more resources to support endemic countries for collaborative international studies and to develop effective control programs for its detection, characterization and containment. Just to highlight the most important features: *P. vivax* is endemic in most of the world's malarial areas and exerts in all these areas a socio-economic burden that is anything but negligible; the transmission of this parasite is mostly driven by the reactivation of hypnozoites (relapses) and the observed increase of *vivax* infection after *falciparum* malaria treatment make mandatory the development and implementation of an effective radical cure for malaria in areas of coendemicity; the report of severe/fatal clinical forms and the proven transmission among the Duffy-negative people of sub-Saharan Africa deny two of the most believed dogmas related to *P. vivax* infection (*P. vivax* is a benign form of malaria; Duffy-negative people are resistant to *vivax* infection); the emergence and spread of chloroquine resistance, especially in southeast Asia [24].

Above all, drug resistance is considered one of the biggest challenges in the fight and control against malaria; it has contributed to the spread of malaria to non-endemic countries and the re-emergence of the disease in areas where it had been eradicated. The combination of these epidemiological and biological features of the *P. vivax*, including the resistance mechanisms, justify an updated picture of the situation. A comparison between what is known about *P. falciparum* and *P. vivax* in term of drug resistance is also presented in this concise review, in order to highlight the lack of biological knowledge about this important human plasmodial parasite.

DRUG RESISTANCE IN *P. VIVAX*

P. vivax resistance to chloroquine (*Pv*CQR) has been detected in the late 1980s in isolates from Papua New Guinea [20, 25] and Indonesia [26], about 30 years later than the emergence of chloroquine resistance in *P. falciparum*. Same situation has been described for other antimalarial drugs, like primaquine, mefloquine and pyrimethamine/sulfadoxine [27]. Mode of action of the pyrimethamine and sulfadoxine and the mechanism for resistance to these drugs are the only ones well documented in *P. vivax*. There are several antimalarials targeting the blood forms of *P. vivax*, such as chloroquine, amodiaquine, piperaquine, artesunate, artemether, lumefantrine, dihydroartemisinin, but chloroquine is still the first-line drug for the treatment of *P. vivax*.

From the discovery of the first isolate of chloroquine-

resistant (CQR) *P. vivax* in Papua New Guinea in 1989 [20-26], this resistance has spread throughout Southeast Asia and the world, even though its progress is slower than for *P. falciparum* resistance [21-28]. Due to the emergence and spread of *Pv*CQR, the treatment of malaria caused by *P. vivax* is expected to become more difficult in the coming years. Despite having been reported more than 30 years ago, mechanisms underlying *Pv*CQR have not been completely understood due to the difficulties to maintain a continuous *in vitro* culture system for this parasite. Antimalarial drug resistance is based on the accumulation of specific mutations occurring in genes encoding essential enzymes or proteins involved in the parasite biology. The development of resistance to chloroquine is a serious issue because this drug is the recommended one as first-line treatment for this parasite. In any case, *Pv*CQR is limited if considering the worldwide use of chloroquine. The radical cure of the parasite, the erythrocyte forms and liver forms responsible for relapse, is based on a combination of chloroquine with primaquine. This treatment, used for sixty years, sometimes failed in different malarious areas in Southeast Asia and primaquine itself has constraints and side effects that limit its use [22]. Unfortunately, as other important aspects of the biology of this parasite, antimalarial resistance mechanisms in *P. vivax* remain no deciphered yet.

Therapeutic protocol based on artemisinin-based combination therapies (ACTs) are currently the treatment of choice for uncomplicated malaria attacks caused by *P. falciparum*. Recent studies have shown evidence of a faster *P. vivax* parasites clearance of ACTs respect to chloroquine with a considerable reduction in recurrence [29, 30]. Notably, the combination of dihydroartemisinin and piperaquine (DHA-PPQ) results in a rapid reduction of parasitemia and in an optimal prophylaxis strategy against potential relapses. The efficacy of this therapeutic approach depends on the combination of a fast acting (DHA) and a longer acting drug (PPQ) highly effective in the prevention of recurrences up to 56 days [31]. A first study in Thailand in 2000 [32] showed that artesunate and artemether quickly eliminated parasites in the blood of patients compared with other antimalarials. More recent studies [33-34] have shown the efficacy of the combination artemether-lumefantrine (AL, Coartem® and Riamet®) in the treatment of *P. vivax* infections. The advantage of a common therapeutic approach, i.e. using ACT to treat all malaria cases, would be of paramount importance in areas where *P. vivax* and *P. falciparum* coexist (e.g. in Southeast Asia and South America). As described by Bassat *et al.* in 2014, the efficacy of AL with other ACTs whose partner drugs have a longer half-life (DHA-PPQ, artemether-mefloquine) were compared. Data showed that *vivax* relapses are only delayed and not eliminated [34]. Using ACT would also limit the use of chloroquine, preserving its effectiveness against *P. vivax*. However, AL combination is not active against hypnozoites, and does not protect against recurrence and exposes patients to the risk of new infections in a shorter period if it is not associated with treatment with primaquine [35]. As described by Commons *et al.*, 2019, in areas where *P. falciparum* and *P. vivax* coexist, there is a high risk of subsequent *P. vivax* parasitemia (by day 63 more than 15%) after treatment of *falciparum* malaria with an effective ACT [36]. The recurrence of *vivax* parasitemia depends on the reactivation of the dormant liver stages, demonstrating that ACTs result efficacy for the treatment of the erythrocytic stages of the two parasites, but not on the hypnozoites. Previous studies have been proved the ability of primaquine to act as a schizonticidal drug on the asexual blood stages of *P. vivax* [37], and as a gametocytocidal drug on *P. falciparum* [38]. For this reason, Commons *et al.* hypothesized that the administration of an artemisinin-combination regimen along with primaquine would decline the incidence of recurrent *P. falciparum* and *P. vivax* infections, and reduce the risk of transmission in patients with *falciparum* monoinfection [36]. Mixed infections of *falciparum*/*vivax* are thus an important issue for the treatment of malaria patients in co-endemic countries; and the study of potential interactions between the two species could provide more information about determinants of drug resistance *in vivo*. A unified treatment strategy for asexual forms of two infections (*falciparum* and *vivax*) offers significant advantages in areas where the two are co-endemic.

The unanswered question is: how long does one have to follow a patient to see if he has a *vivax* relapse? According to several studies, generally, the follow-up ranges between 4-6 weeks, as in the case of *P. falciparum* infections, up to 6 months. Nevertheless, this period is not enough to capture long-latency relapses, which can appear 8-9 months after the first exposure [39]. Finally, even in countries where chloroquine remains highly effective, the administration of an artemisinin-based regimen could remove more rapidly the *vivax* gametocytes biomass, reducing the risk of transmission [40].

THE CHALLENGE OF EVALUATING THE DRUG RESISTANCE IN *P. VIVAX*

The resistance of the malaria parasite is a complex phenomenon that involves many parameters and can be measured by different approach. In general, as for example is for *P. falciparum,* three approaches are used to identify and evaluate the level of drug resistance in plasmodial isolates: *in vivo* tests, *in vitro* tests and molecular markers analysis.

In vivo tests (efficacy therapeutic tests, ETTs) can be used in which clinical and parasitological symptoms in malaria patients treated with these drugs are followed long enough to see whether parasite re-appearance occurs. *In vivo* tests are the gold standard, because they provide clinical evidence of treatment outcome, but, unfortunately, *in vivo* tests are difficult to implement in the frame of control programs, since following cohorts of patients for about a month is rarely possible in endemic area.

In vitro tests (or phenotypic analysis) include putting a culture of the parasite in the presence of various concentrations of drugs to determine the effective dose and to define thresholds for each drug.

Finally, molecular markers analysis (in general, the assessment of polymorphisms present in plasmodial

genes involved in drug resistance) could represent a useful way to survey the emergence and spread of drug resistance surveillance in endemic areas [41].

In vivo tests (ETTs) for *P. vivax* are limited by the same parasite biology. Indeed, *P. vivax* has intra-hepatocyte forms (hypnozoites) that escape most drugs, which can cause re-appearance of the parasite several weeks or months after infection. It then becomes very difficult to know whether a patient was properly treated and relapsed, was infected with a resistant parasite that escaped drug treatment, or was infected by a parasite dormant form, which are protected from the drug. Actually, the outcome of a treatment is very often challenging in *vivax* endemic areas, since it is hard to known if a parasitemia re-appearance in a given patient might be due to a real treatment failure or to the hypnozoite reactivation or a new infection.

Highly standardized methods are available for *P. falciparum* continuous cultures since long time, making affordable a wide range of biological studies, as for example the evaluation of resistance of this parasite to the different antimalarials. In the case of *P. vivax*, the situation is different: we do not have a continuous culture of *P. vivax in vitro*, and therefore, *in vitro* tests is in general somewhat difficult. The main problem is probably that *P. vivax* favors young erythrocytes, i.e., reticulocytes, which represent only one percent of red blood cells in human blood. It also appears that, even if we add a constant amount of reticulocytes to the culture, the parasite has difficulty reproducing and multiplying *in vitro*.

The only way of maintaining *P. vivax* strains *in vitro* is to alternate cultures in flasks with the inoculation of the parasite in monkeys. *P. vivax* infects several species of non-human primates, including chimpanzees, gibbons, and "squirrel monkeys" (Saimiri sciureus), without problems [42], but the research groups able to breed and manipulate monkeys in the laboratory are very few in the world.

In 2007, a group of researchers from Thailand presented an *in vitro* method in which *P. vivax* survives for a few parasitic cycles [43], but the protocol turned to be complex and difficult to reproduce. Subsequently, in 2015, the same protocol has been improved allowing the *in vitro* cultivation for over 26 months, even if with a low parasite density [44]. More recently, different protocols of "short-term culture" for *P. vivax* isolates have been presented in the literature but the protocols are still limited in efficacy especially when compared to the high-standardized protocol for *P. falciparum* culturing [45, 46].

In summary, the situation about *P. vivax* culturing is frustrating after more than 100 years of attempts (the first protocol of *vivax* cultures was published in 1912 by Bass and Johns) and the lack of *in vitro* cultures of *P. vivax* makes it difficult to monitor the sensitivity of this parasite to drugs and constitutes the main gap in biology knowledge of this parasite [47].

The analysis of molecular marker polymorphisms in general supports *in vitro* and *in vivo* tests assays and could be a useful way to try to identify resistance of *plasmodium* parasites to drug treatments [41]. However, in *P. vivax,* the value of these markers depends on the ability to analyze them in a patient where treatment has failed, and the ability to distinguish whether a patient was properly treated and relapsed, was infected with a resistant parasite that escaped drug treatment, or a hepatocyte which was infected with a dormant form of the parasite, which was protected from the drug treatment. Currently there is not a truly reliable method to assess the sensitivity or resistance of *P. vivax* to treatment. Meanwhile, several observations of treatment failures raise concern about the development of resistance in *P. vivax*.

The difficulties in detecting and studying *P. vivax* resistance than *P. falciparum* depends on the basic biology and epidemiology of the two parasites. The major and latest differences obtained towards understanding the drug resistance in *P. falciparum* and *P. vivax* are shown in *Table 2*.

The search for the identification of reliable molecular markers in *P. vivax* has been focused in the recent years on the *P. vivax* putative transporter protein gene (*pvcrt-o, P. falciparum* orthologues CQR-genes), multidrug resistance gene (*pvmdr-1,* CQR) and dihydrofolate reductase and dihydropteroate synthase (*pvdhfr/pvdhps* genes, PYR/SUL-R)

In the case of *P. falciparum*, CQR is measured by a decrease in the action of the drug at the parasite's food vacuole. Mutations of genes *pfcrt* and in part, *pfmdr1* are involved in this process. Shortly after the identification of the gene *crt* in *P. falciparum* and demonstration of its involvement in CQR, the orthologous gene (*crt-o*) in

Table 2
Differences in drug resistance knowledge between *Plasmodium vivax* and *Plasmodium falciparum*

Plasmodium spp.	Antimalarial drugs with known resistance	Identified molecular marker genes for drug resistance
Plasmodium vivax	CQ, PQ, QN, PYR/SUL	*crt-o* = CQR? *mdr1* = CQR?
Plasmodium falciparum	CQ, QN, AQ, MQ, PPQ, PYR/SUL, PG, ARTder, ATQ	*dhfr/dhps* = PYR/SUL, PG *crt, mdr1, nhe,* = CQ, QN, MQ, AQ *pm2* = PPQ *cytb* = ATQ *k13* = ARTder

Drugs – CQ: chloroquine; PQ: primaquine; QN: quinine; AQ: amodiaquine; MQ: mefloquine; PPQ: piperaquine; PRG: proguanil; PYR/SUL: pyrimethamine/sulfadoxine; ATQ: atovaquone; ARTder: artemisinin derivatives (dihydroartemisinin, artesunate, artemether).
Genes – *mdr1:* multidrug resistance1; *crt:* chloroquine resistance transporter; *nhe*: Na^+/H^+ exchanger; *dhfr/dhps:* dihydrofolate reductase/dihydropoteroate synthase; *cytb*: cytochrome b; *pm2*: plasmepsin 2; *k13*: kelch-*on chromosome*-13.

P. vivax was identified [48]. Studies with several different strains of the parasite found in endemic areas have not shown an association between the polymorphism of this gene and resistance to CQ. A 2006 study in Brazil showed a decreased response to CQ for a *P. falciparum* strain transfected with the *crt P. vivax* (heterologous expression system), suggesting a possible involvement of this gene in CQR [49].

More recently, Sà *et al.*, investigated again the possible role of the *crt-o* in *P. vivax* CQR and described an upregulation of this gene through crossing different parasite population with different sensitivity to CQ [50]. Despite the studies above mentioned, the involvement of *crt-o* gene in *vivax* CQR remain to be deciphered yet.

The *mdr1* gene in *P. falciparum* plays an important role in modulating resistance against several drugs, most of which belong to the class of quinolines, but also to artemisinin derivatives [51]. The decrease in antimalarial sensitivity is related to the presence of polymorphic loci in the gene or an increase in the copy number of the gene in the genome of the parasite, a condition known as "multidrug resistance effect". For example, the Thailand-Myanmar, *P. falciparum* isolates have amplification of the gene *Pfmdr1* associated with decreased sensitivity to mefloquine, quinine, lumefantrine, halofantrine, and artemisinin derivatives [52]. In 2005, Brega *et al.* were the first to demonstrate the role of *P. vivax mdr1* gene in drug resistance [53]. While early studies have not shown a relationship between the presence of mutations of this gene and sensitivity of the parasite to quinoline [54, 55], studies conducted more recently in Southeast Asia (Thailand, Indonesia [56, 57] and Papua New Guinea [58]) have sought to clarify the role of *Pvmdr1* in antimalarial drug resistance, taking into consideration either the gene polymorphism or the number of copies and comparing the molecular results with the *in vitro* and *in vivo* sensitivity results. We can summarize the results of these studies as follows: chloroquine and mefloquine exert a competitive evolutionary pressure on *Pvmdr1*, identical to that observed with *P. falciparum*; the polymorphism at the codon 976 (Y976F) of the *Pvmdr1* gene can be used as an indicator to monitor *P. vivax* resistance to chloroquine; amplification of the gene *Pvmdr1* in multidrug-resistance shows an effect similar to that observed in *P. falciparum*. This appears to be limited to areas with endemic zones characterized by the circulation of these drugs. Amplification of copy number was observed in isolates from Thailand but not in isolates from Papua New Guinea, where mefloquine is not used [56]. Finally, in 2017 a study carried out in *P. vivax* field isolates from Mauritania, Sudan and Oman investigated single nucleotide polymorphisms in *Pvmdr1* and, for the first time, in *PvMCA1-cd* (spelling) gene, to look for a potential role of these two genes in *P. vivax* drug resistance [57].

Pyrimethamine and sulfadoxine (S/P) are inhibitors of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). Due to the quick rise of *P. falciparum* resistance and the occurrence of rare but severe adverse events, currently this drug combination is rarely used for malaria treatment. However, this association is still recommended by WHO in intermittent preventive treatment in pregnant women (ITPs) and it is also used in association with artemisinin derivatives. Resistance of *P. vivax* to S/P appeared very quickly and DHFR/DHPS inhibitors have not been used for the treatment of *P. vivax* malaria because preliminary tests showed low effectiveness against *P. vivax*. *P. vivax* was considered intrinsically resistant to these two drugs. But recent studies have shown a correlation between the point mutations in the *dhfr* gene of *P. vivax* isolates from different geographical areas and resistance to antifolate: Thailand, India, Madagascar, Comoros [58] and Papua New Guinea [59]. The mechanism of resistance to S/P is the only well-known mechanism for this parasite because the situation is similar to that of *P. falciparum:* mutations in the genes encoding the DHFR and DHPS enzymes are responsible for a change in the 3-D structure of these proteins and, therefore, these mutations lead to a decrease in the affinity of the mutated enzyme vis-a-vis antifolates. Pécoulas *et al.*, in 1998, isolated and cloned the DHFR-TS domain of the *dhfr* gene of *P. vivax* and different alleles of the gene have been identified [60, 61] and more recently, *P. vivax dhps* gene has been identified and characterized [62, 63]. Nevertheless, further studies on *dhfr/dhps* polymorphisms are needed to properly assess the extent of genetic variation in these *P. vivax* resistance markers.

After a long search to identify a specific locus implicated in artemisinin resistance, the kelch propeller domain of the *k13* gene on chromosome 13 was recently identified as a molecular marker of artemisinin resistance in *P. falciparum*: several mutations in the kelch propeller domain have now been associated with *in vitro* ring stage survival assays and delayed parasite clearance rates in patients treated with artemisinins [64]. A recent study identified the *Pfk13* ortholog for *P. vivax*, *Pvk12*, showing that non-synonymous mutations in this gene are already circulating at very low frequencies in Cambodia [65]. More recent studies conducted in Southeast Asia confirmed the limited polymorphism of *Pvk12* making the role of this gene in artemisinin resistance unclear [66, 67].

ARE HYPNOZOITES RESERVOIR OF RESISTANCE?

The presence of these dormant liver forms enormously complicates control of this parasite in endemic areas as well as in non-endemic areas for the management of *vivax* imported cases. Knowledge of the biology of hypnozoites is very limited and they escape most drugs. Several articles report that the introduction of artemisinin derivatives in Asia has caused a drop in cases of *P. falciparum* in different endemic areas without having the same efficacy on *P. vivax* [68]. The hystorical drug able of acting on hypnozoites and preventing relapse is primaquine (PQ). However, this drug has two weaknesses: hemolysis risk in the case of red blood cell of the patient is G6PD deficient, and a bad compliance due to its long course of treatment (15 days). *P. vivax* resistance to primaquine has been already reported also [69, 70].

Hypnozoites and their reactivation allow *P. vivax* to survive in temperate zones characterized by a marked seasonality of vector populations. Several questions on

the biology of hypnozoites are currently unanswered, which has direct implications for the control of this pest. What is the "signal alarm" for the hypnozoites? A Finnish study sought to provide an answer to this question, assuming that the saliva of *Anopheles* injected during the bite by the mosquito can be responsible for the triggering [71].

The ability to artificially induce the reactivation of hypnozoites is a fascinating perspective that might provide a new and effective control strategy for this elusive human parasite.

CONCLUSION

WHO recognized in its recent agenda that more attention has to be paid to *P. vivax* infections to move forward in the elimination efforts. Even though TQ does not overcome all shortcomings of PQ, the TQ single dose anti-relapse treatment for hypnozoites of *P. vivax* as well as of *P. ovale* markedly improves the patient compliance to the treatment regimen for these malaria species [72]. The changes in the WHO agenda might have given the final impulse to keep TQ in the development pipeline and finally bring it to the market. Currently no other drugs are available or in pipeline for anti-relapse therapy [73].

Considering that development from the early clinical phase to market approval takes ~10 years, most probably no alternative to 8-AQ for anti-relapse therapy will be available in the near future. It is ultimately conceivable that in the near future TQ will replace current treatment regimens with PQ and play a crucial role in the treatment protocol included in programs for the control of *P. vivax* malaria [72].

The European Commission and the major international stakeholders are now the only ones who can effectively support research programs of the magnitude needed to overcome the bottlenecks in knowledge in the fight against *P. vivax*. Yet, obtaining financing in these frameworks is very uncertain, and the money offered is rarely compatible with these objectives. In an international scenario in which funding made available for *falciparum* malaria control programs had an increase stop in 2017 and even a decrease in 2018 [7] and it is necessary to define priorities in the use of public or private funds, malaria control projects focused on *P. vivax* have little chance of winning.

In conclusion, despite the completion of the whole genome sequencing, several steps forward in the knowledge of the biology of this parasite and the availability of a new drug for radical treatment, *P. vivax* malaria is still to be considered among the neglected human diseases.

It is of pivotal importance to invest in international control programs targeting *P. vivax* otherwise in the near future we could assist to the paradox of seeing different malaria co-endemic countries, that have successfully controlled/eliminated *P. falciparum*, still fighting against *P. vivax*.

Acknowledgements

The authors are particularly indebted to Marie Godfrey and Alessandro Bettini for the language revision.

Authorship contributions

CS, SP, MLE and EP wrote the manuscript. FS edited Tables and Figures. CS, SP, MLE, EP and FS reviewed the manuscript and approved the final version of the manuscript.

Conflict of interest statement

The Authors declare no conflicts of interest.

Received on 2 March 2020.
Accepted on 28 July 2020.

REFERENCES

1. Krotoski WA, Garnham PC, Bray RS, Krotoski DM, Killick-Kendrick R, Draper CC, Targett GA, Guy MW. Observations on early and late post-sporozoite tissue stages in primate malaria. I. Discovery of a new latent form of *Plasmodium cynomolgi* (the hypnozoite), and failure to detect hepatic forms within the first 24 hours after infection. Am J Trop Med Hyg. 1982;31(1):24-35.
2. Cogswell FB. The hypnozoite and relapse in primate malaria. Clin Microbiol Rev. 1992;5(1):26-35. doi: 10.1128/cmr.5.1.26
3. Petersen E, Severini C, Picot S. *Plasmodium vivax* malaria: a re-emerging threat for temperate climate zones? Travel Med Infect Dis. 2013;11(1):51-9. doi: 10.1016/j.tmaid.2013.01.003
4. Piperaki ET, Daikos GL. Malaria in Europe: emerging threat or minor nuisance? Clin Microbiol Infect. 2016;22(6):487-93. doi: 10.1016/j.cmi.2016.04.023
5. Howes RE, Battle KE, Mendis KN, Smith DL, Cibulskis RE, Baird JK, Hay SI. Global Epidemiology of Plasmodium vivax. *Am J Trop Med Hyg*. 2016;95(6 Suppl):15-34. doi: 10.4269/ajtmh.16-0141
6. Hupalo DN, Luo Z, Melnikov A, Sutton PL, Rogov P, Escalante A, Vallejo AF, Herrera S, Arévalo-Herrera M, Fan Q, Wang Y, Cui L, Lucas CM, Durand S, Sanchez JF, Baldeviano GC, Lescano AG, Laman M, Barnadas C, Barry A, Mueller I, Kazura JW, Eapen A, Kanagaraj D, Valecha N, Ferreira MU, Roobsoong W, Nguitragool W, Sattabonkot J, Gamboa D, Kosek M, Vinetz JM, González-Cerón L, Birren BW, Neafsey DE, Carlton JM. Population genomics studies identify signatures of global dispersal and drug resistance in *Plasmodium vivax*. Nat Genet. 2016;48(8):953-8. doi: 10.1038/ng.3588
7. World Health Organization. World Malaria Report 2019. Geneva: WHO; 2019.
8. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med. 1976;295(6):302-4. doi: 10.1056/NEJM197608052950602
9. Twohig KA, Pfeffer DA, Baird JK, Price RN, Zimmerman PA, Hay SI, Gething PW, Battle KE, Howes RE. Growing evidence of *Plasmodium vivax* across malaria-endemic Africa. PLoS Negl Trop Dis. 2019;13(6):e0007525. doi: 10.1371/journal.pntd.0007525

10. Gunalan K, Niangaly A, Thera MA, Doumbo OK, Miller LH. *Plasmodium vivax* infections of Duffy-negative erythrocytes. Historically undetected or a recent adaptation? Trends Parasitol. 2018;34(5):420-9. doi: 10.1016/j.pt.2018.02.006
11. Zimmerman PA. *Plasmodium vivax* infection in Duffy-negative people in Africa. Am J Trop Med Hyg. 2017;97(3):636-8. doi: 10.4269/ajtmh.17-0461
12. Baldari M, Tamburro A, Sabatinelli G, Romi R, Severini C, Cuccagna G, Fiorilli G, Allegri MP, Buriani C, Toti M. Malaria in Maremma, Italy. Lancet. 1998;351(9111):1246-7. doi: 10.1016/S0140-6736(97)10312-9
13. Armengaud A, Legros F, Quatresous I, Barre H, Valayer P, Fanton Y, D'Ortenzio E, Schaffner F. A case of autochthonous *Plasmodium vivax* malaria, Corsica, August 2006. Travel Med Infect Dis. 2008;6(1-2):36-40. doi: 10.1016/j.tmaid.2007.09.042
14. Santa-Olalla Peralta P, Vazquez-Torres MC, Latorre-Fandós E, Mairal-Claver P, Cortina-Solano P, Puy-Azón A, Adiego Sancho B, Leitmeyer K, Lucientes-Curdi J, Sierra-Moros MJ. First autochthonous malaria case due to *Plasmodium vivax* since eradication, Spain, October 2010. Euro Surveill. 2010;15(41):pii=19684.
15. Eurosurveillance editorial team. World Health Organization announces European Region malaria free. Euro Surveill. 2016;21(17):pii=30216. doi: 10.2807/1560-7917.ES.2016.21.17.30216
16. Andriopoulos P, Economopoulou A, Spanakos G, Assimakopoulos G. A local outbreak of autochthonous *Plasmodium vivax* malaria in Laconia, Greece – a re-emerging infection in the southern borders of Europe? Int J Infect Dis. 2013;17(2):e125-e128. doi: 10.1016/j.ijid.2012.09.009
17. Ponçon N, Tran A, Toty C. A quantitative risk assessment approach for mosquito-borne diseases: malaria re-emergence in southern France. Malar J. 2008;7:147. doi: 10.1186/1475-2875-7-147
18. Romi R, Boccolini D, Vallorani R, Severini F, Toma L, Cocchi M, Tamburro A, Messeri G, Crisci A, Angeli L, Costantini R, Raffaelli I, Pontuale G, Thiéry I, Landier A, Le Goff G, Fausto AM, Di Luca M. Assessment of the risk of malaria re-introduction in the Maremma plain (Central Italy) using a multi-factorial approach. Malar J. 2012;11:98. doi: 10.1186/1475-2875-11-98
19. European Centre for Disease Prevention and Control. Multiple reports of locally-acquired malaria infections in the EU – 20 September 2017. Stockholm: ECDC; 2017.
20. Rieckmann KH, Davis DR, Hutton DC. *Plasmodium vivax* resistance to chloroquine? Lancet. 1989;2(8673):1183-4. doi: 10.1016/s0140-6736(89)91792-3
21. Price RN, von Seidlein L, Valecha N, Nosten F, Baird JK, White NJ. Global extent of chloroquine-resistant Plasmodium vivax: a systematic review and meta-analysis. Lancet Infect Dis. 2014;14(10):982-91. doi: 10.1016/S1473-3099(14)70855-2
22. World Health Organization. Guidelines for the Treatment of Malaria. 3rd edition. Annex 6, Treatment of *Plasmodium vivax, P. ovale, P. malariae* and *P. knowlesi* infections. Geneva: WHO; 2015. Available from: www.ncbi.nlm.nih.gov/books/NBK294430/.
23. Lacerda MVG, Llanos-Cuentas A, Krudsood S, Lon C, Saunders DL, Mohammed R, Yilma D, Batista Pereira D, Espino FEJ, Mia RZ, Chuquiyauri R, Val F, Casapía M, Monteiro WM, Brito MAM, Costa MRF, Buathong N, Noedl H, Diro E, Getie S, Wubie KM, Abdissa A, Zeynudin A, Abebe C, Tada MS, Brand F, Beck HP, Angus B, Duparc S, Kleim JP, Kellam LM, Rousell VM, Jones SW, Hardaker E, Mohamed K, Clover DD, Fletcher K, Breton JJ, Ugwuegbulam CO, Green JA, Koh GCKW. Single-dose tafenoquine to prevent relapse of *Plasmodium vivax* malaria. N Engl J Med. 2019;380(3):215-28. doi: 10.1056/NEJMoa1710775
24. Price RN, Commons RJ, Battle KE, Thriemer K, Mendis K. New developments in *Plasmodium vivax* malaria: severe disease and the rise of chloroquine resistance. Trends Parasitol. 2020; 6:560-70.
25. Whitby M, Wood G, Veenendaal JR, Rieckmann K. Chloroquine-resistant *Plasmodium vivax*. Lancet. 1989;2(8676):1395. doi: 10.1016/s0140-6736(89)92002-3
26. Baird JK, Basri H, Purnomo, Bangs MJ, Subianto B, Patchen LC, Hoffman SL. Resistance to chloroquine by *Plasmodium vivax* in Irian Jaya, Indonesia. Am J Trop Med Hyg. 1991;44:547-52. doi: 10.4269/ajtmh.1991.44.547
27. World Health Organization. Global report on antimalarial efficacy and drug resistance: 2000-2010. Geneva: WHO; 2010.
28. Gonçalves LA, Cravo P, Ferreira MU. Emerging *Plasmodium vivax* resistance to chloroquine in South America: an overview. Mem Inst Oswaldo Cruz. 2014;109(5):534-9. doi: 10.1590/0074-0276130579
29. Visser BJ, Wieten RW, Kroon D, Nagel IM, Bélard S, van Vugt M, Grobusch MP. Efficacy and safety of artemisinin combination therapy (ACT) for non-*falciparum* malaria: a systematic review. Malar J. 2014;13:463. doi: 10.1186/1475-2875-13-463
30. Tavul L, Hetzel MW, Teliki A, Walsh D, Kiniboro B, Rare L, Pulford J, Siba PM,Karl S, Makita L, Robinson L, Kattenberg JH, Laman M, Oswyn G, Mueller I. Efficacy of artemether-lumefantrine and dihydroartemisinin-piperaquine for the treatment of uncomplicated malaria in Papua New Guinea. Malar J. 2018;17(1):350. doi: 10.1186/s12936-018-2494-z
31. Sinclair D, Gogtay N, Brand F, Olliaro P. Artemisinin-based combination therapy for treating uncomplicated *Plasmodium vivax* malaria. Cochrane Database Syst Rev. 2011 Jul 6;(7):CD008492. doi: 10.1002/14651858.CD008492.pub2
32. Pukrittayakamee S, Chantra A, Simpson JA, Vanijanonta S, Clemens R, Looareesuwan S, White NJ. Therapeutic responses to different antimalarial drugs in *vivax* malaria. Antimicrob Agents Chemother. 2000;44(6):1680-5. doi: 10.1128/aac.44.6.1680-1685.2000
33. Eibach D, Ceron N, Krishnalall K, Carter K, Bonnot G, Bienvenu AL, Picot S. Therapeutic efficacy of artemether-lumefantrine for *Plasmodium vivax* infections in a prospective study in Guyana. Malar J. 2012;11:347. doi: 10.1186/1475-2875-11-347
34. Bassat Q. The use of artemether-lumefantrine for the treatment of uncomplicated *Plasmodium vivax* malaria. PLoS Negl Trop Dis. 2011;5(12):e1325. doi: 10.1371/journal.pntd.0001325
35. Senn N, Rarau P, Manong D, Salib M, Siba P, Reeder JC, Rogerson SJ, Genton B, Mueller I. Effectiveness of artemether/lumefantrine for the treatment of uncomplicated *Plasmodium vivax* and *P. falciparum* malaria in young children in Papua New Guinea. Clin Infect Dis. 2013;56(10):1413-20. doi:10.1093/cid/cit068.
36. Pukrittayakamee S, Vanijanonta S, Chantra A, Clemens R, White NJ. Blood stage antimalarial efficacy of primaquine in *Plasmodium vivax* malaria. J Infect Dis. 1994;169(4):932-5. doi: 10.1093/infdis/169.4.932
37. Smithuis F, Kyaw MK, Phe O, Win T, Aung PP, Oo AP, Naing AL, Nyo MY, Myint NZ, Imwong M, Ashley E, Lee SJ, White NJ. Effectiveness of five artemisinin com-

bination regimens with or without primaquine in uncomplicated *falciparum* malaria: an open-label randomised trial. Lancet Infect Dis. 2010;10(10):673-81. doi: 10.1016/S1473-3099(10)70187-0

38. Commons RJ, Simpson JA, Thriemer K, Hossain MS, Douglas NM, Humphreys GS, Sibley CH, Guerin PJ, Price RN. Risk of Plasmodium vivax parasitaemia after *Plasmodium falciparum* infection: a systematic review and meta-analysis. Lancet Infect Dis. 2019;19(1):91-101. doi: 10.1016/S1473-3099(18)30596-6
39. Rijal KR, Adhikari B, Ghimire P, Banjara MR, Das Thakur G, Hanboonkunupakarn B, Imwong M, Chotivanich K, Day NPJ, White NJ, Pukrittayakamee S. Efficacy of primaquine in preventing short- and long-latency *Plasmodium vivax* relapses in Nepal. J Infect Dis. 2019;220(3):448-56. doi: 10.1093/infdis/jiz126
40. Douglas NM, Anstey NM, Angus BJ, Nosten F, Price RN. Artemisinin combination therapy for *vivax* malaria. Lancet Infect Dis. 2010;10(6):405-16. doi: 10.1016/S1473-3099(10)70079-7
41. Baird JK. Resistance to therapies for infection by *Plasmodium vivax*. Clin Microbiol Rev. 2009;22(3):50834. doi: 10.1128/CMR.00008-09
42. Borlon C, Russell B, Sriprawat K, Suwanarusk R, Erhart A, Renia L, Nosten F, D'Alessandro U. Cryopreserved *Plasmodium vivax* and cord blood reticulocytes can be used for invasion and short term culture. Int J Parasitol. 2012;42(2):155-60. doi: 10.1016/j.ijpara.2011.10.011
43. Udomsangpetch R, Kaneko O, Chotivanich K, Sattabongkot J. Cultivation of *Plasmodium vivax*. Trends Parasitol. 2008;24(2):85-88. doi: 10.1016/j.pt.2007.09.010
44. Roobsoong W, Tharinjaroen CS, Rachaphaew N, Chobson P, Schofield L, Cui L, Adams JH, Sattabongkot J. Improvement of culture conditions for long-term *in vitro* culture of *Plasmodium vivax*. Malar J. 2015;14:297. doi: 10.1186/s12936-015-0815-z
45. Rangel GW, Clark MA, Kanjee U, Lim C, Shaw-Saliba K, Menezes MJ, Mascarenhas A, Chery L, Gomes E, Rathod PK, Ferreira MU, Duraisingh MT. Enhanced *ex vivo Plasmodium vivax* intraerythrocytic enrichment and maturation for rapid and sensitive parasite growth assays. Antimicrob Agents Chemother. 2018;62(4):e02519-17. doi: 10.1128/AAC.02519-17
46. Chaorattanakawee S, Lon C, Chann S, Thay KH, Kong N, You Y, Sundrakes S, Thamnurak C, Chattrakarn S, Praditpol C, Yingyuen K, Wojnarski M, Huy R, Spring MD, Walsh DS, Patel JC, Lin J, Juliano JJ, Lanteri CA, Saunders DL. Measuring *ex vivo* drug susceptibility in *Plasmodium vivax* isolates from Cambodia. Malar J. 2017;16(1):392. doi: 10.1186/s12936-017-2034-2
47. Noulin F, Borlon C, Van Den Abbeele J, D'Alessandro U, Erhart A. 1912-2012: a century of research on *Plasmodium vivax in vitro* culture. Trends Parasitol. 2013;29(6):286-94. doi: 10.1016/j.pt.2013.03.012
48. Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, Fidock DA, Su X, Collins WE, McCutchan TF, Wootton JC, Wellems TE. Evidence for different mechanisms of chloroquine resistance in 2 Plasmodium species that cause human malaria. J Infect Dis. 2001;183(11):1653-61. doi: 10.1086/320707
49. Sá JM, Yamamoto MM, Fernandez-Becerra C, de Azevedo MF, Papakrivos J, Naudé B, Wellems TE, Del Portillo HA. Expression and function of pvcrt-o, a *Plasmodium vivax* ortholog of pfcrt, in *Plasmodium falciparum* and *Dictyostelium discoideum*. Mol Biochem Parasitol. 2006;150(2):219-28. doi: 10.1016/j.molbiopara
50. Sá JM, Kaslow SR, Moraes Barros RR, Brazeau NF, Parobek CM, Tao D, Salzman RE, Gibson TJ, Velmurugan S, Krause MA, Melendez-Muniz V, Kite WA, Han PK, Eastman RT, Kim A, Kessler EG, Abebe Y, James ER, Chakravarty S, Orr-Gonzalez S, Lambert LE, Engels T, Thomas ML, Fasinu PS, Serre D, Gwadz RW, Walker L, DeConti DK, Mu J, Bailey JA, Sim BKL, Hoffman SL, Fay MP, Dinglasan RR, Juliano JJ, Wellems TE. *Plasmodium vivax* chloroquine resistance links to pvcrt transcription in a genetic cross. Nat Commun. 2019;10(1):4300. doi: 10.1038/s41467-019-12256-9
51. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S. Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number. Lancet. 2004;364(9432):438-47. doi: 10.1016/S0140-6736(04)16767-6
52. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA. Decreasing pfmdr1 copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis. 2006;194(4):528-35. doi:10.1086/507115
53. Brega S, Meslin B, de Monbrison F, Severini C, Gradoni L, Udomsangpetch R, Sutanto I, Peyron F, Picot S. Identification of the *Plasmodium vivax* mdr-like gene (*pv*mdr1) and analysis of single-nucleotide polymorphisms among isolates from different areas of endemicity. J Infect Dis. 2005;191(2):272-7. doi: 10.1086/426830
54. Sá JM, Nomura T, Neves Jd, Baird JK, Wellems TE, del Portillo HA. *Plasmodium vivax*: allele variants of the mdr1 gene do not associate with chloroquine resistance among isolates from Brazil, Papua, and monkey-adapted strains. Exp Parasitol. 2005;109(4):256-9. doi: 10.1016/j.exppara.2004.12.005
55. Picot S, Brega S, Gérôme P, et al. Absence of nucleotide polymorphism in a *Plasmodium vivax* multidrug resistance gene after failure of mefloquine prophylaxis in French Guyana. Trans R Soc Trop Med Hyg. 2005;99(3):234-7. doi:10.1016/j.trstmh.2004.09.007
56. Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, Prasetyorini B, Piera KA, Barends M, Brockman A, Lek-Uthai U, Anstey NM, Tjitra E, Nosten F, Cheng Q, Price RN. Chloroquine resistant *Plasmodium vivax: in vitro* characterisation and association with molecular polymorphisms. PLoS One. 2007;2(10):e1089. doi: 10.1371/journal.pone.0001089
57. Sow F, Bonnot G, Ahmed BR, Diagana SM, Kebe H, Koita M, Samba BM, Al-Mukhaini SK, Al-Zadjali M, Al-Abri SS, Ali OA, Samy AM, Hamid MM, Ali Albsheer MM, Simon B, Bienvenu AL, Petersen E, Picot S. Genetic diversity of *Plasmodium vivax* metacaspase 1 and *Plasmodium vivax* multi-drug resistance 1 genes of field isolates from Mauritania, Sudan and Oman. Malar J. 2017;16(1):61. doi: 10.1186/s12936-017-1687-1
58. Imwong M, Pukrittakayamee S, Looareesuwan S, Pasvol G, Poirreiz J, White NJ, Snounou G. Association of genetic mutations in *Plasmodium vivax* dhfr with resistance to sulfadoxine-pyrimethamine: geographical and clinical correlates. Antimicrob Agents Chemother. 2001;45(11):3122-7. doi: 10.1128/AAC.45.11.3122-3127.2001
59. Tjitra E, Baker J, Suprianto S, Cheng Q, Anstey NM. Therapeutic efficacies of artesunate-sulfadoxine-pyrimethamine and chloroquine-sulfadoxine-pyrimethamine in *vivax* malaria pilot studies: relationship to *Plasmodium vivax* dhfr mutations. Antimicrob Agents Chemother. 2002;46(12):3947-53. doi: 10.1128/aac.46.12.3947-3953.2002
60. de Pécoulas PE, Tahar R, Ouatas T, Mazabraud A, Basco

LK. Sequence variations in the *Plasmodium vivax* dihydrofolate reductase-thymidylate synthase gene and their relationship with pyrimethamine resistance. Mol Biochem Parasitol. 1998;92(2):265-73. doi: 10.1016/s0166-6851(97)00247-8

61. Marfurt J, de Monbrison F, Brega S, Barbollat L, Müller I, Sie A, Goroti M, Reeder JC, Beck HP, Picot S, Genton B. Molecular markers of *in vivo Plasmodium vivax* resistance to amodiaquine plus sulfadoxine-pyrimethamine: mutations in pvdhfr and pvmdr1. J Infect Dis. 2008;198(3):409-17. doi: 10.1086/589882
62. Menegon M, Majori G, Severini C. Genetic variations of the *Plasmodium vivax* dihydropteroate synthase gene. Acta Trop. 2006;98:196-9.
63. Imwong M, Pukrittayakamee S, Cheng Q, Moore C, Looareesuwan S, Snounou G, White NJ, Day NP. Limited polymorphism in the dihydropteroate synthetase gene (dhps) of *Plasmodium vivax* isolates from Thailand. Antimicrob Agents Chemother. 2005;49(10):4393-5. doi: 10.1128/AAC.49.10.4393-4395.2005
64. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V, Bouchier C, Ma L, Lim P, Leang R, Duong S, Sreng S, Suon S, Chuor CM, Bout DM, Ménard S, Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A, Barale JC, Fairhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Ménard D. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature. 2014;505(7481):50-5. doi: 10.1038/nature12876
65. Popovici J, Kao S, Eal L, Bin S, Kim S, Ménard D. Reduced polymorphism in the Kelch propeller domain in *Plasmodium vivax* isolates from Cambodia. Antimicrob Agents Chemother. 2015;59(1):730-3. doi:10.1128/AAC.03908-14
66. Wang M, Siddiqui FA, Fan Q, Luo E, Cao Y, Cui L. Limited genetic diversity in the *Pv*K12 Kelch protein in *Plasmodium vivax* isolates from Southeast Asia. Malar J. 2016;15(1):537. doi: 10.1186/s12936-016-1583-0
67. Duanguppama J, Mathema VB, Tripura R, Day NPJ, Maxay M, Nguon C, von Seidlein L, Dhorda M, Peto TJ, Nosten F, White NJ, Dondorp AM, Imwong M. Polymorphisms in *Pv*kelch12 and gene amplification of *Pv*plasmepsin4 in *Plasmodium vivax* from Thailand, Lao PDR and Cambodia. Malar J. 2019;18(1):114. doi: 10.1186/s12936-019-2749-3
68. Howes RE, Battle KE, Mendis KN, Smith DL, Cibulskis RE, Baird JK, et al. Global epidemiology of *Plasmodium vivax*. Am J Trop Med Hyg. 2016;95:15-34
69. Fernando D, Rodrigo C, Rajapakse S. Primaquine in *vivax* malaria: an update and review on management issues. Malar J. 2011;10:351. doi:10.1186/1475-2875-10-351
70. Dijanic C, Nickerson J, Shakya S, Dijanic A, Fabbri M. Relapsing malaria: a case report of primaquine resistance. Case Rep Infect Dis. 2018;2018:9720823. doi: 10.1155/2018/9720823
71. Huldén L, Huldén L, Heliövaara K. Natural relapses in *vivax* malaria induced by *Anopheles mosquitoes*. Malar J. 2008;7:64. doi: 10.1186/1475-2875-7-64
72. Hounkpatin AB, Kreidenweiss A, Held J. Clinical utility of tafenoquine in the prevention of relapse of *Plasmodium vivax* malaria: a review on the mode of action and emerging trial data. Infect Drug Resist. 2019;12:553-70. doi:10.2147/IDR.S151031
73. Medicines for Malaria Venture. MMV Annual Report 2016. Medicines for Malaria Venture. Published June 14, 2018. Available from: www.mmv.org/newsroom/publications/mmv-annual-report-2016.