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# TREATMENT OF VIVAX MALARIA IN VIETNAM

by

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A thesis submitted to the Open University U.K

For the degree of Doctor of Philosophy in the field of Life Sciences

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Dec, 2019

## Abstract

Globally, relapse caused by *Plasmodium vivax* malaria is estimated to contribute approximately 50% to the overall number of vivax infections. In South-East Asia, relapse rates commonly exceed 50%, making relapse as the main source of vivax illness. Recurrent episodes of febrile illness and hemolysis inflict a significant public health burden particularly in vulnerable groups such as pregnant women and young children. Multiple relapses may contribute substantially to the delayed morbidity and unappreciated mortality relative to falciparum malaria. Therefore, the radical cure of *P. vivax* malaria requires a combination of both blood schizontocides to achieve acute clinical cure, and hypnozoitocides to prevent relapses in the future. The World Health Organization (WHO) recommends the standard 14-day primaquine regimen in curing and preventing relapse from vivax malaria. Another 8-aminoquinoline derivative with prolonged elimination half-life, tafenoquine, also propose an alternative choice in radical therapy. The main research question is: what is the most effective treatment to achieve radical cure of *Plasmodium vivax* in Vietnam?

In order to answer this question a series of studies and clinical trials were conducted in Vietnam and our findings point to these conclusions:

1/ The short 7-day course of primaquine is an available, safe and highly efficacious antirelapse treatment that could improve the adherence of patients and hence the effectiveness of radical cure of vivax relapse.

2/ The single dose of 300 mg tafenoquine, an 8-aminoquinoline drug, has similar antirelapse activity compared to primaquine. When administered with chloroquine, this hypnozoitocidal agent is a potential candidate in the malaria elimination era.

3/ The prevalence and variants of G6PD deficiency determinants of hemolytic toxicity of 8-aminoquinolines in malaria endemic regions represent important factors to be considered when implementing radical cure of latent vivax malaria.

## Acknowledgements

The work described in this thesis has a considerable contribution from the participation of malaria patients admitted to the Phuoc Long Medical Center, to the Commune Health Stations of Dak O and Bu Gia Map in Binh Phuoc Province and the Krong Pa Medical Center in Gia Lai Province.

First of all, I am extremely grateful to **Professor Tran Tinh Hien**, my supervisor. His demanding supervision, constructive comments and endless enthusiasm have encouraged me in conducting these projects. Next, my great appreciation goes to my director **Professor Guy Thwaites** and **Professor Kevin Baird**, Head of Eijkman-Oxford Clinical Research Unit, for invaluable scientific advice, insightful discussion. My PhD thesis would be far from completion without their help.

I would like to thank my colleagues from Malaria group, Oxford University Clinical Research Unit, Ho Chi Minh city and all the staff in study sites (Binh Phuoc and Gia Lai province) for their collaboration, management the malaria patients and collection of clinical and laboratory data.

Finally, my deepest gratitude goes to my wife, Ms. Tuyet for her affection and my two little daughters for sharing all the aspect of life especially during the PhD time.

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# Chapter 1

## Introduction

### 1.1 Epidemiology of vivax malaria

#### 1.1.1 History of vivax malaria

Human malaria is a parasitic disease that is endemic in most tropical and subtropical ecosystems worldwide. Malarial parasites belong to the genus *Plasmodium* and infect many vertebrate hosts, including several species of non-human primate. These parasitic protozoa have complex life-cycles that involve sexual reproduction in the mosquito vector and asexual stages in the vertebrate host [1]. *Plasmodium vivax* (*P. vivax*) originated from a malarial parasite of non-human primates as a result of a host switch, probably from a macaque. *Plasmodium cynomolgi*, a parasite found in macaques, is the closest sister species to *P. vivax* [2]. Another parasite, *Plasmodium simium* was sequenced to show that it is identical to that of *P. vivax*. Although *P. simium* was previously considered to be a monkey-specific species in South America, the transmission from a monkey reservoir to human beings could pose challenges in eliminating malaria in these regions [3].

#### 1.1.2 Current vivax malaria situation in the world

*P. vivax* is a major public health challenge for Central and South America, the Middle East, Central, South and Southeast Asia, Oceania and East Africa, where 2.85 billion people are currently at risk of infection and 70-80 million clinical cases are reported each year. Important variances in the biology of *P. vivax* and *P. falciparum* render their epidemiology distinctly different. This leads to the wider geographic distribution of *P.*

*vivax* into temperate climate regions [4]. The most apparent is the ability of *P. vivax* to cause relapses weeks to months following the primary infection by activation of dormant liver-stage parasites, known as hypnozoites. There also exist differences in blood-stage dynamics that impact *P. vivax* epidemiology. Natural immunity is acquired at a younger age than against *P. falciparum*, making infants the primary risk group for severe vivax malaria in heavily endemic settings. While, in low-transmission settings, where *P. vivax* often persists against elimination efforts, all age groups appear at risk of severe disease [5, 6]. The propensity for *P. vivax* to invade young blood cells (reticulocytes) and the number of reticulocytes are generally around 0.5 – 1.5% of the total circulating erythrocytes result in relatively low levels of parasitaemia. Even in low-transmission settings, most infections appear to be microscopically sub-patent and asymptomatic [4].

The geographic distribution of *P. vivax* infection and differences in relapse phenotype are illustrated and described alongside estimates of *P. vivax* burden, as well as severe, lethal, and chloroquine-resistance vivax malaria to demonstrate that control and elimination strategies developed for *P. falciparum* cannot simply be transferred to *P. vivax*.

*P. vivax* depends on the Duffy antigen to invade red blood cells. Individuals who do not express the Duffy antigen have, therefore, been considered refractory to *P. vivax* infection and the prevalence of Duffy negative populations must be considered in predictions and maps of *P. vivax* endemicity [4]. Levels of *P. vivax* endemicity vary widely among the World Health Organization (WHO) regions. Outside of Africa, *P. vivax* is the dominant species, with relatively high prevalence of infection in the South-East Asian and Western Pacific regions. Relatively high endemicity also occurs in most

of the Americas, but in much less densely populated areas. Data from which to estimate the geographic extent of *P. vivax* transmission in Africa are limited. High *P. falciparum* endemicity in much of that continent coupled with high prevalence of Duffy negativity overshadowed collection of *P. vivax* specific data as a priority. However, *P. vivax* infection in Africa has been increasing from several surveys suggesting the *P. vivax* endemicity in this continent may have been underestimated previously [7].

Compared with similar predictions for *P. falciparum*, relatively low prevalence occurs throughout endemic world. There are other reasons why PvPR (parasite rate) could be lower than Pf PR. Although the prevalence of *P. vivax* in heavily endemic areas may reach or even exceed that of *P. falciparum*, *P. vivax* can often go undetected [8]. Low parasite densities lead to high rates of false-negative diagnoses by microscopy or rapid diagnostic tests (RDTs). Microscopy diagnosis underestimates the true prevalence of blood-stage *P. vivax* in both high- and low-transmission settings. *Plasmodium vivax* in mixed infections is also often underdiagnosed; lower densities of *P. vivax* in peripheral blood translate into *P. falciparum* being diagnosed before *P. vivax* is spotted by the microscopist. Another consideration with regard to true versus observed prevalence is the invisibility of the dormant liver stages to any diagnosis. These parasites could be highly prevalent in many endemic settings. All of these findings recommend caution in comparing prevalence estimates of *P. vivax* and *P. falciparum* based largely on blood-stage infections diagnosed by relatively insensitive means. These may underestimate true prevalence of *P. vivax* far more than *P. falciparum*.

### **1.1.3 Relapse epidemiology**

The hypnozoites cause multiple clinical attacks from a single bite of a *P. vivax*-infected mosquito. In contrast, *P. falciparum* infection of the liver yields a single blood-stage infection [4]. In other words, “infection” as an event in *P. falciparum* is singular and clear, whereas the same in *P. vivax* takes on complex plurality and ambiguity in an epidemiological sense. Recurrence of asexual *P. vivax* parasites in peripheral blood may derive from three distinct sources: relapse (from hypnozoites), recrudescence (from sub-patent asexual parasitaemia), or reinfection (by new mosquito inoculation of sporozoites). Relapse may well be the predominant origin of most *P. vivax* clinical attacks throughout the endemic world. Geographical variation in the rate and timing at which a “strain” of *P. vivax* may relapse has long been known. Temperate and subtropical strains of the parasite exhibit either a long incubation period or a long delay between the primary infection and relapse (around 8-10 months). Tropical strains are characterized by short incubation times and short relapse intervals. The Americas, South East Asia, and Western Pacific WHO regions all harbor strains that relapse quickly and repeatedly following primary infection. The Indian subcontinent and sub-Saharan Africa have variable relapse frequencies and periodicity, but show moderate time to relapse overall. Longer times to relapse are shown in areas around the Mediterranean and Central America, with the longest periods of latency in China and the Korean Peninsula [9, 10]. Regionally specific relapse patterns should be considered when assessing control and elimination strategy and tactics. Effects may be slowest to materialize in regions characterized by long-latency strains or high rates of relapse (even in areas with shorter latency).

Since the 1990s, Viet Nam has gained a remarkable success in controlling the morbidity and mortality associated with malaria. In annual reports from National Malaria Control Programme, the significant decline of malaria cases by 97% and deaths by 99.8% has been notified between 1991 and 2014 [11]. These reductions both in morbidity and mortality might have been attributable to a relatively strong malaria control programme, accessible health care facilities, and socio-economic improvements [12]. Currently, malaria transmission, with highest incidence, has been reported mostly in forested, border areas in Central and Central-Southern Viet Nam [13]. Following the successful control efforts against *P. falciparum*, the frequency of *P. vivax* relative to *P. falciparum* infection has increased in recent years, from 20% in 1991 to nearly 50% in 2015 [11]. Located in the Greater Mekong Subregion (GMS) and belonging to the Western Pacific Region of WHO, the relapse pattern of *P. vivax* malaria has been classified as frequent. In consequence, after the initial episode of acute vivax infection, the subsequent reappearance of parasitaemia usually occurs within 3 to 8 weeks [5]

## **1.2 Malaria parasite**

### **1.2.1 Parasite life cycle**

*Plasmodium vivax* has unique attributes to support its survival in varying ecologies and climates. These include dormant hypnozoite forms in the liver, an invasion preference for reticulocytes, caveola-vesicle complex structures in the infected erythrocyte membrane and rapidly forming and circulating gametocytes. These characteristics make this species very different from *P. falciparum*, which combine to make the *P. vivax* relatively difficult to control and eliminate. In general terms, the life cycle of *P. vivax* is like that of all of the other primate malaria species in that it requires an

invertebrate and a vertebrate host for survival and sexual reproduction; a female mosquito of a susceptible *Anopheles* species serves as definitive host whereas primates are intermediate host. When the female mosquito bites, she releases salivary fluid and along with it a typically small number of sporozoites (e.g., < 10) from her salivary glands. In the dermal tissues, the sporozoites are motile and capable of penetrating small blood vessels, and there begin stimulating a host immune response [14]. In the circulating blood, they are swept into the liver sinusoid vessels where they penetrate through the professional phagocytes known as K upffer cells into the Space of Disse to begin the exoerythrocytic or liver-stage cycle of growth. Once there, the sporozoite then penetrates a hepatocyte, rounds up and differentiates into a small trophozoite (~4  m in diameter) growing in size over the next few days eventually differentiating into a multinucleated schizont in 5 days. By 6 or 7 days, of primary growth and development, a fully mature schizont 40–60  m in diameter has differentiated into thousands of individual invasive single nucleated merozoites surrounded by a parasitophorous membrane capsule. The plasma membrane of an infected hepatocyte breaks down, and blebs of the parasitophorous membrane full of merozoites called merosomes break off and flow into the circulation of the liver sinusoid vessels. These merosomes are carried into the faster flowing general blood circulation and break apart releasing the merosomes-incarcerated merozoites, which then attach to and invade red blood cells (RBCs) to start the erythrocytic cycle of asexual reproduction, also called blood schizogony. The newly invaded merozoite immediately differentiates into an erythrocytic trophozoite and begins remodelling the anucleate RBC to provide a suitable environment for it to grow larger over a period of 48 hours feeding upon the haemoglobin of the parasitised RBC. Thirty-eight or 40 h into this cycle of growth, the



nucleus divides in two to create a schizont and over the next 8 or so hours continues to divide by schizogony to form 12–16 or more differentiated merozoites in the case of *P. vivax*. When the host cell membrane ruptures after 48 h of parasite intracellular growth, the merozoites are released and invade new RBCs to begin the entire asexual blood cycle again. The merozoites in some schizonts are preprogrammed to differentiate into sexual-stage male or female micro- and macro-gametocytes, respectively, and not into asexual trophozoites upon entry into a new RBC. The insect or sexual stage of the life cycle begins when a feeding female *Anopheles* mosquito takes up some of these circulating male and female gametocytes in her blood meal. In the midgut of the blood-engorged mosquito, the gametocytes lose their RBC membrane outer cover and become sexually active. The nucleus of the microgamete fractures into eight nuclei and eight flagellating bodies are formed. When one penetrates a macrogamete, a diploid zygote is formed that over a period of 24–36 h metamorphoses into an ookinete, another tissue invasive parasite stage. The ookinete penetrates through the mosquito midgut lining to lodge under the basal membrane where it transforms into an oocyst that undergoes multiple nuclear divisions to form a capsule of several thousand elongated sporozoites. At maturity, the capsule breaks open, releasing the thousands of sporozoites into the haemocoel of the mosquito, which then migrate to and penetrate the salivary glands to lodge in the glandular spaces waiting until the mosquito probes dermal tissue seeking a blood meal [14].

### **1.2.2 The Hypnozoite**

When a sporozoite of a relapsing malaria parasite enters the liver, it will differentiate into an early small liver trophozoite of about 4.0 or 5.0  $\mu$  in size and then it may enter

either of two very different developmental pathways. Firstly, there can be immediate growth in the host liver cell all the way through to schizogony and the production of thousands of merozoites that will initiate the erythrocytic cycle, as above. Alternatively, the small trophozoites become dormant hypnozoites and may remain in this quiescent metabolic state for weeks, months or up to 2 years in host liver [15].

### **1.2.3 The Reticulocyte as a Host Cell**

For *P. vivax* dependent on reticulocyte host cells, an efficient process must ensue, enabling these organisms with the ability to effectively find and latch onto the limited number of young RBCs amidst a virtual sea of more mature erythrocytes. Generally, reticulocytes only make up 0.5–2.0% of the erythrocytes in circulation. A few proteins have so far been identified and characterized as being required during the invasion process of the reticulocyte by the merozoite. The reticulocyte-binding proteins have been defined as critical proteins that select these host cells in the circulation. The parasite's Duffy Binding Protein has also been recognized for several decades as a critical RBC adhesion; only recently have *P. vivax* infections been associated with individuals with Duffy-negative RBC phenotypes [16, 17]. Restricting an infection to these young RBCs, which typically represent 0.5–2% of the circulating RBCs, may limit the rise in parasitaemia, but this strategy may also enable *P. vivax* to alter the infected RBCs (iRBCs) to remain or become more flexible and able to circulate through small capillary vessels and more likely to survive passage through the sinusoidal vasculature of the spleen.

#### **1.2.4 The Sexual Life Strategies of *P. vivax***

*P. vivax* gametocytes are known to develop early in an acute primary infection, within 5 days of the clinical onset. In fact, gametocytes are known to be produced earlier, perhaps, within 8 days after mosquito inoculation before they can be seen by light microscopy as mosquitoes can become infected at this time. Gametocyte densities become greater as blood-stage infections progress, seeming to come in waves at 5-day intervals and the production of gametocytes continues as the infection progresses on into chronicity becoming asymptomatic or more mildly symptomatic [18]. This ability to form infective gametocytes early and continuously, in addition to the periodic renewal of blood infections (and gametocyte propagation) by reactivated hypnozoites, makes *P. vivax* transmission fast, efficient and persistent. Like the asexual stages, the *P. vivax*-gametocyte-infected erythrocyte, which continues to circulate in the blood and not become immobilized in a tissue site as *P. falciparum* gametocytes do, remains highly flexible and capable of passing through small capillaries or splenic sinusoidal vessels despite containing a large parasite body [19]. There are many Anopheline mosquito species in the tropical, sub-tropical and, most illustratively, in the temperate latitudes quite far north that can act as efficient and effective transmitters of *P. vivax* [20]. The biological attributes of sporozoite development over a large temperature range in an array of susceptible mosquito vector species that over winter combined with delayed relapse genotypes/phenotypes allow the successful transmission of *P. vivax* [21, 22].

### **1.3 Pathophysiology of vivax malaria**

There are a number of differences in pathobiology between *P. vivax* and *P. falciparum* that are important in understanding the pathophysiology of vivax malaria.

#### **1.3.1 Parasite biomass**

*Plasmodium falciparum* invades RBC of all ages, and progresses to high parasite burdens if uninhibited by treatment or host immunity, with parasite biomass a major independent determinant of the risk of death [23]. In contrast, *P. vivax* has a very strong predilection for infecting RBCs that have emerged from the bone marrow within the last 14 days. This property contributes to the lower parasite biomass seen in *P. vivax* infections. Unlike *P. falciparum* infections, parasitaemia in vivax malaria rarely exceed 2% of circulating RBCs [24].

#### **1.3.2 Relapse**

A fundamental difference between *P. falciparum* and *P. vivax* is the ability of *P. vivax* to relapse from dormant hypnozoites and cause repeated episodes of clinical and subclinical infections. Differences in relapse patterns may be a major contributor to the geographic variation in vivax morbidity and disease severity. In tropical regions, *P. vivax* is characterized by frequent relapses 3–6 weeks apart, resulting in recurrent infections often with heterologous strains to which there is little cross-immunity. Frequent recurrent infections prevent adequate time for the patient to achieve haematological recovery from each bout of haemolysis. In contrast, in temperate areas, relapses are fewer and delayed, and the associated impact of each recurrence is less [25].

### **1.3.3 Greater inflammatory response in *P. vivax* than *P. falciparum***

*Plasmodium vivax* has a lower pyrogenic threshold than *P. falciparum*. Cytokine production, endothelial activation and pulmonary inflammatory responses are higher during and after *P. vivax* infections than in *P. falciparum* infections of similar parasite biomass [24]. Although the inflammatory process correlates of the lower pyrogenic threshold have been reported, the underlying mechanism has not identified.

In falciparum malaria, plasma concentrations of the pro-inflammatory cytokine tumor necrosis factor (TNF) and the anti-inflammatory cytokine interleukin-10 (IL-10) are directly related to disease severity. Although *P. vivax* is capable of eliciting greater concentrations of both pro- and anti-inflammatory cytokines than *P. falciparum*, the relationships to disease severity are different in vivax malaria. While plasma concentrations of the pro-inflammatory cytokines TNF and gamma interferon (IFN- $\gamma$ ) are directly related to disease severity, plasma concentrations of IL-10 are inversely related to vivax disease severity, suggesting a deficiency in the anti-inflammatory response in severe vivax malaria, with an unopposed pro-inflammatory response [26]. Plasma concentrations of superoxide dismutase, an enzyme produced in response to oxidative stress, have also been associated with vivax disease severity, but a role in pathogenesis has not been determined [27]. Another putative toxin that appears unique to *P. vivax* is a lipid found in the cholesterol/triglyceride fraction of plasma at the time of paroxysmal fever. This lipid has greater activity than phospholipids, and together with host cytokines, is known to mediate in vitro aggregation of leucocytes, mostly neutrophils. This lipid may also contribute to the greater pyrogenicity of *P. vivax* [28].

#### **1.3.4 Cytoadherence and rosetting**

A central mechanism in the pathophysiology of severe falciparum malaria is the cytoadherence of late stages of *P. falciparum* to activated microvascular endothelium resulting in sequestration and microvascular obstruction [29]. Since all stages of *P. vivax* are visible in peripheral blood, albeit with partial depletion of mature stages, sequestration is not thought to occur to a significant degree in vivax malaria or cause end-organ dysfunction in the same manner as *P. falciparum* [30]. Recent in vitro data show that *P. vivax*-infected RBCs do cytoadhere to endothelial cells, via ICAM-1 and chondroitin sulphate-A (CSA), with a similar strength but a 10-fold lower frequency than *P. falciparum*-infected RBCs [31]. Another study showed no cytoadherence to ICAM-1 but did confirm cytoadherence to the glycosaminoglycans, CSA and hyaluronic acid [32]. Rosetting, adherence of non-infected to infected RBCs, has been linked to the pathophysiology of severe falciparum malaria. Rosetting has been described ex vivo in vivax malaria, however, its role in vivax pathophysiology is unknown [33, 34].

#### **1.3.5 Endothelial activation and altered thrombostasis**

Concentrations of circulating endothelial activation markers are as high (ICAM-1 and E-selectin) or higher (angiopoietin-2) in uncomplicated vivax malaria than in falciparum malaria. Endothelial dysfunction and impaired NO bioavailability are significant contributors to severe falciparum malaria, but their importance in severe vivax malaria is not known [35]. Other consequences of endothelial activation and altered thrombostasis may be more important in *P. vivax* infection. The role of altered haemostatic pathways, intravascular coagulation and endothelial inflammation through

increased formation of ultra-large Von Willebrand factor and platelet aggregates in severe vivax malaria is not known. However, with the reports of thrombotic microangiopathy in some cases, vivax-associated kidney disease, these processes may contribute to at least a proportion of vivax associated acute kidney injury, coma, anaemia and/or thrombocytopenia [36].

## **1.4 Diagnosis of vivax malaria**

### **1.4.1 Clinical diagnosis**

The spectrum of disease associated with *P. vivax* infection ranges from asymptomatic parasitaemia and uncomplicated febrile illness through to severe and fatal malaria [24]. Clinical diagnosis of vivax malaria is difficult because signs and symptoms are non-specific and impacted by endemicity and host immunity. Common symptoms include malaise, fever, chills, diaphoresis, headache, arthralgia, myalgia, cough, abdominal pain, nausea, vomiting, and diarrhea, which can also occur with many common systemic febrile illnesses such as meningitis, pneumonia or gastroenteritis. Splenomegaly, anaemia, leukopenia and thrombocytopenia are also common, but non-specific. Infection of a non-immune host results in a prodromal period followed by an acute fever. Prodromal symptoms can include increasing headache, anorexia, malaise, myalgias and/or gastrointestinal symptoms for one or more days, sometimes with periodicity. A low-grade prodromal fever without paroxysmal symptoms may also occur. The ‘paroxysm’ has long been recognized as the periodic febrile response to *Plasmodium* infection occurring following rupture of schizont-infected red cells. The paroxysm is classically, but not invariably, preceded by a ‘cold stage’ of chills and a rigor. A chilly sensation proceeds to shivering and then intense muscle tremors,

chattering of teeth and sometimes violent shaking. The temperature rises before the rigor ceases. Defervescence is accompanied by sweating and fatigue [24]. Respiratory symptoms have long been recognized as common in vivax malaria. Cough, usually non-productive, occurs in approximately half of the adults with vivax malaria, in residents of both vivax- endemic and -non-endemic areas [37]. Tachypnoea may occur because of high fever, anaemia and/or pulmonary pathology.

Depending on where infection is acquired, the differential diagnosis should also include dengue fever, typhoid fever (particularly in south central Asia), infectious mononucleosis, rickettsiosis, influenza, and bacteremia and region-specific arboviral infections.

#### **1.4.2 Microscopic diagnosis**

Diagnosis of vivax malaria should be established by microscopic examination of Giemsa-stained thick and thin smear blood samples obtained every 8-12 h over a 24-48 h period [38]. Clinical signs and symptoms alone, though frequently used, can neither differentiate malaria infection from other causes of febrile illness, nor distinguish between *P. falciparum* and *P. vivax* or malaria caused by another plasmodia. Examination of at least 200 fields of a thick blood film under oil immersion magnification (x 1,000) should be undertaken before a negative diagnosis is made [39]. The limit of detection for expert microscopists is considered to be about 10-20 parasites/uL. The density of parasitaemia in patients with acute vivax malaria depends upon many factors, including naïve versus a state of semi-immunity, age, delay in seeking treatment, self-treatment behavior before presentation, and likely a variety of host and parasite factors. The parasite density in *P. vivax* malaria is typically and order



of magnitude lower than *P. falciparum* in most clinical settings where both these species occur, thus increasing the risk of false negative microscopy diagnosis with acute vivax malaria [8].

### **1.4.3 Antigen-capture rapid diagnostic testing**

Malaria rapid diagnostic tests (RDTs) composed of immunochromatographic lateral flow devices, are qualitative diagnosis tools to detect antigens derived from parasite antigens in patient blood. The principal advantage of RDTs is their ease of use and sustainability in resource-challenged settings. RDTs are available from many commercial sources at relatively low cost (usually < US\$1/test) [40]. Most of these kits are stable at ambient temperature storage for many months. In 2002, WHO began to develop malaria RDT evaluation programme to ensure the quality control among different kits of RDTs. The parasite detection score is a measure of inter-test and inter-lot consistency. For *P. falciparum*, the detection score indicates an RDT result confirming the presence of *P. falciparum*, when tested against cultured and wild-type *P. falciparum* samples. While, the *P. vivax* detection score indicates *P. vivax*-positive/*P. falciparum* negative results when tested on wild-type *P. vivax* samples [40]. In general, the kits perform better with *P. falciparum* infection than that with *P. vivax* (e.g., 74% versus 37% of test brands scored > 75% “parasite detection score” at a density of 200 parasites/uL, respectively) [41]. Some tests detect *P. falciparum* histidine-rich protein 2 (HRP-2) in addition to a pan-genus antigen (lactate dehydrogenase [pLDH]). Reaction to both indicates presence of *P. falciparum*, either alone or mixed with any other species, whereas reaction to pLDH alone indicates absence of *P. falciparum* and presence of any other species. Other RDTs do offer a *P.*

*vivax*-specific diagnosis employing aldolase antigen capture with satisfactory performance [41]. If available, an antigen-capture RDT, which can distinguish *falciparum* from non-*falciparum* malaria, may be helpful for establishing early diagnosis when microscopic diagnosis capabilities are not available. Currently, the only US Food and Drug Administration (FDA) approved RDT is the Binax NOW Malaria Test, a card-based diffusion immunochromatographic assay which detects the presence of *P. falciparum*-specific histidine-rich protein 2 (HRP-2) and a pan-malarial antigen common to all four human species. When used in endemic locations where prevalence of malaria is high, the overall sensitivities and specificities for *P. falciparum* exceeded 95%, while performance for diagnosis of *P. vivax* is much lower, sensitivity 69% and specificity 100%, and does not meet the WHO recommended minimum panel detection score [41]. Because RDT sensitivity is directly related to parasite density, health care providers cannot rely on a negative RDT result to rule out malaria, particularly for *vivax* malaria. However, a positive result may be helpful in making management decisions before a microscopic diagnosis can be made [38]. In Vietnam, the current available malaria rapid diagnostic test using in health facilities is SD Bioline Malaria Ag *P. falciparum*/*P. vivax*, which is supported by the Global Fund. The test measures the presence of HRP-2 antigen specified for *P. falciparum* and pLDH specified for *P. vivax*.

#### **1.4.4 Molecular diagnosis**

The most widely applied and validated molecular diagnostic is a nested polymerase chain reaction (PCR) amplification of small subunit ribosomal RNA gene sequences. The term nested refers to an initial amplification using primers of genus-wide sequences

followed by primers of species-specific character to provide the definitive diagnosis. This technique, and other PCR-based approaches to diagnosis, requires relatively advanced laboratory equipment and technologically skilled execution. Typically, blood blots dried onto filter paper in the field are transported to a laboratory where extraction of DNA and its analysis by PCR is performed. It is also relatively expensive at tens of dollars per test on most platforms. The advantage of PCR is increased sensitivity to detect parasitaemia up to three orders of magnitude lower (depending on blood volume sampled) compared with microscopy or RDT. Its absolute sensitivity in operational use is approximately 1.0 parasite/uL, increasing to 0.02 parasites/uL if > 0.25 ml of venous blood can be collected [42].

#### **1.4.5 Loop-mediated isothermal amplification (LAMP)**

LAMP of parasite-specific DNA is a more recent technology more suited to active case detection in endemic settings. The technique does not require expensive thermocyclers or gel electrophoresis, the readout being a visual color change in a small test tube [43]. Recent evaluation of a commercially available kit showed the technique to be comparable to standard nested PCR technique and superior to expert microscopy. The procedure can be completed in about 1 hour at the site of collection [44]. Deployment of LAMP-based diagnostics to low resource settings and further evaluation of sensitivity and specificity outside of the research setting is necessary before widespread use or replacement of microscopic diagnosis with LAMP can be recommended.

#### **1.5 Treatment of vivax malaria**

The goals of antimalarial treatment in *P. vivax* are to reduce the immediate risk to the patient, eradicate peripheral asexual parasitaemia, prevent the recurrent infection, and

interrupt the cycle of transmission. The ability of *P. vivax* to form dormant liver stages (hypnozoites) capable of causing relapsing infections weeks to months after the initial blood-stage infection, provides a major challenge to the complete eradication of parasites from the body (radical cure). Since no single drug achieves all of these aims, a combination of antimalarials is required targeting a variety of specific key elements of the parasite life cycle. Treatment of infection by *P. vivax* imposes far greater complexity, nuance, and risk to the patient relative to treatment of uncomplicated falciparum malaria. Treating both liver and blood stages, as opposed to blood stages alone, greatly amplifies the complexity of the chemotherapeutic problem [38].

### **1.5.1 Treatment of asexual erythrocytic stages of *P. vivax***

#### **1.5.1.1 Treatment of uncomplicated vivax malaria**

Chloroquine has been the treatment of choice for *P. vivax* malaria for almost 70 years [38]. By the 1940s, clinical studies confirmed chloroquine to be an extremely potent antimalarial agent, effective against all plasmodia species, and its slow elimination from the blood allowing curative regimens to be achieved with short-course treatment regimens. Clinical experience in a huge number of patients demonstrated the drug to be safe and well-tolerated, including its use in pregnant women and young infants. Chloroquine is rapidly absorbed after oral administration, its bioavailability exceeding 90% [45]. It has an extremely long and complex terminal elimination phase because of its extensive distribution in tissue, with high concentrations occurring in the liver, spleen, kidney, and lung. This tissue penetration accounts for its enormous apparent volume of distribution (~200-300 l/kg), and it is this rather than the elimination process that determines the blood concentration profile of this drug. Its reported half-life varies

from 70 to 300 h, resulting in therapeutic drug concentrations being detected in the blood for between 3 weeks and 3 months after administration in some patients. Against sensitive strains of *P. vivax*, chloroquine produces a very rapid clinical and parasitological response, patients usually clearing their fever and peripheral parasitaemia within 48 h. Chloroquine, similar to all blood schizontocidal agents effective against *P. vivax*, is gametocytocidal with rapid clearance of sexual stages during acute infection. Its slow elimination provides a period during which blood concentrations exceed the minimally effective concentration of the parasites, ensuring complete clearance of all erythrocytic stages of the parasite and providing a prolonged period of post-treatment prophylaxis (PTP) during which the drug can suppress new invasion of the blood stream by merozoites from reinfection or relapse from hypnozoites [38]. Although chloroquine has dominated antimalarial treatment of *P. vivax* for over half a century, this position is under threat from the emergence and spread of chloroquine-resistant strains of the parasite. The first cases of chloroquine-resistant *P. vivax* were documented in 1989, almost 30 years after reports of the emergence of chloroquine-resistant *P. falciparum* [46]. In areas where *P. vivax* is known to be chloroquine (CQ) sensitive, the WHO recommends 3 days of CQ or an artemisinin combination treatment plus 2 weeks of primaquine (PQ) (provided the affected individual is not G6PD deficient) [45]. CQ remains a first-line treatment in most parts of the world due to its wide availability, low cost, and long terminal elimination half-life. However, in co-endemic malarious areas, this necessitates a separate treatment approach for *P. falciparum* and *P. vivax*. Artemisinin combination therapies (ACTs) are the treatment of choice for CQ resistant *P. vivax*. WHO-recommended ACTs include artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, and

dihydroartemisinin-piperaquine. Artemisinin in combination with effective partner drug have shown excellent cure rates in *P. vivax* infection [47]. ACTs with partner drugs with longer elimination periods provide incidental suppressive prophylaxis against relapse for about a month, but relapse risk thereafter remains relatively high.

#### **1.5.1.2 Treatment of severe vivax malaria**

Severe and fatal vivax malaria has been reported from Indonesia, Papua New Guinea, India, and Brazil [48]. The main manifestations are anemia and respiratory distress, although series of patients with coma, shock, and renal and hepatic dysfunction associated with vivax malaria have also been described. *Plasmodium vivax* is very sensitive to artemisinin and its derivatives. In the absence of comparative drug trials, physicians have tended to adopt a similar treatment approach for severe vivax malaria, namely administration of parenteral artesunate, if unavailable, artemether, and if that is also not available then quinine, along with broad spectrum antibiotic cover and supportive care. Intravenous artesunate also leads to a rapid clinical response in patients with severe vivax malaria, but there have been no randomized clinical trials in severe *P. vivax* malaria. Specific antimalarial treatment recommended in severe vivax malaria includes the following in order of preference:

- Artesunate: 2.4 mg/kg body weight, intravenously or intramuscularly given on admission (time = 0), then at 12 and 24 hours, and then once a day. This is the treatment of choice.
- Artemether: 3.2 mg/kg body weight, intramuscularly given on admission, then 1.6 mg/kg body weight per day.

- Quinine: 20 mg quinine salt/kg body weight on admission (intravenous infusion in 5% dextrose/dextrose saline over a period of 4 hours) followed by maintenance dose of 10 mg/kg body weight 8 hourly (maximum infusion rate 5 mg salt/kg/hour).

Parenteral antimalarials should be administered for at least 24 hours. Once the patient can accept oral therapy, full course of oral ACT should be given to the patients.

### **1.5.2 Treatment of liver stages of *P. vivax***

Six 8-aminoquinolines have been used as antimalarial agents in humans [49]. Although most are no longer in use, all were evaluated first in animals and then in humans to assess their efficacy and toxicity in the treatment of malaria. All the 8-aminoquinolines that have been studied in detail are rapidly metabolized in humans in vivo, with only a small fraction (< 1%) eliminated unchanged in the urine. The commonest side-effect of these drugs is abdominal pain; the discomfort is proportional to the dose taken but is reduced (even at higher doses) when the drug is administered with food. The main concern throughout the development and use of 8-aminoquinolines has been the risk for haemolysis of G6PD-deficient individuals [50]. Pamaquine has pre-erythrocytic, radical curative and gametocytocidal properties. It has weak but significant activity against asexual stages of *P. vivax* and *P. malariae* but is ineffective against *P. falciparum* trophozoites. It was less effective in terms of radical curative activity than primaquine and was more toxic. Pentaquine was an effective hypnozoitocidal drug, but side-effects similar to those of pamaquine were observed frequently in carefully controlled studies, and its development was not continued. War in the Pacific in 1941 created an urgent strategic need in the United States for a drug to prevent relapse of

malaria. Studies during and after World War II focused on 8-aminoquinolines, because, in the 1920s, pamaquine (the prototypical 8-aminoquinoline) had proven effective but too toxic. Primaquine became available to American troops during the Korean War [49].

### **1.5.2.1 Primaquine**

For over 60 years, clinicians, policy makers, and patients have relied on primaquine for the radical cure of *P. vivax* [51]. Primaquine is the only licensed antimalarial with proven hypnozoitocidal activity, but can result in significant hemolysis, particularly in those with glucose-6-phosphate dehydrogenase deficiency (G6PDd). Primaquine is indicated for radical cure of *P. vivax* or *P. ovale* malaria; for presumptive anti-relapse therapy (terminal prophylaxis) in people extensively exposed to *P. vivax* or *P. ovale*; to reduce onward transmission of *P. falciparum* malaria in programme to eliminate *P. falciparum* malaria and in areas threatened by resistance of *P. falciparum* to artemisinin [45].

#### **1.5.2.1.1 Structure and mechanism of action**

Primaquine is an 8-aminoquinoline, which is highly active against the exoerythrocytic forms (hypnozoites) and the sexual stages of malaria parasites (gametocytes). It has weak activity against the asexual blood stages of *P. vivax* and has negligible activity against *P. falciparum*. The precise mechanism of action of primaquine is not fully understood [52]. Several possible modes of action have been proposed. One hypothesis is that the active metabolite impairs the mitochondrial metabolism of parasites, interfering with the function of ubiquinone as an electron carrier in the respiratory chain [53]. Another theory is that the highly reactive metabolites generate intracellular



reactive species, which cause oxidative damage [54]. There is no evidence for acquired resistance to its hypnozoitocidal or gametocytocidal activities [55, 56].

#### **1.5.2.1.2 Pharmacokinetics**

Primaquine is rapidly absorbed from the gastrointestinal tract, reaching peak concentrations within 1–4 h, with a bioavailability of about 96% [57]. Primaquine is biotransformed by two main routes: by monoamine oxidase (MAO) to the predominant, but inactive, metabolite carboxyprimaquine, which is relatively slowly eliminated; and via cytochrome P450 (CYP2C19, CYP2D6 and CYP3A4) in the liver, which generate the reactive intermediates responsible for antimalarial effects and haemolytic toxicity [58]. Genetic polymorphisms that decrease CYP2D6 enzyme activity reduce bioactivation of primaquine and may result in treatment failure. Primaquine is extensively distributed in the body. About 75% of primaquine in plasma is bound to proteins, and high concentrations occur in erythrocytes [45]. Primaquine crosses the placenta, but the concentrations of primaquine in breast milk are very low [59]. Because of lack of the data on its safety of haemolysis in a G6PD-deficient fetus or in infants < 6 months, primaquine is not advised in pregnant and child-bearing women. Both primaquine and carboxyprimaquine are excreted mainly through the biliary tract and can be found in faeces within 24 h of administration [60]. Primaquine is also excreted in the urine as unchanged drug. The pharmacokinetics of a single oral dose of 15 mg did not appear to be altered in patients with severely impaired renal function and end-stage renal dysfunction [61].

### **1.5.2.1.3 Antimalarial action**

Primaquine is active against pre-erythrocytic (liver) stages of all the parasites, the asexual and sexual stages of *P. vivax*, *ovale*, *malariae* and *knowlesi*, and the sexual stages of *P. falciparum* but is only very weakly active against the asexual blood stages of *P. falciparum* [49]. Primaquine is the only currently available antimalarial agent that kills the latent persistent stages of *P. vivax* and *P. ovale* (hypnozoites) in the liver [62]. As primaquine accelerates gametocyte clearance and reduces transmissibility, it is also used as a gametocytocidal drug against *P. falciparum* malaria [45].

#### **1.5.2.1.3.1 Prevention relapse of vivax malaria**

It is difficult to evaluate the efficacy of any antimalarial agent in preventing vivax relapse, as in endemic areas relapses cannot be distinguished reliably from re-infections. Relapse rates vary considerably by area, as do relapse intervals [15].

*P. vivax* infections relapse as a result of activation of persistent liver stages, hypnozoites. The incidence of relapses and the intervals between them depend on several factors, including the parasite strain (geographical origin), the size of the sporozoite inoculum and the immunity and age of the human host [9]. Three general patterns of infection can be distinguished: tropical (*P. vivax* Chesson strain), with frequent relapses at approximately 3-week intervals after administration of a rapidly eliminated antimalarial agent; temperate (Saint Elizabeth or Madagascar strain), in which a primary infection is usually followed by an interval of 8–9 months before relapse; and long or “hibernans”, in which there is no primary infection and the first illness occurs 8–9 months after sporozoite inoculation [9].

The dose of primaquine recommended globally was chosen largely on the basis of studies on the sensitive Korean *P. vivax* [63]. After a very high rate of relapse was observed in soldiers returning to the USA from the Korean War in 1950, all soldiers were given a radical curative regimen of 15 mg/day for 2 weeks during their return by sea, which was highly effective. The *P. vivax* Chesson strain from New Guinea was found to be more “resistant” to 8-aminoquinolines [64], but the recommendation for use of a higher primaquine dose (22.5 mg/day) was applied initially only in Oceania, although it might have been better to recommend higher doses for all areas in which frequent relapse parasites were found, including South-East Asia. It is possible that all “temperate strains” are equally sensitive to primaquine and that all “tropical strains” are equivalent but have higher “activatable” hypnozoite burdens and so require a higher dose (i.e. 0.5 mg/kg for 14 days) [62].

Most *P. vivax* endemic countries recommend a primaquine dosing regimen of 0.25 mg base/kg/day for 14 days, although there is a wide range of dosing regimens across countries [45]. The main problems with the standard 14-day course of 15 mg/day of primaquine following chloroquine are: poor adherence, since people become rapidly asymptomatic after treatment with chloroquine and are poorly motivated to complete primaquine treatment [65]; the risk of adverse events; the lack of availability of cheap, rapid and practical tests for G6PD deficiency. The efficacy of various regimens has been evaluated in different studies. Recurrence rates after primaquine (15 mg base/day for 14 days) were 4.6% at 3 months [66] and 17.5% at 6 months [67] in Thailand. Studies with similar primaquine doses reported a recurrence rate of 0% at 3 months (Thailand) [68], and 8.1% at 6 months (compared to 16.4% without primaquine (India)) [69], and 13.6% (Peru) [70] with chloroquine as the partner drug. In some areas of

South East Asia and Oceania, high-dose primaquine (0.5 mg base/kg/day for 14 days) is used. With this high dose course, the recurrence rates decreased by less than 5% in Thailand [71], by 3.5% in Vietnam [72]. In Papua New Guinea, this regimen reduced the risk of recurrence by 28% [73]. One study evaluated high dose primaquine in combination with chloroquine showed that the recurrence rate was 1.8% over 11 months [74].

#### **1.5.2.1.3.2 Adverse effects**

While primaquine is generally well tolerated, it may cause dose-related gastrointestinal discomfort, including abdominal pain, nausea and vomiting [75]. This often happens in dose over 1 mg base/kg. Administration with food greatly improves tolerability [50]. Hypertension and cardiac arrhythmia have been reported rarely. The most important adverse effect is haemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, and the degree of haemolysis is proportional to the dose, duration of exposure, and degree of G6PD deficiency [76]. Leukopenia, methaemoglobinaemia with cyanosis and granulocytopenia may also occur [76].

#### **1.5.2.2 Tafenoquine**

Tafenoquine, also known as WR238605, is an investigational 8-aminoquinoline derivative. Tafenoquine was first discovered at the Walter Reed Army Institute of Research in 1978, and jointly developed by Medicines for Malaria Venture (MMV) and GlaxoSmithKline Pharmaceuticals (GSK). Like primaquine, tafenoquine has activity against the hypnozoite of *P. vivax* lifecycle. Nevertheless, it has a long elimination half-life (about 2-3 weeks) [77]. This novel drug is co-administered as a single dose with standard doses of chloroquine for the radical cure of *P. vivax* malaria [78]. This may be

a promising alternative to primaquine and an important practical advantage in order to increase the patient's adherence [79, 80]. The shorter treatment course is an important practical advantage in people who do not have G6PD deficiency. However, the key safety issue for both primaquine and tafenoquine is the capacity to induce haemolysis in G6PD deficient patients [81]. Particularly, tafenoquine could cause sustained toxicity because of its slow elimination.

#### **1.5.2.2.1 Structure and mechanism of action**

Tafenoquine (TQ) is a prodrug that needs activation to quinone TQ metabolite through metabolism by CYP2D6 [82]. The mechanism of action of TQ is not yet precisely known. The spontaneous oxidation of metabolites also generates hydrogen peroxide and hydroxyl radicals. It is hypothesized that the reactive oxygen species generated through *P. falciparum* ferredoxin-NADP<sup>+</sup> reductase and diflavin reductase enzymes leads to parasite kill. Similar to the blood schizonticide CQ, TQ inhibits heme polymerase in blood stage of the parasites [83]. This may explain the reason why TQ has activity against asexual blood stage of parasites, unlike PQ that does not inhibit the polymerization of hemozoin.

#### **1.5.2.2.2 Pharmacokinetics**

The time to peak concentration of TQ is 13.8 hours. This could be due to a combination of prolonged absorption at distal gastrointestinal tract and the drug's slow clearance [84]. Bioavailability of TQ increases when the drug is taken with high-fat meal [85]. From a population kinetics study of TQ in Thai soldiers, it was demonstrated that food affects the amount of TQ absorbed, rather than the rate of absorption [86]. The concentration of TQ in whole blood is approximately twofold higher than the

corresponding concentration in plasma. Like PQ, the activation of TQ needs metabolism by cytochrome P450 2D6 (CYP2D6) liver microsomal enzyme [87].

#### **1.5.2.2.3 Efficacy in radical cure**

Different doses of Tafenoquine were evaluated in clinical trials to find out the efficacious formula. High quality evidence on the efficacy of TQ for radical cure is obtained from double-blind, multicenter, randomized, placebo-controlled Phase IIb study [88]. Single dose of TQ at 300 mg and 600 mg, following the standard CQ therapy for clinical cure, prevented relapse of vivax malaria by 91.9% and 96.5%, whereas 15 mg PQ administered for 14 days prevented relapse only by 77.3%. In a meta-analysis, a lower efficacy of single dose of 300 mg TQ has been shown by preventing 74% of participants free from recurrence after 6 months of follow-up in Southeast Asian region [89]. Tafenoquine has good efficacy in preventing relapses up to six months by clearing vivax hypnozoites when used at a total dose of 300 mg or more, raising the possibility of single-dose treatment and directly observed therapy [90].

#### **1.5.2.2.4 Safety of Tafenoquine**

Like other 8-aminoquinoline, the most frequent complaint of tafenoquine is gastrointestinal abnormalities (nausea and abdominal pain) [91]. Mild vortex keratopathy, corneal deposits were also detected in 93% of subjects taking tafenoquine. These disorders were not associated with any effect on visual acuity and were fully resolved in all subjects by 1 year [92]. There is no evidence of a relationship between tafenoquine dosage and the occurrence of abnormal laboratory parameters, including aminotransferase, hemoglobin, white blood cell counts, platelets and bilirubin levels [93]. Major complication with tafenoquine administration is the acute haemolysis in

G6PD deficient subjects [94]. Prolongation of the QT interval is also one of the concerns in patients treated with antimalarial drugs. In the DETECTIVE trial, QT prolongation occurred in 2% of patients taking tafenoquine, while the phenomenon was observed in 8% and 4% of patients taking primaquine and chloroquine, respectively [88].

## **1.6 Glucose-6-Phosphate Dehydrogenase Deficiency and Radical cure of *Plasmodium vivax***

### **1.6.1 Epidemiology of Glucose-6-Phosphate Dehydrogenase Deficiency**

The presence of G6PDd in patients with *P. vivax* infection results in significant challenges to achieving radical cure. G6PDd is the most common enzymopathy with at least 400 million individuals affected worldwide [95]. G6PD deficiency is widespread across malaria endemic regions. At the continental scale, the high allele frequencies seen in Africa meant that 28.0% of the overall deficient population was in sub-Saharan Africa, while only 4.5% of the global population burden was in the Americas, and 67.5% across the whole of Asia [96]. The prevalence of G6PD deficiency and predominant G6PD deficiency variants can vary by ethnicity [97]. In Asia, the prevalence of G6PD deficiency is as high as 20%, while in Americas and Africa, this number can vary from < 1% to 15% [96, 98, 99]. More than 185 clinically relevant variants of G6PDd have been reported with a spectrum of associated enzyme deficiencies. In Asian populations, different variants have been identified, such as the Mahidol variant across Myanmar and Thailand; Viangchan variant across Mekong region; Kaiping variant among Chinese populations and the Vanua Lava variant in central and eastern Indonesia, genetic diversity was high among these populations. The

proportion of “unidentified” variants was also greatest among Asian populations, emphasizing the inadequacy of molecular methods for diagnosing phenotypic deficiency: a limited number of primers cannot reliably identify all possible cases of deficiency [96]. The high prevalence of these mutants is likely to have been driven by their ability to provide some degree of protection from malaria.

### **1.6.2 Physiology of Glucose-6-Phosphate Dehydrogenase Deficiency**

Glucose-6-phosphate dehydrogenase is an essential enzyme in the pentose phosphate pathway (PPP), the only pathway for human RBC to maintain the cells’ redox potential by reducing NADP<sup>+</sup> to NADPH. The enzyme consists of two dimers encoded by a gene on the long arm of the X chromosome. Non-synonymous mutations in the gene can decrease enzyme activity or reduce the stability of the enzyme, resulting in different degrees of G6PD deficiency. Because the gene is on the X chromosome, males are hemizygous and are either classified as G6PD deficient or normal by phenotype. Females have two gene copies which are expressed alternately in RBCs. Females therefore can be homozygous deficient (two gene copies with a deleterious mutation), heterozygous with one gene copy encoding a normal G6PD variant and one gene copy encoding a deficient G6PD variant (and a phenotype ranging from normal to deficient), or homozygous normal with both gene copies expressing G6PD variants with normal G6PD activity. Through the process of X-chromosome inactivation (also called Lyonization), females express only one of their two copies of the gene in each cell. This occurs randomly in the precursors of RBCs early in the embryonic stage and results in different ratios of gene expression in mature RBCs amongst females but is constant within an individual [100]. As consequence females heterozygous for G6PD can



manifest a range of intermediate G6PD activities between typical normal and deficient G6PD activities that reflects the average enzymatic activity of these two cellular populations.

### **1.6.3 Clinical features of Glucose-6-Phosphate Dehydrogenase Deficiency**

Most people with G6PDd do not exhibit symptoms unless exposed to oxidative stress. Oxidative stress can be triggered by medicines, infections, fava bean consumption, or even strenuous physical exercise [101]. The main clinical manifestations of G6PDd are acute haemolytic anaemia, chronic non-spherocytic haemolytic anaemia (CNSHA), neonatal jaundice and favism [102, 103]. The World Health Organization (WHO) classifies G6PDd into five different categories according to the severity of enzyme deficiency. A Class I deficiency is defined as severe deficiency and is associated with CNSHA. A Class II deficiency is also defined as severe deficiency and the enzyme activity is 1%-10% of normal activity. Individuals with a Class III deficiency are moderately deficient and their enzyme activity is 10%-60% of normal activity. Class IV and Class V individuals have normal and increased activity with an enzyme activity of 60%-150% and over 150%, respectively [102]. The two most common G6PDd variants are the G6PD A- and G6PD Mediterranean variants. G6PD A- is common across the African continent and is categorized as a Class III deficiency, whereas G6PD Mediterranean is common among Italians, Arabs, Persians, and Jews, and is categorized as a Class II deficiency. In Southeast Asia, the most common variant in Myanmar and Thailand is G6PD Mahidol (Class III), whereas in Laos and Cambodia, the most common variant is G6PD Viangchan (Class II) [95].

#### **1.6.4 Diagnosis of Glucose-6-Phosphate Dehydrogenase Deficiency**

In the Great Mekong Subregion (GMS), many malaria endemic villages are remote, difficult to access and have very weak health infrastructures [104]. Therefore, when haemolysis occurs in such settings, the local health facilities are unlikely to be able to provide appropriate medical care, potentially resulting in the loss of life. G6PD testing is particularly crucial under such conditions. Implementing routine testing for G6PD deficiency is challenging and the WHO guidelines also state that if testing is not available, an individual risk-benefit assessment should guide the decision whether to administer without testing or withhold radical treatment altogether [105]. G6PD testing is currently available in a number of formats. Diagnostic assays can be grouped into genotypic test assays, sequencing methods as well as phenotypic assays [106]. Genotypic assays and sequencing methods can provide a precise option for diagnosing G6PD mutations but require long and complicated test procedures, a well-equipped laboratory and high trained staff. Phenotypic tests can be grouped into qualitative, quantitative and cytochemical test assays. Phenotypic tests are based on the direct or indirect detection of  $\text{NADPH} + \text{H}^+$ , formed as a result of G6PD activity [107]. Qualitative test formats indicate activity above a test's inherent activity threshold level. While qualitative test formats are easier to perform and interpret compared to quantitative test methods and cytochemical tests, the reduction of a quantitative value (G6PD activity) to a binomial outcome poses difficulty in differentiate the females with heterozygous G6PD alleles because of technical reasons. A quantitative test assays will accommodate different threshold activities and within limits is able to identify heterozygous females as individuals with intermediate G6PD activity. Most quantitative test assays to date require a good laboratory infrastructure and well-trained

staff. Handheld devices that do not rely on laboratory and can provide results within several minutes are currently being introduced.

Only cytochemical assays can effectively distinguish between G6PD normal and G6PD deficient RBCs on a cellular level and can effectively identify heterozygous women with a high percentage of G6PDd red blood cells that are accordingly at risk for severe haemolysis [108, 109]. There are flow cytometry-based assays that allow the measurement of G6PD activity in labelled RBCs. The main drawbacks of this format are the complexity of the respective test assays and interpretation, the need for costly machinery and the long turn-around time that make these assays unsuitable for PoC testing [110].

The gold standard test is by spectrophotometry and flow cytometry. Although these tests quantify enzyme activity, they are expensive and require a well-functioning laboratory infrastructure. However, in the setting of the impoverished rural tropics, the requirements for laboratory skills, refrigeration, specialized equipment, and high costs have excluded its availability to the vast majority of patients suffering malaria. A number of qualitative tests have been introduced over the last couple of years with superior operational characteristics and comparable diagnostic performance. Since 1979, the fluorescent spot test (FST) is recommended as the most suitable method for screening in the field [111]. However, the FST is not suitable for remote locations without laboratory facilities and requires experience in its interpretation. Expert consensus defined practicality criteria for point-of-care (PoC) G6PD diagnostics that included simplicity of use, ease of interpretation, no specialized equipment or cold chain, and relatively low cost. Expert consensus also acknowledged that the availability of such robust devices where most malaria patients live is a key to the control and

elimination of endemic *P. vivax* malaria [106, 112]. In recent years, novel technologies are changing the landscape and three new PoC devices have been implemented. Two companies, Accessbio (New Jersey, USA) and Alere (Maine, USA), have developed different types of PoC diagnostics for G6PD deficiency that focus on rapid diagnosis, with limited need for technical training and infrastructure. The format of the qualitative, lateral flow RDT is similar to many malaria RDTs on the market. The G6PD RDT is based on the reduction of colourless nitro blue tetrazolium dye to dark coloured formazan [113]. The appearance of a purple/blue coloration indicates a G6PD normal result. While the BinaxNOW G6PD test (Alere, USA), available since 2008, has good performance in controlled laboratory settings, performance of this test in less controlled settings was less satisfactory [114]. BinaxNOW presents a major drawback with the need to perform the test within a temperature ranging from 18 to 25°C, imposing limited usefulness in tropical malaria-endemic countries [115]. In 2013, Accessbio (USA) released the CareStart G6PD test onto the market. The assessment of the CareStart G6PD revealed performance characteristics essentially similar to the current screening standard, the FST [116]. However, the current CareStart does not include a control-line, a short-coming that affects result validity.

The degree of G6PD activity can help to guide clinical management, but the relevant threshold varies with the intended use. All of these tests have a threshold of around 30% enzyme activity, a threshold sufficient to discriminate G6PDd homozygous females and hemizygous males, from G6PD normal but inadequate for heterozygous females, with intermediate enzyme activities above 30% of normal but below a pre-defined considered safe threshold such as 70% from normal. The threshold of current qualitative Point-of-care tests is considered sufficient to guide PQ treatment, however

heterozygous females are at substantial risk of drug induced haemolysis and there are concerns regarding the safety of prescribing the long acting TQ in patients with enzyme activity of less than 70% [117]. Diagnosis of this higher threshold will require more discriminative quantitative tests. Biosensors, the second type of PoC device developed, are handheld devices that in conjunction with a disposal strip provide a quantitative result. These tests directly measure G6PD activity from collected blood based on electro-chemical properties of the sample. In 2015, AccessBio (USA) launched the CareStart Biosensor, which at present, is the only product of this type on the market.

Several factors can influence the performance of a G6PD test and its ability to correctly classify a patient as either normal or deficient, starting with the cut-off definition. These include biological conditions such as concomitant haemoglobinopathies, recent haemolysis events that leave a patient with a relatively high proportion of young cells with high G6PD activity that can produce a false normal result, and high leukocyte counts that also lead to a false normal G6PD result [112]. Because they are enzyme activity tests, the G6PD assays are particularly sensitive to specimen handling and reaction conditions.

The evaluation of a novel quantitative assay requires comparison with an appropriate quantitative reference method. Additional tests such as genotyping and flow cytometry provide complementary information on underlying G6PD variants and heterozygosity. Several quantitative assays have emerged that either directly or indirectly measure NADPH formation as a result of G6PD activity, however the majority of test evaluations have used spectrophotometry as a reference method. Absolute values can vary between different assays and hence results need to be transformed into population specific values relative to the population specific adjusted male median. Presentation

of the results without the corresponding adjusted male median (AMM) value significantly confounds the comparison of the assay results across different studies.

The G6PD activity is defined as one International Unit (U) is the amount of G6PD activity that will convert 1 micromole of NADP<sup>+</sup> per minute under predetermined substrate and reaction conditions. Activity may be expressed in either a standard number of cells (U/10<sup>12</sup> RBCs) or amount of Hemoglobin (U/g Hb) [102]. Typically, G6PD activity in a population is bimodal, with a minor group of individuals clustered around 10% or less G6PD activity and most clustered in the 60% to 150% range.

## Chapter 2

### The Clinical Manifestations of *Plasmodium vivax* malaria in Vietnam

#### 2.1 Introduction

*Plasmodium vivax* malaria is a significant public health issue in many parts of the world. In 2015, *P. vivax* is estimated to have been responsible for 13.8 million malaria cases globally, and accounted for approximately half the total number of malaria cases outside Africa. Most cases of *P. vivax* malaria occur in the WHO South-East Asia Region (74%) [118]. Clinical diagnosis of vivax malaria is difficult because signs and symptoms are non-specific and impacted by endemicity and host immunity. Common symptoms include fever, malaise, chills, diaphoresis, headache, arthralgia, myalgia, cough, abdominal pain, nausea, vomiting, and diarrhea, which can also occur with many common systemic febrile illnesses such as meningitis, pneumonia or gastroenteritis. Splenomegaly, anemia, leucopenia and thrombocytopenia are also common, but non-specific [24]. The spectrum of disease associated with *P. vivax* infection ranges from asymptomatic parasitaemia and uncomplicated febrile illness through to severe and fatal malaria. Vivax malaria was historically described as benign and self-limiting infection because individual clinical episodes were less likely to cause severe illness than *Plasmodium falciparum*. More recently, *P. vivax* has been shown to cause severe anemia, respiratory failure, malnutrition, and possibly coma [119]. In malaria elimination era, with the successful in controlling *P. falciparum*, the incidence of vivax cases has decreased more slowly than that of the falciparum because of key differences in parasite and vector biology [120]. Vivax becomes predominate and persists as the principal cause of malaria. Since the 1990s, the malaria burden in Vietnam has been

efficiently reduced (> 90%) by the national control program and malaria is now confined to the remote and forest areas populated mainly by poor ethnic minorities [121]. In 2015, there were totally over 19,000 malaria cases in Vietnam, in which *P. vivax* accounted for 51% [122]. For that reason, *P. vivax* becomes an important problem in malaria control programme. Although there are studies related to *P. vivax*, most of them described the other fields outside the clinical manifestations, we conduct a study to describe the clinical and laboratory signs and symptoms of an episode of acute vivax malaria in Vietnam in order to have an accurate and appropriate diagnosis as well as a correct treatment.

## **2.2 Methodology**

### **2.2.1 Study sites**

The descriptive longitudinal study was a part of two clinical trials 1) to evaluate the safety and efficacy of short course of primaquine and 2) to assess the haemolysis risk of single dose tafenoquine (Chapter 3 and 4). This study was carried out in patients with *P. vivax* malaria in the health facilities of Bu Gia Map district, Binh Phuoc province and Krong Pa district, Gia Lai province from October 2014. Binh Phuoc and Gia Lai Province are the remote areas that have the highest incidence of malaria cases out of the country. Binh Phuoc Province is located 200 km North West of Ho Chi Minh city. It consists of about 932,000 people belonging mainly Kinh and S'tieng ethnic group. A minor part of the population is Tay and Nung people who are immigrants from the Northern provinces. The habitations of local people are surrounded by cashew nut or coffee tree gardens. Gia Lai Province is located in the central of Vietnam, the



mountainous region. It has many communes living nearby forest and mountain. The habitants are about 1.36 million people with mainly the J'rai ethnic group.

**Sample size:** The sample size of this descriptive study was calculated based on the following equation

$$n \geq \left( \frac{1.96}{m} \right)^2 \hat{p}(1 - \hat{p})$$

m: sampling error = 0,05

p: proportion of *P. vivax* malaria presenting a specific symptom or sign = 0.5

Z = confidence level = 1.96 with a two-sided significance level of 5%, and a power of 80%

n = sample size = 385 participants

### 2.2.2 Study procedures

All of the patients were included in the study as they attended to at the malaria diagnosis facilities; men and women were selected according to the criteria.

- Inclusion criteria: All the following had fulfilled: a. > 6 months of age; b. > 5 kg of weigh; c. Mono *P. vivax* infection; d. Fever (axillary temperature  $\geq 37.5^{\circ}\text{C}$ ) or history of fever in the last 48 hours; e. Informed consent (signed by the patient or his/her parents if underage).

- Exclusion criteria: To have at least one of the following: a. Pregnant or lactating female; b. Inability to tolerate oral treatment; c. Severe/Complicated malaria; d. Hb < 9 g/dL; d. Another conditions affect the results of the study (serious underlying cardiac, renal or hepatic disease, severe malnutrition, HIV, severe febrile condition other than malaria).

Malaria diagnosis: Diagnosis, species and number of parasites were determined by Giemsa stained thick and thin blood smears.

Clinical evaluation: After the diagnosis, anamnesis and clinical evaluation were done and by a physician. A case sheet proforma was prepared and the data (demographic profile, clinical features, investigation, treatment and complications) from all the case records were filled up and later on were analyzed.

Laboratory tests: At the enrollment in the study, capillary blood sample was obtained for the following tests: Field Blood Haemoglobin, parasite microscopy. Microscopy will be used to confirm the presence of parasitaemia and to estimate the parasite density. Standard thick (6  $\mu$ L blood on a 12-mm diameter template) and thin malaria films were stained with Giemsa (3% in 40-50 min). Blood films were considered negative if no parasites were detected after examining 200 high-power fields (magnification x 1000) on the thick film. Parasite counts were obtained from thick or thin films by counting the number of asexual parasites on a minimum of 40 high-power fields on the thick film or by assuming  $5 \times 10^6$  erythrocytes per  $\mu$ L for thin film counts. A venous blood sample was also obtained for the Complete Blood Count if available.

Treatment: All of the participants will be treated with a 3-day regimen of chloroquine with the total dosage of 25 mg base/kg [105]. Parasite blood smears will be examined every day until 2 consecutive smears are negative.

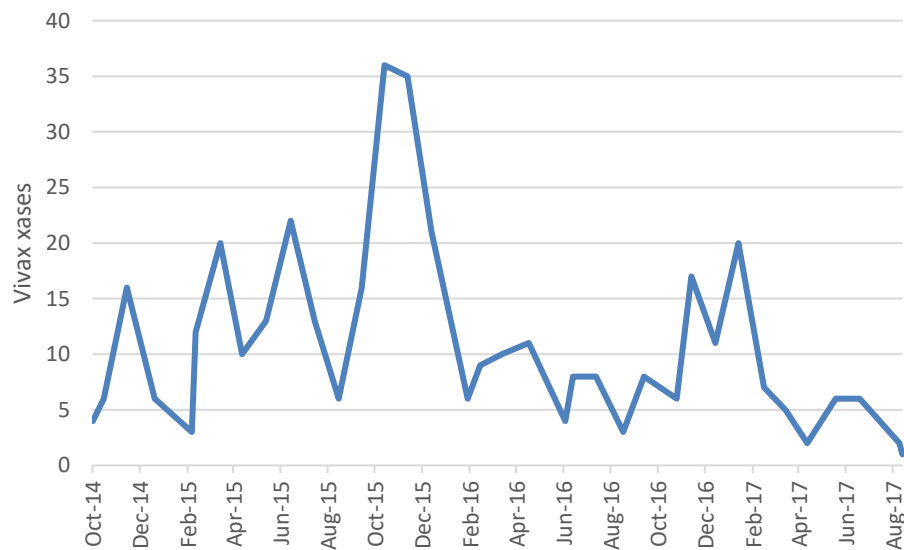
### **2.2.3 Statistical analysis**

All analyses were performed with the statistical software R3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). Frequencies of various symptoms and signs were determined. Age and laboratory alterations were calculated by Median + Range.

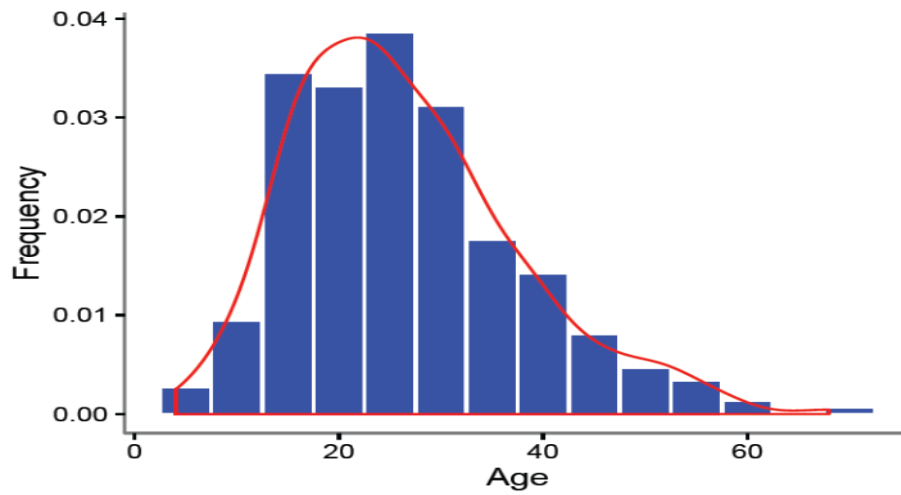
## 2.2.4 Results

### 2.2.4.1 Patient characteristics

From October 2014 up to November 2017, a total of 389 cases with *P. vivax* diagnosis were admitted to the study, of which 326 (84%) were males and 63 (16%) were females. The number of admissions due to malaria fluctuated during the study period, and reached the highest in the end of 2015 and the beginning of 2016 (Fig.2.1). 345 (88.76%) patients carried out works related with agriculture. The minority ethnic was accounted for three fourth of malaria cases. The median age is 24 years old with the range between 4 and 68 years old. Twenty-seven of 389 (6.9%) patients were children less than 15 years of age. The age distribution of patients is shown in Fig.2.2.



**Figure 2.1:** Seasonal malaria incidence pattern



**Figure 2.2:** Age distribution of study population

#### **2.2.4.2 Symptoms and physical findings**

Signs and symptoms analysis on admission is shown in Table 2.1. The evolution time of the disease from the start of the symptoms to the moment of clinical evaluation was from 0 to 21 days and the mean of nearly 2 days. Headache were reported in 203 (68.81%) cases, chill in 45 (15.25%) cases and heavy sweating in 32 (10.85%) cases. Other symptoms only presented in small percentage like diarrhea, abdominal pain and insomnia.

**Table 2.1:** Symptoms and clinical signs at admission

	Number of patients	%
<b>Symptoms</b>		
Fever	389	100
Headache	203	52.2
Chill/Rigor	45	11.6
Heavy sweating	32	8.2
Anorexia	31	8.2
Fatigue	29	7.5
Nausea	19	4.9
Dizziness	17	4.4
Muscle pain	12	3.1
Vomiting	11	2.8
Coughing	11	2.8
Diarrhea	5	1.3
Abdominal pain	3	0.8
Insomnia	3	0.8
<b>Signs</b>		
Jaundice	3	0.8
Hepatomegaly	1	0.3
Splenomegaly	2	0.5

On general physical examination at admission, 389 (100%) cases had the temperature higher than 37.5<sup>0</sup>C. The median temperature of the patients was 38.7<sup>0</sup>C, of which half of the cases (51.35%) had the temperature above 39<sup>0</sup>C. Jaundice and hepatomegaly were recorded in only 3 cases and 1 case, respectively. Two cases with spleen enlargement were described.

#### **2.2.4.3 Laboratory abnormalities**

Laboratory abnormalities are shown in Table 2.2 and 2.3. The median of haemoglobin concentration was 13.5 g/dL with range from 9.3 to 16.8 g/dL. Anaemia was observed in 112 (28.81%) patients based on age-adjusted reference value [123]. Of 143 patients whose blood samples were tested for full blood count, thrombocytopenia was common (82.4%) although in most cases it was mild-to-moderate (50,000 to 150,000

platelets/ $\mu\text{L}$ ); only 9 patients (6.7%) had low platelets levels (25,000 to 50,000 platelets/ $\mu\text{L}$ ) without clinical bleeding manifestations. Leukopenia (WBC < 4,000/ $\mu\text{L}$ ) was present in 10 (6.99%) patients.

**Table 2.2:** Sex and age hemoglobin values (g/dL)

Age	No.	Hemoglobin $X \pm SD$	Anemia No. (%)	Reference value (lower value)
6m - < 5 y	1	10.4	1 (100)	11.0
5 – 11	22	11.55 $\pm$ 1.2	9 (52.94)	11.5
12 – 14	18	12.58 $\pm$ 0.89	2 (22.22)	12.0
>15 (women)	53	12.54 $\pm$ 1.38	16 (40)	12.0
>15 (men)	295	13.82 $\pm$ 1.35	57 (25)	13.0

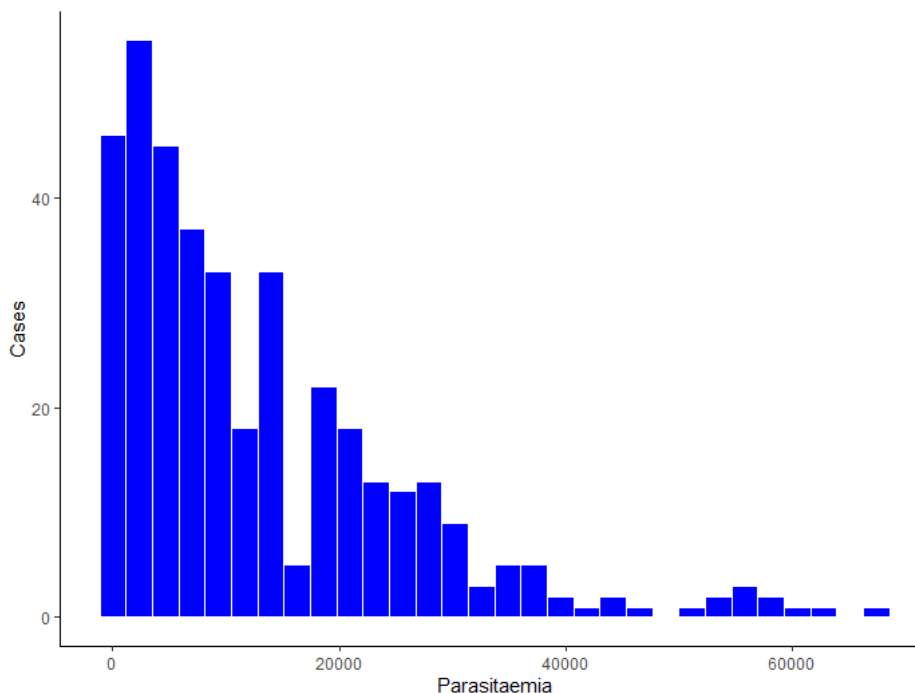
**Table 2.3:** Hematological values in 143 patients

Laboratory value	No. of patients (143)	Percent of total
White blood cells (/ $\mu\text{L}$ )		
< 2,000	0	0
2,001 – 4,000	10	6.99
4,001 – 10,000	122	85.31
10,000+	11	7.7
Platelets (/ $\mu\text{L}$ )		
< 25,000	0	0
25,001 – 50,000	9	6.29
50,001 – 75,000	27	18.81
75,001 – 150,000	82	57.34
150,001+	25	17.56

#### 2.2.4.4 Parasite densities

The parasite densities ranged from 517 to 58,740 parasites per microliter of blood, and the median parasite density was 7,508. Approximately 60% (n=230) of subjects had asexual parasitaemia less than 20,000/ $\mu\text{L}$  at admission (Figure 2.3). Of the 389 patients,

292 (75.5%) cases had gametocytes on peripheral blood smears at the time of admission.



**Figure 2.3:** Asexual parasitaemia distribution among participants

#### **2.2.4.5 Treatment response and complications**

In all the participants, fever resolved promptly after a standard course of chloroquine treatment. In our study, the clinical course of vivax malaria was usually benign. All of the patients recovered fully with no severe complications. The mean fever clearance time and parasite clearance time were 1.53 and 2.08 days, respectively. However, 5 cases still had mild fever until Day 3 post treatment and 6 cases had positive parasite blood smear on Day 3.

### **2.3 Discussion**

The study showed that the majority of the patients were males and 88% of them worked in agriculture-related work. Males were affected than females, which is possibly due to

increased outdoor activities and increased exposure to mosquitoes in males as compared to females. Information regarding age and gender related prevalence of malaria is scarce but most of the studies reported generally a high burden in males compared to females, our study also showed similar results [124, 125]. In areas where major epidemiological shifts toward elimination have occurred, the adult males were predominant in vivax patient population that will sustain transmission [126]. From this study, most of the participants presented with vivax infection during the rainy season. This may be caused by the favorable humid climate and warm stagnant water with aquatic vegetation for mosquito vector breeding, which is comparable with other study [127].

The common signs, fever and headache, were present in nearly 100% of the patients. This is similar compared to other studies [128, 129] and also agrees with classic findings [119]. Infection of a non-immune host results in a prodromal period followed by an acute fever. The temperature rises before the rigor ceases, usually reaching a peak 1 – 3 hours after the rigor finishes, commonly 39.5-40.5<sup>0</sup>C but occasionally over 41.5<sup>0</sup>C [24]. In our study, 51.35% of cases had the temperature above 39<sup>0</sup>C. In both adults and children, *P. vivax* has a lower pyrogenic threshold (the parasite density required to evoke a fever) compared to *P. falciparum*, which median parasitaemia in uncomplicated vivax malaria lower than that seen in uncomplicated falciparum malaria [24]. However, the parasite blood smears of the study participants had a median of 7,500 parasites/ $\mu$ L, different from other studies [130, 131]. Headache, anorexia, vomiting, myalgias, abdominal pain and diarrhea commonly occur during the paroxysm of vivax malaria [24]. Respiratory symptoms have long been recognized as common in vivax malaria, but in our study, coughing was only recorded in 11 cases. The signs of vivax malaria



were not typical, usually jaundice, hepatomegaly or anaemia. Malaria is the most frequent cause of spontaneous splenic rupture, which occurs commonly in *P. vivax* parasite infection [132]. The spleen enlargement is a widely prevalent feature of malaria. However, the average enlargement of the spleen is considered to be greater in highly endemic malaria regions [133]. As the endemicity of malaria has decreased in Viet Nam, the occurrence of splenomegaly in participants was relatively low. The frequency of clinical signs was significantly lower than the frequency of symptoms, which confirms what is already well known. In comparison with *P. falciparum* malaria, fever was significantly less frequent in *P. vivax* cases, whereas pallor was more frequent in *P. vivax* infected patients. Uncommon symptoms, such as vomiting, cough, and diarrhea were mostly reported in *P. vivax* patients. Patients presented with hepatosplenomegaly all with *P. vivax* infections [134].

Hematological changes, which are one of the most common complications, play an important role in increasing mortality. Anaemia is known to be associated with malaria in endemic areas, although malaria may not be the prime cause of it. The pathogenesis of anaemia in malaria is complex and multifactorial. It is often thought to result from a combination of hemolysis of parasitized red blood cells, accelerated removal of both parasitized and unparasitized red blood cells, depressed as well as ineffective erythropoiesis with dyserythropoietic changes, and anaemia of chronic disease. Other contributing factors may include decreased red blood cell deformability, splenic phagocytosis and/or pooling, leading to an increased rate of clearance from the circulation [135]. Anaemia was observed in 85 (28.81%) patients. The degree of anaemia in an individual is influenced by a wide range of factors including premorbid haemoglobin concentration, level of immunity, duration of infection before treatment,

the number of prior episodes of malaria, as well as the presence of comorbid conditions. Moreover, the anaemia status can be related with helminth infections and iron deficiency anaemia, especially in ethnic minority community [136]. Anaemia associated *P. vivax* infection is amongst the commonest manifestations of severe vivax disease and is more strongly skewed towards young children [24]. This percentage of anaemia is lower than other studies with 73% and 35.77% of the cases were anaemic, respectively [125, 137].

Despite not being a criterion for severe malaria, thrombocytopenia is one of the most common complications of *P. vivax* malaria. Platelets count under 150,000/ $\mu$ L occurs in 24 – 94% of patients with vivax malaria [24, 135, 137-139]. Thrombocytopenia was the most frequent laboratory abnormality found in our study, presenting in 82.44%, but severe thrombocytopenia was not seen and clinical bleeding did not occur. Our finding is comparable to other studies [128, 137, 140, 141]. The speculated mechanisms leading to thrombocytopenia are: coagulation disturbances, splenomegaly, bone marrow alterations, antibody-mediated platelet destruction, oxidative stress [138]. This phenomenon improves with disease resolution and the platelet count is generally normal in one or two weeks [142]. However, the trend of disease with *P. vivax* malaria is changing. All complications seen in falciparum positive cases are being seen in vivax positive cases also. Thrombocytopenia may not be a cause of mortality by itself, but it can be a marker of increased severity and need of aggressive management [140]. The presence of thrombocytopenia in malaria patients is not a distinguishing feature between *P. falciparum* and *P. vivax* infection [142]. Furthermore, in tropical countries, malaria and dengue fever are prevalent and the challenge of making correct differential

diagnosis between two diseases may be difficult. Clinicians in the tropical areas should think of malaria and dengue whenever a patient present with acute febrile illness and or thrombocytopenia [143].

In this study, almost 75% of patients at the time of *P. vivax* infection diagnosis had gametocytes in their blood. This is similar to that found in other research [125, 129]. *P. vivax* gametocytes are present earlier in the progression of a primary or recrudescent infection than *P. falciparum* and mature faster, and may, therefore, be transmitted earlier in the course of infection before being diagnosed or treated [126, 144].

Chloroquine is the first-line treatment for *P. vivax* malaria in most endemic countries, but chloroquine resistance was also recorded in many places, such as Indonesia and Oceania [145]. The standard chloroquine total dose of 25 mg base/kg is administered as three daily doses. This typically clears chloroquine-sensitive blood-stage *P. vivax* parasites within 48 hours [126]. In our study, there were 6 cases (2%) that had positive asexual parasite blood smear after 48 hours after chloroquine treatment and 5 mild fever cases on day 3. Clearance of parasitaemia (assessed by microscopy) in all patients by day 3, or in 95% of patients by day 2, was 100% predictive of chloroquine sensitivity in the study population as defined by day 28 recurrence [145]. In central of Vietnam, confirmed *P. vivax* chloroquine resistance was described for the first time in 2009 with optimal measured chloroquine blood concentration [125]. The mean fever clearance time and parasite clearance time of our study were 1.53 days and 2.08 days, respectively while other study showed more rapid fever clearance time and parasite clearance time [146]. In southern Vietnam, in another study, chloroquine has been proved efficacious for the treatment of *P. vivax*, but dihydroartemisinin-piperaquine provided more rapid symptomatic and parasitological recovery [146].

There were some limitations in this study. Firstly, all subjects were male which may have impacted findings. Secondly, one exclusion criterion of our study was if haemoglobin concentration was below 9 g/dL, which occurred in 9 patients. This exclusion of these anaemic subjects could underestimate the prevalence of severe anaemia of vivax patients in malaria endemic regions. Furthermore, this study did not investigate the biochemistry laboratory values, so that the clinical features of vivax patients are thus not fully described.

This study focuses on clinical and laboratory findings in vivax malaria. Although these clinical and laboratory alterations in association with malaria are not new to the subject, this data adds more detailed information to the current knowledge in manifestation of vivax malaria.

## Chapter 3

### Improving the radical cure of vivax malaria: A placebo-controlled comparison of short and long course of primaquine regimen

#### 3.1 Introduction

In 2018, while *Plasmodium falciparum* is the most prevalent malaria parasite in the WHO African Region, 53% of the *Plasmodium vivax* malaria burden comes from the WHO South-East Asia Region [4, 147]. Historically, vivax malaria has been considered as a benign infection, there are now more evidence to prove it can cause death and severe cases [119, 148, 149]. The main distinct biological characteristic in the life cycle of *P. vivax* is the appearance of dormant liver stages, called hypnozoites. After the initial infection, the hypnozoites stay in the liver, then under certain circumstance they can be awakened and cause recurrent illnesses in the following weeks or months [9, 150]. This stage in life cycle is refractory to most of antimalarial drugs and relapses is the main source of vivax malaria in endemic areas [73].

Primaquine, an 8-aminoquinoline, has been used as the only drug that can kill hypnozoites, thus preventing relapses [49, 62, 151]. However, the coverage of this drug is limited due to the risk of acute haemolysis in patients with glucose -6-phosphate dehydrogenase deficiency (G6PDd) [50]. The efficacy of PQ depends on the total dose whereas the adverse reactions are mostly associated with the daily intake [151]. The World Health Organisation has recommended 0.5 mg/kg/day for 14 days of PQ in G6PD normal patients to prevent relapses in areas where frequent relapsing strains of *P. vivax* are prevalent [45]. However, the adherence of the prolonged treatment in

malaria endemic settings is low. This can result in a significant reduction in PQ effectiveness [68, 152, 153]. Short courses with higher daily dose may improve the adherence and thus effectiveness without compromising efficacy [71, 154, 155]. The primary objective of this study was to assess the safety, tolerability and efficacy of a 7-day high dose (1 mg/kg/day) PQ regimen for vivax malaria in patients screened as G6PD-normal.

## **3.2 Methods**

### **3.2.1 Trial design**

This was a multi-site, randomised, double-blinded, placebo-controlled, non-inferiority trial in G6PD-normal patients with uncomplicated vivax malaria. There were 4 countries participating this trial; including Indonesia, Ethiopia, Afghanistan and Viet Nam. Ethical and drug regulatory approvals were obtained from the Oxford Tropical Research Committee, the relevant national and local committees and authorities. The study protocol has been published previously [156]. The result from this multi-site trial has been published in *The Lancet* [157]. This chapter only described the results derived from Vietnam participants. From Viet Nam site, eligible patients presenting to Dak O and Bu Gia Map Commune Health Station in Binh Phuoc province and Krong Pa Medical Centre in Gia Lai province were screened to enroll into the study. Sharing as the role of site principal investigator, I had the oversight responsibility in conduct of the study. I supervised the study performance and ensured the participants' well-being and safety were protected; all the study procedures were conducted at the research sites in accordance with the protocol and International Conference on Harmonisation – Good Clinical Practice (ICH-GCP).

### **3.2.2 Participants**

Patients presenting with uncomplicated vivax malaria (mono-infection) who had fever or history of fever within the last  $\leq 48$ h, were aged  $> 6$  months and weighed  $\geq 5$ kg, haemoglobin concentration  $\geq 9$  g/dl, and normal G6PD status as assessed by the fluorescent spot test (FST) were eligible for enrolment. Patients were excluded if they were pregnant or lactating, could not tolerate oral treatment, had a history of previous haemolytic episodes or blood transfusion within the last 90 days, or had signs of severe malaria. Other exclusion criteria included any hypersensitivity to study drugs, or concomitant medication with the potential to cause haemolysis or interference with the pharmacokinetics of the study drugs.

Before enrolment, written informed consent was obtained from the patient or their guardian as well as assent if aged 12 to 18 years. Patients with G6PDd were excluded from the main trial, but enrolled into a parallel observational arm and treated with chloroquine (CQ) plus supervised PQ (0.75 mg/kg) once a week for 8 weeks.

### **3.2.3 Randomisation and masking**

Eligible patients were treated with a blood schizontocidal drug (Chloroquine in Vietnam) and assigned randomly in a 2:2:1 ratio to a 7-day PQ regimen (PQ7), a 14-day regimen (PQ14) or placebo. The allocation ratio of 2:2:1 was based on the sample size requirements for the non-inferiority comparison of a 7-day primaquine regimen with a 14-day regimen, and the superiority comparisons of the 7-day and 14-day primaquine regimens with the placebo. Randomisation was done using STATA version 14.1 (StataCorp, College Station, TX, USA), which generated blocks of 20 for each dosing band. The independent statistician responsible for generating the randomisation

list and for selecting code letters for PQ/placebo was otherwise uninvolved in the conduct of the trial and did not visit any of the study sites. Identical PQ/placebo tablets were manufactured by Centurion Laboratories (Vadodara, India) and blister packed (Bilcare Research, Pune, India). Randomisation to the body weight-based regimen allocation provided in sequentially numbered boxes was performed at enrolment. Participants and all the study team were masked to treatment assignments.

### **3.2.4 Procedures**

All patients enrolled were admitted to the hospital and treated with chloroquine (total dose of 25 mg base/kg) for 3 days as the first line treatment for acute *P. vivax* infection. The study drug (primaquine or placebo) was started on day 0. Patients in the PQ7 were treated with either 7 days of daily primaquine 1.0 mg/kg per day followed by 7 days of daily placebo (PQ7), while patients in PQ14 were treated with 14 days of daily primaquine 0.5 mg/kg per day (7 mg/kg total dose) (PQ14), and those in the placebo group received 14 days of daily placebo (90% starch, calcium phosphate). Primaquine was administered as 15 mg tablets. Treatment was fully supervised and dosed according to body weight. In children weighing less than 23 kg, tablets were dissolved in 5 ml of syrup and administered as a suspension.

At enrolment, a medical history was taken, a physical examination was performed and antimalarial treatment with chloroquine was initiated. After completion of treatment, patients were asked to return weekly until day 42 and then monthly for one year. At each visit, a medical history was taken, a symptom questionnaire was done, and any adverse events (AEs) or serious adverse events (SAEs) were recorded. Patients were encouraged to report to the study staff if they became ill. Those who missed their



scheduled follow up visits were contacted by study staff and encouraged to return to the study centre for review. Blood films were examined immediately for all symptomatic patients, but otherwise stored for later examination. Recurrent parasitaemia with any Plasmodium species within 28 days was considered a treatment failure and treated with artemisinin combination therapy (ACT). After 28 days, patients with recurrent symptomatic vivax parasitaemia were prescribed the same treatment allocated at enrolment. Patients presenting with their third or subsequent symptomatic *P. vivax* recurrence were treated with open-label supervised primaquine (0.5 mg/kg for 14 days).

At the initial screening, venous blood was collected and tested for G6PDd, using the qualitative FST (R&D Diagnostics; Athens, Greece). Standard thick (using 6µl blood on a 12 mm diameter template) and thin malaria films were prepared at each visit for Giemsa staining (3%, 40-50 min) according to procedures based on the Research Malaria Microscopy Standard [39]. Blood films were declared negative if no parasites were detected after examining 200 high power fields (1000x magnification) on the thick film. Parasite counts were obtained from thick or thin films at each of the study sites by counting the number of asexual parasites on a minimum of 40 high power fields or per 2000 erythrocytes. Parasite densities were estimated based on calculated volumes of blood examined per 40 high power fields on the thick film or by assuming  $5 \times 10^6$  erythrocytes per  $\text{mm}^3$  for thin film counts. Microscopists underwent onsite training in study laboratory procedures and continuous quality control was implemented at all sites. Haemoglobin concentration was checked at each visit (HemoCue; Angelholm, Sweden).

### **3.2.5 Outcomes**

The primary endpoint was defined as the incidence rate of symptomatic *P. vivax* parasitaemia (mono-infection or mixed) over 12 months. The primary analysis was the difference in incidence rate between patients treated with 7-day and 14-day primaquine. The secondary efficacy endpoint included the incidence rate and incidence risk (time-to-event analysis) of any (symptomatic and asymptomatic) *P. vivax* parasitaemia, comparing the treatment arms to each other and with the placebo group. Other efficacy endpoints were the incidence risks of *P. vivax* at 28 and 42 days, and the proportions of patients with *P. vivax* parasitaemia and/or fever on days 1, 2, and 3. The number of recurrences avoided per 1000 patients and the number needed to treat (NNT) to avoid one recurrence were derived from the rate difference.

Safety endpoints were defined as the incidence risk of severe anemia (Hb < 7 g/dl) or transfusion, an acute drop in Hb > 5 g/dl within 7 days, Hb concentration on day 3 and 7, the median time to Hb nadir, grade 3 or 4 AEs within 42 days and SAEs. The relationship between treatment and AEs or SAEs was determined by the site investigator.

### **3.2.6 Sample size and statistical analysis**

The primary objective of this trial is to demonstrate the non-inferiority of PQ7 regimen to the standard PQ14 regimen with respect to incidence rate of vivax symptomatic parasitaemia over 12 months. The sample size calculation is based on the assumption that incidence rate of 0.2 recurrences per person-year (PPY) in both primaquine arms, a non-inferiority margin of 0.07 recurrences per person-year and a one-sided significance level of 2.5%. From this assumption, total sample size of 1200 eligible

patients, randomly assigned to receive a primaquine regimen, followed for one year provided a power of 80% to show the non-inferiority that the two-sided 95% confidence interval for the difference in incidence rate of malaria between the two arms excluded an excess rate of 0.07 recurrences per person-year or more in favour of the PQ14 regimen. In the control arm (placebo), a further 300 patients were also followed for one year. With 1500 patients in which 600 patients in each primaquine arms (PQ7 and PQ14) and 300 patients in the control arm, the study has 95% power to detect a difference with the assumption that an incidence rate of 0.2 recurrences PPY in each of the primaquine arms and 0.6 recurrences PPY in the control arm. The losses to follow-up and major protocol violations was expected to be no more than 20%, a planned total of 1875 G6PD normal patients (750 per primaquine arms and 375 in the control arm) will be randomized in this trial. For each of the study sites including Vietnam site, the target sample size of 375 patients was required as follow 150 patients for each primaquine arms and 75 patients in the control arm. The total of 375 patients per study sites also has the power of 80% to detect the non-inferiority between PQ7 and PQ14 regimens; and the power of 95% to detect the superiority between the primaquine arms and the control arm.

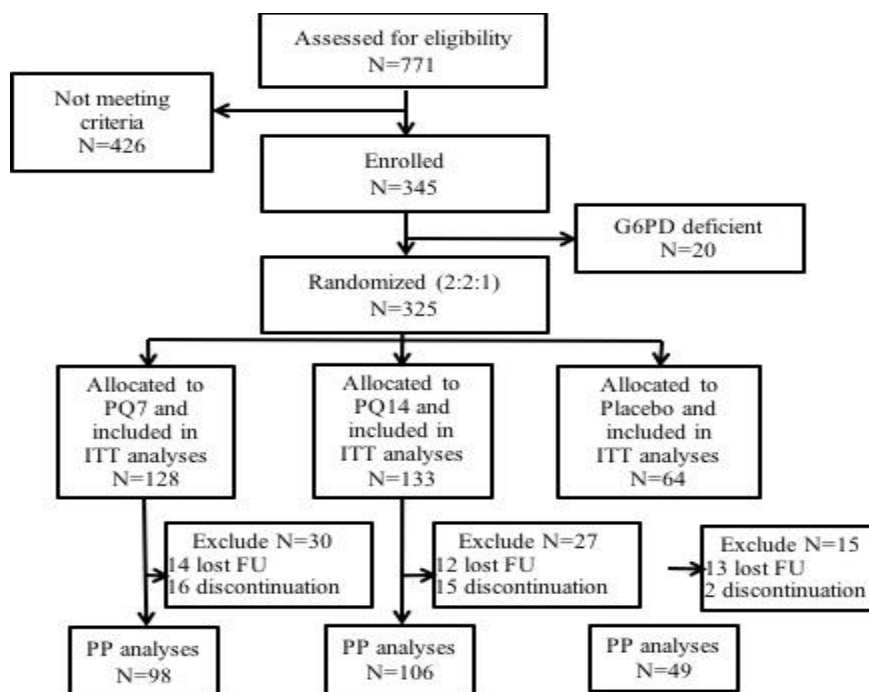
Statistical analyses were performed with the use of R software, version 3.6.1. The primary outcome was analysed on an intention-to-treat population which included all randomized patients who fulfilled the enrolment criteria. The distribution of the demographic and clinical variables collected at baseline were presented for each arm using mean and standard deviation (SD) for normally distributed continuous variables, median (25th – 75th percentile) for non-normally distributed continuous variables, and frequency (per cent) for categorical variables. The incidence rate of symptomatic and

asymptomatic recurrent *P. vivax* for each treatment arm and the control arm were calculated by the total number of recurrent *P. vivax* infections divided by the total number of person-years at risk. The incidence rate of symptomatic and asymptomatic recurrent *P. vivax* was compared between the 7-day and 14-day primaquine treatment arms by calculating the absolute incidence rate difference and two-sided 95% confidence interval. The 7-day treatment was considered non-inferior to the 14-day treatment if the upper limit of the confidence interval is lower than 0.07 recurrences per person-year. The incidence risk and the absolute risk difference (two-sided 95% CIs) of recurrent *P. vivax* within 12 months follow-up were calculated using survival analysis Kaplan-Meier estimates as well as Cox regression analysis for the time to first recurrent *P. vivax*. Comparisons were made between the treatment arms and control groups and statistical comparisons of the groups assessed using the log rank test. The incidence risk of severe anaemia and blood transfusion within 12 months follow-up and the absolute risk difference between groups were calculated. For serious adverse events including serious haematological events and gastrointestinal tolerability, the absolute difference between treatment groups in the proportion of patients (two-sided 95% CI) with each outcome were calculated.

### **3.3 Results**

Overall, a total of 11585 patients were screened for inclusion into the study between July 2014 and Nov 2017. Of whom, 2336 patients were randomized into either PQ7 (n = 935), PQ14 (n = 937), or placebo (n = 464). By country, about half (42.8% [n = 1000]) of the 2336 subjects were from Indonesia; the rest were either from Ethiopia (24.8% [n = 580]), Afghanistan (18.5% [n = 431]), or Vietnam (13.9% [n = 325]). At Vietnam site,

the study screened 771 patients for the eligibility of the study, of whom 426 did not meet the enrolment criteria and 20 were G6PD deficient. Subsequently, 325 subjects were enrolled into the study and assigned to receive either PQ7 (n=128), PQ14 (n=133), or placebo (n=64) (Figure 3.1).



**Figure 3.1:** Trial profile in Vietnam

G6PD=glucose-6-phosphate dehydrogenase; ITT=intention-to-treat; FU=follow-up; PP=per protocol  
Discontinuation includes: Consent withdrawn, Ineligible selection, Non-compliance with study protocol, Study terminated.

In Vietnam, the subjects were enrolled from two locations: Binh Phuoc province (n = 219) and Gia Lai province (n = 106). A majority of participants (85.8%) were male with the median age of 24 years old. While in other study sites, more than half of the participants (62.8%) were male, and the median age of enrolled subjects was younger (16 years old). At enrollment, the average temperature of subjects was 38.8°C (SD 1.01), and the geometric mean asexual *P. vivax* parasite density was 7,407 (95%CI:

6,407 – 8,562) parasites/ $\mu$ L. The proportion of gametocytes appearance at admission was about 75% of the subjects. The mean level of haemoglobin concentration of participants was 13.5 g/dL (SD 1.53). About half of the participants (57.8%) did not have fever at enrollment. Baseline characteristics were not significantly different among three groups (Table 3.1). Among subjects enrolled into the study, one patient (1.6%) in the placebo group failed to complete the initial 14-day treatment, while the numbers in PQ7 and PQ14 groups were 7/128 (5.5%) and 4/133 (3.0%), respectively. Related to the vomiting of study drugs within one hour of dosing, 1.6% (2/128), 1.5% (2/133) of participants in PQ7 and PQ14 were recorded, respectively while there was no reported case in the placebo group.

**Table 3.1:** Baseline characteristics

Characteristics	Placebo (n = 64)	PQ7 (n = 128)	PQ14 (n = 133)	Total (n = 325)
Study sites				
- Dak O & Bu Gia	43	87	89	219
Map	21	41	44	106
- Krong Pa				
Age (years)	23 (16-31)	24 (17-30)	24 (17-32)	24 (17-31)
Male	59 (90.6%)	104 (81.2%)	117 (88.0%)	280 (85.8%)
Weight (kg)	54.0 (47.2-60.0)	54.0 (47.0-60.0)	53 (48.0-60.0)	54 (47.0-60.0)
Temperature ( $^{\circ}$ C)	38.9 (1.04)	38.7 (1.01)	38.8 (0.99)	38.8 (1.01)
Haemoglobin (g/dL)	13.6 (1.54)	13.5 (1.58)	13.5 (1.50)	13.5 (1.53)
<i>Plasmodium vivax</i> parasites per $\mu$ L*	9534 (7088 – 12823)	7345 (5771 – 9348)	6612 (5258 – 8315)	7407 (6407 – 8562)

Gametocyte presence	45 (70.3%)	96 (75%)	101 (75.9%)	242 (74.5%)
Gametocytes per $\mu\text{L}^*$	169 (101 – 283)	187 (132 – 265)	177 (129 – 243)	180 (146 – 221)

Data are presented as n (%), median (IQR), or mean (SD), unless otherwise indicated.

\*Data are geometric mean (95% normal range).

Overall, chloroquine had a high efficacy against the parasite blood stage and was similar among three groups. A majority of patients (92.0% [n = 299]) became afebrile within 24 hours. And, the peripheral parasitaemia was cleared within two days of the treatment in 87.4 % (n = 284) of the participants (Table 3.2).

**Table 3.2:** Parasite and fever clearance

	<b>Placebo (n = 64)</b>	<b>PQ7 (n = 128)</b>	<b>PQ14 (n = 133)</b>
Fever on Day 1	19/64 (29.7)	46/128 (35.9)	45/133 (34.1)
Fever on Day 2	5/64 (7.8)	12/128 (9.4)	9/133 (6.8)
Fever on Day 3	1/64 (1.6)	2/128 (1.6)	1/133 (0.8)
Asexual <i>P. vivax</i> parasitaemia on Day 1	57/64 (89.1)	112/128 (87.5)	120/133 (90.2)
Asexual <i>P. vivax</i> parasitaemia on Day 2	10/64 (15.6)	16/128 (12.5)	15/133 (11.3)
Asexual <i>P. vivax</i> parasitaemia on Day 3	1/64 (1.6)	0/128 (0)	0/133 (0)
Fever was defined as either an axillary temperature > 37.5°C.			

Among the participants received the treatment, no reappearance of *P. vivax* parasitaemia within 28 and 42 days after the enrollment was reported in three groups.

The cumulative risk in both asymptomatic and symptomatic *P. vivax* recurrence at day

28 and 42 was 0% in all the participants regardless the treatment group they belonged to.

After one year, the incidence rate of symptomatic recurrent *P. vivax* malaria was 0.132 (95% CI, 0.065 – 0.199) episodes per person-year (PPY) following PQ7, 0.143 (95% CI, 0.075 – 0.211) episodes PPY following PQ14 and 1.105 (95% CI, 0.825 – 1.384) episodes PPY in the placebo group (Table 3.3). Between the PQ7 and PQ14 groups, the incidence rate difference (IRD) was -0.0105 (95% CI, -0.0106 - -0.00848;  $p = 0.8277$ ). This difference (IRD = -0.0105) was within the pre-defined non-inferiority margin of 0.07. The incidence rate ratio (IRR) was estimated as 0.926 (95% CI: 0.462 - 1.854;  $p=0.8278$ ). The number of recurrences avoided per 1,000 patients was 971 for PQ7 (NNT=1.03, 95% CI: 0.79 – 1.46) and 962 for PQ14 (NNT=1.04, 95% CI: 0.8 – 1.46). The incidence rate of any recurrent vivax malaria (both symptomatic and asymptomatic) at 1 year was 0.194 (95% CI, 0.113 – 0.275) episodes PPY following PQ7 and 0.143 (95% CI, 0.075 – 0.211) episodes PPY following PQ14. These rates were significantly lower than in the placebo group (1.454 [95% CI, 1.134 – 1.775] episodes PPY) with IRR = 0.13 (95% CI, 0.08 – 0.21;  $p<0.001$ ) for PQ7 versus placebo and IRR = 0.098 (95% CI, 0.058 – 0.166;  $p<0.001$ ) for PQ14 versus placebo (Table 3.4).



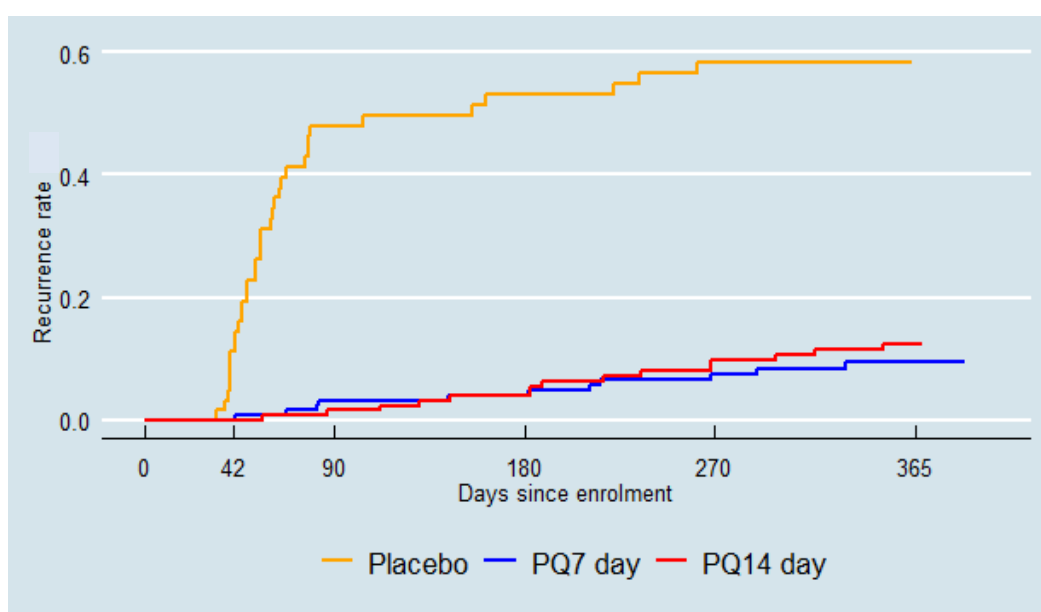
**Table 3.3:** Recurrence data per treatment group

	<b>Placebo (n = 64)</b>	<b>PQ7 (n = 128)</b>	<b>PQ14 (n = 133)</b>
Follow-up time (person-years)	54.32	113.34	118.93
Symptomatic <i>P. vivax</i> recurrence (episode)	60	15	17
Any <i>P. vivax</i> recurrence (episode) (symptomatic & asymptomatic)	79	22	17
Incidence rate of symptomatic <i>P. vivax</i> recurrence (episodes PPY; 95% CI)	1.105 (0.825-1.384)	0.132 (0.065-0.199)	0.143 (0.075-0.211)
Incidence rate of any <i>P. vivax</i> recurrence (episodes PPY; 95% CI)	1.454 (1.134-1.775)	0.194 (0.113-0.275)	0.143 (0.075-0.211)
Cumulative risk of symptomatic <i>P. vivax</i> recurrence after one year (95% CI)	58.4% (43.7-69.2)	9.5% (4.00 – 14.6)	12.4% (6.3 – 18.1)

**Table 3.4:** Incidence rate ratio between treatment arms

	<b>PQ7 vs Placebo</b>		<b>PQ14 vs Placebo</b>		<b>PQ7 vs PQ14</b>	
	IRR	p	IRR	p	IRR	p
Symptomatic <i>P. vivax</i> parasitemia	0.12 (0.068-0.211)	< 0.01	0.129 (0.076-0.222)	< 0.01	0.926 (0.462-1.854)	0.83
Any <i>P. vivax</i> parasitemia	0.13 (0.08-0.21)	< 0.01	0.098 (0.058-0.166)	< 0.01	1.358 (0.721-2.557)	0.34

In the time to first event analysis, the cumulative risk of symptomatic *P. vivax* at one year was 9.5% (95% CI, 4.00 – 14.6) after PQ7 and 12.4% (95% CI, 6.3 – 18.1) after PQ14. These cumulative risks in both primaquine treatment groups were similar ( $p = 0.506$ ). Both of these risks are significantly lower than the risk of 58.4% (95% CI, 43.7 – 69.2) in the placebo group (HR = 0.10 [95% CI, 0.05 – 0.20];  $p < 0.001$  and HR = 0.356 [95% CI, 0.263 – 0.483];  $p < 0.001$ ), respectively (Figure 3.2).



**Figure 3.2:** Kaplan Meier curves for symptomatic *P. vivax* recurrence.

From Vietnam site, during the trial, a total of 4 serious adverse events were reported. In PQ7 group, there was one haemolytic serious adverse event. This patient was a G6PDd male who had been enrolled erroneously into the PQ7 group. The event occurred 5 days after initiation of the treatment. His Hb level dropped from 15.3 to 6.4 g/dL accompanied with dark urine colour. He required hospitalization and a blood transfusion. His clinical condition and Hb concentration improved later on. The other serious adverse event was associated with gastrointestinal disturbance. This was a patient with diarrhea symptom appeared on day 7 after the treatment. The patient was

required to admit into the hospital for fluid supplement therapy and close monitoring. The study drugs were temporarily discontinued. Symptoms resolved within 5 days and he continued to complete the entire regimen afterwards. In PQ14 group, one subject with the diagnosis of undifferentiated carcinoma in nasopharynx during the follow up time (Month 5) was recorded. While in placebo group, one subject with severe *P. falciparum* malaria infection appeared at month 6 of follow up time, needed hospitalised and received intravenous artesunate treatment (Table 3.5).

**Table 3.5:** Severe adverse events and adverse events (grade 3 – 4)

	<b>Placebo (n=64)</b>	<b>PQ7 (n=128)</b>	<b>PQ14 (n=133)</b>
<b>≤ Day 42</b>			
SAE (Primaquine-related)	0	2 (1.6%)	0
SAE (Primaquine unrelated)	0	0	0
AE (Grade 3 and 4)	0	1 (0.8%)	0
<b>≤ 1 year</b>			
SAE (Primaquine-related)	0	2 (1.6%)	0
SAE (Primaquine unrelated)	1 (1.6%)	0	1 (0.8%)

Adverse events within 42 days with a severity grade of 3 or 4 were reported in one subject that received the 7-day primaquine course. This event was related to gastrointestinal symptoms. This patient was suffered from vomiting, watery diarrhea after 4 days of treatment initiation and recovered within one day. All of them belonged to the PQ 7 arm. These events involved gastrointestinal discomfort,

During the period of treatment, 87 (33.3%) of 261 patients in the primaquine group reported any gastrointestinal discomfort, which was higher compared with the placebo group (4/64 [6.3%],  $p < 0.001$ ). This number in the 7-day primaquine group (72/128

[56.3%]) was significantly greater than one in the 14-day group (15/133 [11.3%],  $p < 0.05$ ) or the placebo group (4/64 [6.3%],  $p < 0.05$ ) (Table 3.5). Most of the events were mild in severity, appeared within 5 days after the treatment and improved after 3 – 5 days of temporary study drug discontinuation.

**Table 3.6:** Symptoms elicited from daily questionnaires during treatment time  
(Day 1– 14)

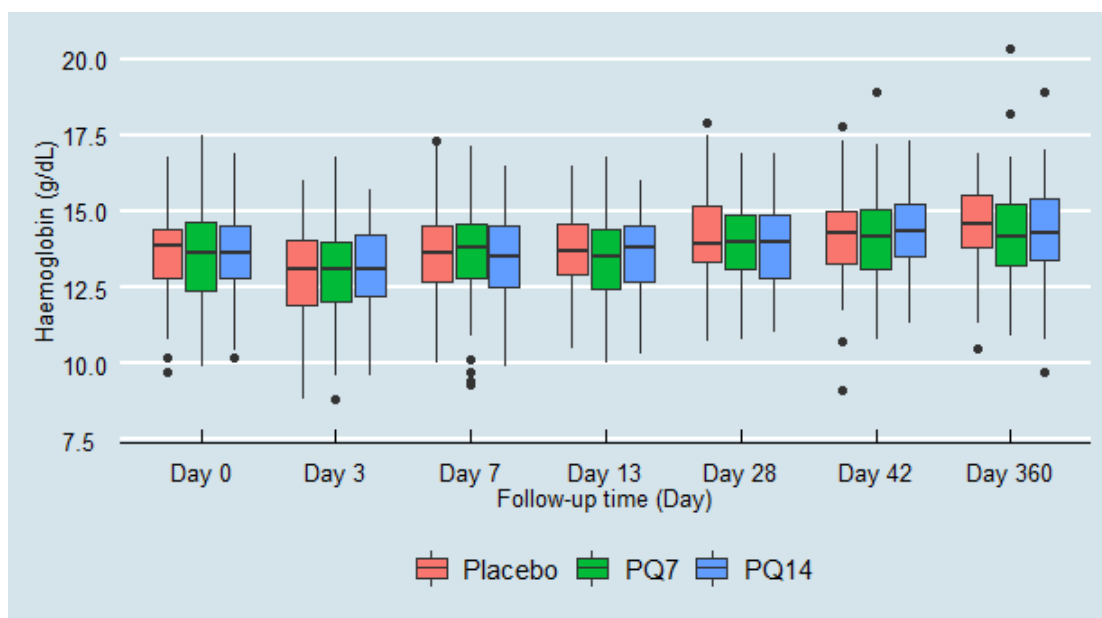
	Placebo (n=64)	PQ7 (n=128)	PQ14 (n=133)	p value	
				Placebo vs PQ	PQ7vsPQ14
<b>≤ 1 hour</b>					
Vomiting study drug	0	2 (1.6%)	2 (1.5%)	NA	> 0.05
<b>Day 1-3 [1]</b>					
Fever	20 (31.3%)	48 (37.5%)	44 (33.1%)	> 0.05	> 0.05
Headache	14 (21.9%)	35 (27.3%)	24 (18.0%)	> 0.05	> 0.05
Vomit	1 (1.6%)	3 (2.3%)	1 (0.8%)	> 0.05	> 0.05
Abdominal pain	1 (1.6%)	4 (3.1%)	2 (1.5%)	> 0.05	> 0.05
Diarrhoea	1 (1.6%)	2 (1.6%)	0	> 0.05	NA
Poor appetite	1 (1.6%)	1 (0.8%)	2 (1.5%)	> 0.05	> 0.05
Itching	0	0	2 (1.5%)	NA	NA
Skin rash	0	1 (0.8%)	2 (1.5%)	NA	> 0.05
Myalgia / Arthralgia	0	2 (1.6%)	0	NA	NA
Dizziness	0	1 (0.8%)	0	NA	NA
Shortness of breath	0	0	0	NA	NA
Passing dark urine	0	0	0	NA	NA
Any GI symptoms [2]	4 (6.3%)	10 (7.8%)	5 (3.8%)	> 0.05	< 0.05
<b>Day 4-14 [1]</b>					

	Placebo (n=64)	PQ7 (n=128)	PQ14 (n=133)	p value	
				Placebo vs PQ	PQ7vsPQ14
Fever	2 (3.1%)	9 (7.0%)	7 (5.3%)	> 0.05	> 0.05
Headache	0	1 (0.8%)	1 (0.8%)	NA	> 0.05
Vomit	0	15 (11.7%)	1 (0.8%)	NA	< 0.05
Abdominal pain	0	26 (20.3%)	7 (5.3%)	NA	< 0.05
Diarrhoea	0	13 (10.2%)	2 (1.5%)	NA	< 0.05
Poor appetite	0	8 (6.3%)	0	NA	NA
Itching	0	1 (0.8%)	1 (0.8%)	NA	> 0.05
Skin rash	0	2 (1.6%)	1 (0.8%)	NA	> 0.05
Myalgia / Arthralgia	0	0	0	NA	NA
Dizziness	0	0	0	NA	NA
Shortness of breath	0	0	0	NA	NA
Passing dark urine	0	1 (0.8%)	0	NA	NA
Any GI symptoms [2]	0	62 (48.4%)	10 (7.5%)	< 0.01	< 0.05

[1]: Number of patients with daily symptom questionnaires during treatment course.

[2]: Composite of any of the following: nausea, vomiting, anorexia, diarrhoea, or abdominal pain.

The median time to haemoglobin nadir in the placebo group as well as in both primaquine groups was similar with on day 3 after the starting of treatment. The mean absolute decreases in haemoglobin at day 3 were 0.41 g/dL (SD 0.99) in the 7-day primaquine group, 0.50 g/dL (1.04) in the 14-day group, and 0.54 g/dL (0.96) in the placebo group. The corresponding mean percentage change in haemoglobin between day 0 and day 3 was -2.84% (SD 7.36) in the 7-day group, -3.40% (7.73) in the 14-day group, and -3.79% (7.04) in the placebo group. At day 7, the mean absolute increases in haemoglobin in the 7-day and placebo group was 0.20 g/dL (SD 1.24) and 0.04 g/dL (1.27), respectively; while the mean absolute decrease haemoglobin in the 14-day group was 0.01 g/dL (1.07) (Table 3.6; Figure 3.3). The incidence risk of severe anaemia was 0.8% in 7-day regimen; while this was none in the 14-day and placebo group. This patient was erroneously randomized into the PQ 7 group despite deficient G6PD status.



Boxes represent median; 25<sup>th</sup> and 75<sup>th</sup> percentile

**Figure 3.3:** Distribution of haemoglobin during follow up time by treatment group.

**Table 3.6:** Haemoglobin profile by treatment group

	<b>Placebo (n=64)</b>	<b>PQ 7 (n=128)</b>	<b>PQ 14 (n=133)</b>
Incidence risk of severe anaemia (<7 g/dL) or transfusion within 365 days, N (%)	0	1 (0.8)	0
Hb drop > 5 g/dL within 7 days of initial treatment N (%)	0	0	0
Hb nadir within 28 days of treatment initiation, g/dL, mean [SD]	12.72 [1.4]	12.54 [1.4]	12.55 [1.3]
Time to Hb nadir, days, median (range)	3 (3-7)	3 (3-13)	3 (3-13)
<b>Day 0</b>			
Number of patients	64	128	133
Hb on day 0, g/dL, mean [SD]	13.62 [1.5]	13.46 [1.6]	13.54 [1.5]
<b>Day 3</b>			
Number of patients	63	124	129
Hb on day 3, g/dL, mean [SD]	13.08 [1.5]	13.03 [1.5]	13.07 [1.4]
Change in Hb between Day 0 and Day 3, g/dL, mean [SD]	-0.54 [0.96]	-0.41 [0.99]	-0.50 [1.04]
Absolute drop between Day 0 and Day 3 > 5 g/dL, N (%)	0	0	0
Fractional change in Hb between Day 0 and Day 3, %, mean [SD]	-3.79 [7.04]	-2.84 [7.36]	-3.40 [7.73]
Fractional drop between Day 0 and Day 3 > 25%, N (%)	0	0	2 (1.5)
<b>Day 7</b>			
Number of patients	64	117	124
Hb on day 7, g/dL, mean [SD]	13.65 [1.6]	13.67 [1.6]	13.52 [1.4]
Change in Hb between Day 0 and Day 7, g/dL, mean [SD]	0.04 [1.27]	0.20 [1.24]	-0.01 [1.07]
Absolute drop between Day 0 and Day 7 > 5 g/dL, N (%)	0	0	0
Fractional change in Hb between Day 0 and Day 7, %, mean [SD]	0.69 [9.81]	1.90 [9.56]	0.28 [8.12]
Fractional drop between Day 0 and Day 7 > 25%, N (%)	0	0	0
<b>Day 13</b>			
Number of patients	62	119	129

	<b>Placebo (n=64)</b>	<b>PQ 7 (n=128)</b>	<b>PQ 14 (n=133)</b>
Hb on day 13, g/dL, mean [SD]	13.69 [1.4]	13.41 [1.4]	13.59 [1.3]
Change in Hb between Day 0 and Day 13, g/dL, mean [SD]	0.07 [1.19]	-0.07 [1.20]	0.03 [1.04]
Absolute drop between Day 0 and Day 13 > 5 g/dL, N (%)	0	0	0
Fractional change in Hb between Day 0 and Day 13, %, mean [SD]	1.09 [9.0]	0.0 [9.37]	0.70 [8.10]
Fractional drop between Day 0 and Day 13 > 25%, N (%)	0	0	0
<b>Day 28</b>			
Number of patients	51	112	112
Hb on day 28, g/dL, mean [SD]	14.17 [1.4]	13.93 [1.3]	13.87 [1.4]
<b>Day 42</b>			
Number of patients	50	110	100
Hb on day 42, g/dL, mean [SD]	14.24 [1.61]	14.12 [1.45]	14.28 [1.34]

### 3.4 Discussion

The results from Vietnam together with other sites of this placebo-controlled trial confirm the high antirelapse efficacy of primaquine at a total dose of 7 mg/kg in a short course regimen of 7 days. Furthermore, the results from Vietnam shows the high incidence of recurrence vivax parasitaemia if primaquine treatment is not prescribed. This supervised course of 7-day high dose primaquine prevented 971 recurrences per 1,000 patients, and the 14-day regimen prevented 962 recurrences per 1,000 patients. The 7-day regimen was proved to be non-inferior in preventing vivax recurrences to the standard 14-day regimen. Though the haemolytic risks of two primaquine regimens were not different, the frequencies of gastrointestinal intolerance were higher in 7-day short course compared with 14-day course despite both being administered with food per protocol.



In combination with schizonticidal drugs (chloroquine or the artemisinin derivative), primaquine has been recommended as a hypnozoitocidal agent for more than 60 years in order to achieve the radical cure of *P. vivax* malaria [52]. The major safety concern in primaquine usage is the haemolytic toxicity in G6PD deficient patients [158]. The WHO recommends the screening for G6PD status before the use of primaquine. However, in most malaria endemic countries, there is currently no available and reliable G6PD testing, primaquine is rarely used due to the concerns over safety. In Vietnam, the primaquine radical treatment is recommended from national guideline in preventing vivax relapse. However, this administration is usually withheld by most of the healthcare workers because of the lack of G6PD rapid diagnostic test as well as the fear of primaquine-induced haemolysis. The antirelapse efficacy of primaquine is related to the total dosage, while the hemolytic toxicity as well as gastrointestinal disturbance depends on the daily dose administration [159]. While the widely acceptable duration of primaquine regimen is 14-day, the compliance to such a long course of treatment in most malaria endemic regions may be relatively low [153, 160]. In this study, we found not only the non-inferiority anti-relapse efficacy but also the acceptable safety profile between the 7-day primaquine regimen and the 14-day regimen in G6PD normal patients.

The findings from this trial have firstly demonstrated the true relapse pattern of vivax malaria in different endemic regions and the high antirelapse efficacy of primaquine at the total dose of 7 mg/kg compared with no primaquine. If no hypnozoitocidal drugs were prescribed, the cumulative risk of recurrence at one year of follow-up could reach above 50% in Vietnam and other sites. However, at the two Indonesia sites, the incidence risks of symptomatic vivax recurrence were 39.33% (95% CI 30.31–49.92)

and 28.59% (19.39–40.90) after treatment with dihydroartemisinin-piperaquine alone [157]. And when primaquine therapy was applied, this incidence risk of recurrent vivax parasitaemia decreased dramatically to around 15%. This present study showed that the supervised course of 7-day high dose primaquine prevented 971 recurrences per 1,000 patients, and the 14-day regimen prevented 962 recurrences per 1,000 patients. Primaquine is generally recommended as a 14-day course for radical cure of *P. vivax* malaria, the optimum dose and duration is still debated. The long duration of this regimen could reduce the adherence of patients, hence influence the efficacy of the therapy. Previous studies have shown that adherence to a full course of 14 days is often poor [68, 161, 162]. In one study from Thailand, the migrant populations along the borders, poor compliance to primaquine has been well documented [68, 161, 162]. A study in Afghanistan showed that 11/139 (8%) non-adherent subjects contributed 36 recurrences compared to 6/170 (4%) adherent individuals who contributed 19 recurrences [160]. Under supervision, the short 7-day primaquine regimen was shown to be non-inferior compared with the standard 14-day regimen. This shorter regimen could help to enhance the compliance of the patients, hence improve the effectiveness of the therapy in field settings. Owing to the high frequent relapse rate in vivax malaria, there was a high demand to implement this short course of primaquine together with site-specific approaches in order to reach the elimination goal.

Individual dosing of primaquine is limited by abdominal discomfort described as nausea, vomiting, abdominal pain and diarrhea. This is often severe at doses over 1 mg base/kg. In general primaquine is well tolerated at individual doses  $\leq 0.5$  mg base/kg if given together with food [76]. In this trial, patients in the 7-day primaquine group received twice the daily dose in the standard 14-day high dose regimen (0.5 mg/kg/day)

and 4 times higher than that in the 14-day low dose regimen (0.25 mg/kg/day) recommended by national control malaria program [45]. The results from multisite trial illustrated the low rate of vomiting study drug within one hour of administration as well as the comparable percentage of patients reported gastrointestinal discomfort between 1 mg/kg per day and 0.5 mg/kg per day groups. Regards to Vietnam site, there was also similarity in the occurrence of vomiting, however patients with higher daily dose (1 mg/kg per day) were more likely to report abdominal disturbance. These symptoms generally occurred early, were mild in severity and resolved within 72 hours. The gastrointestinal tolerability can be improved by taking primaquine with food [75]. Although shortening the duration of treatment might potentially improve adherence, a higher daily dose increases the risk of haemolysis and gastrointestinal disturbance. While these adverse events can be reduced by the reliable G6PD rapid diagnostic tests and food co-administration, the disadvantages of short course high dose primaquine could affect the tolerability and hence the adherence when implementing in malaria fields.

All of the patients enrolled into the study were screened for G6PD deficiency using the FST, a qualitative assay that identifies individuals with less than 30% enzyme activity [163]. The FST has a sensitivity and specificity above 95% for the diagnosis of G6PD deficiency in adults with less than 30% enzymatic activity [110]. While this approach can exclude G6PDd hemizygous males and homozygous females, heterozygous females with intermediate G6PD activity (30 – 70%) tested as G6PD “normal” and were enrolled. With the usage of primaquine, the haemolytic risk in both 7-day and 14-day course was similar to the placebo. The occurrence of one haemolysis anaemia serious adverse event in the 7-day because of erroneous G6PD diagnosis. The

distribution of hemoglobin changes during the treatment course was identical among three groups. In combination with the reliable G6PD diagnostic tests and the early detection of any signs of haemolysis, the severity of haemolytic anaemia could be prevented when 7-day short course of primaquine is implemented. Tafenoquine, a slowly eliminated 8-aminoquinoline, recently licensed in the USA and Australia, achieves radical cure with only a single dose when combined with standard chloroquine therapy [164]. Although Tafenoquine can enhance the effectiveness of the radical cure, the prolonged exposure results in lengthy haemolysis, even in G6PDd heterozygous females. For this concern, the use of Tafenoquine is only restricted in subjects with > 70% G6PD enzyme activity. The determination of G6PD enzyme activity at 70% threshold demands a highly complex quantitative test. This will add cost and infrastructure to the overall malaria endemic communities. Furthermore, tafenoquine is now only licensed to be prescribed in subjects older than 16 years, while primaquine is safely indicated in patients with G6PD activity more than 30% and age more than 6 months. Furthermore, thank to the rapid elimination and daily administration, the risk of haemolysis of primaquine can be identified and managed timely whenever the hemolytic signs appear.

The findings of this study should of course be considered in context of strengths and weaknesses. The study was based on a large, well characterized cohort who was recruited from four vivax malaria endemic countries. Thus, the estimates presented here are likely to reflect the situation in other vivax endemic regions. To our knowledge, this is the first study that helps to identify the true relapse pattern and the efficacy of primaquine with long follow-up time. The main limitation is the inadequate of sample size. The study did not reach the target sample size although a large number of patients

had been screened (325 participants per 771 screened) mainly due to study early termination. However, the non-inferiority of efficacy between PQ7 and PQ14 regimen, the primary objective, was demonstrated to be within the margin of 0.07. Multi-site analysis as well as single site sub-analysis also showed the comparable of antirelapse efficacy between the two primaquine therapies [157]. In PQ14 group, all of the recurrence episodes (n = 17) were symptomatic, which was different from PQ7 group. Until now, the clear underlying cause could not identified.

In summary, in vivax malaria endemic regions where the incidence risk of recurrent parasitaemia is substantial high (~50% at one year follow-up), primaquine therapy with dose of 7 mg/kg showed the superiority of antirelapse efficacy compared with single schizontocidal treatment. The efficacy of a shorter 7-day regimen of primaquine was non-inferior to the standard 14-day regimen in normal G6PD patients and had an acceptable safety and tolerability profile. The shorter the course of treatment, the more facile the adherence of patients; therefore improve the effectiveness of radical cure in vivax-endemic countries. The available of qualitative G6PD rapid diagnostic test at the 30% threshold could ameliorate the wide-spread coverage this short primaquine regimen.

## Chapter 4

### **Safety and efficacy of Tafenoquine as a radical cure for adult subjects with *Plasmodium vivax* when co-administered with chloroquine**

#### **4.1 Introduction**

*Plasmodium vivax* and *Plasmodium falciparum* are the primary causes of malaria in humans. Both parasite species expose approximately 2.5 billion people to the risk of infection [165]. Although *P. falciparum* causes many deaths, especially in sub-Saharan Africa, *P. vivax* is predominant in South East Asia, South America, the world's most densely populated regions [5]. Vivax malaria infection coupled with its now proven association with severe and fatal outcomes impose its importance in reducing the incidence [165]. The most apparent characteristic of *P. vivax* is the ability to cause relapses weeks to months following a primary infection by activation of dormant liver-stage parasites, known as hypnozoites [166]. The hypnozoites cause multiple clinical attacks from a single bite of a *P. vivax*-infected mosquito. Relapses caused approximately 50% of blood-stage infections and more than 60% of clinical episodes in the first 3 months [73]. Relapse may be the predominant origin of most *P. vivax* clinical attacks throughout the endemic world [73]. The elimination of *P. vivax* infection requires the combination treatment of blood schizonticide to cure the primary blood infection and hypnozoiticide to kill the hypnozoite in the liver [167]. Primaquine, an 8-aminoquinoline, was first licensed for use in the 1950s by the Food and Drug Administration (FDA), United States, for treatment of vivax hypnozoites [168]. Without administration of primaquine in adequate doses, complete cure of patients with *P. vivax* is difficult, and patients often have relapses of clinical cases. Primaquine

treatment has to be continued for 14 days, which often leads to poor compliance. A search for a replacement drug for primaquine in its curative role has been ongoing for the last few decades. Tafenoquine, like primaquine, has the effect on clearing the dormant *P. vivax* parasites in infected patients to prevent a relapse [169]. Tafenoquine is shown promising results for both prevention and radical cure in individual trials [92, 93, 170-173]. With a longer half-life, tafenoquine has the advantage with only single dose of 300 mg to increase the adherence in preventing the relapses compared with 14-day course of primaquine. The efficacy of tafenoquine has been shown to reduce the risk of recurrent malaria at 6 months approximately 70% compared with placebo in patients with vivax infection in two comparative trials [88, 164]. DETECTIVE study identified two tafenoquine doses (300 mg and 600 mg) that, when co-administered with chloroquine, had significantly improved relapse-free efficacy at 6 months compared with chloroquine alone [88].

Like other 8-aminoquinoline antimalarial drugs, tafenoquine can cause hemolytic anemia in glucose-6-phosphate dehydrogenase (G6PD) deficient patients. This Mendelian X-linked gene is one of the most highly polymorphic of the human genome with at least 400 mutations described [97]. G6PD deficiency is widespread across malaria endemic regions [96]. An overall allele frequency of deficiency of 8.0%, varied from 1 to 30%, was predicted across all malaria endemic countries [95]. The hemolytic risk with tafenoquine relative to that with primaquine has been characterized among healthy volunteers with normal G6PD enzyme activity and volunteers who were heterozygous for G6PD deficiency [174]. Our knowledge of the hemolytic risk of tafenoquine when administering in vivax patients is not fully understood. The aim of this research was thus to assess the hemolytic risk of tafenoquine, further to evaluate

the efficacy of tafenoquine in preventing relapse of *P. vivax* malaria in comparison with primaquine.

## **4.2 Methods**

### **4.2.1 Trial design**

This was a phase 3, prospective, double-blind, double-dummy, randomized, controlled trial that was conducted at seven hospitals or clinics in Peru, Brazil, Colombia, Vietnam and Thailand between April 30, 2015, and November 4, 2016. The result from this multi-site trial has been published in The New England Journal of Medicine [89]. In this multi-site trial, my role was site clinical investigator. My major activities were to enroll the eligible subjects, manage and assess the subjects as per protocol. It was conducted in accordance with the International Conference on harmonization Good Clinical Practice guidelines, the tenets of the Declaration of Helsinki (2013), and relevant regulatory requirements. The ethics committee or institutional review board at each participating site provided ethical approval. All participants or their parents or guardians provided written informed consent. Assent was obtained from participants who were younger than 18 years of age.

### **4.2.2 Participants**

Eligible patients were at least 16 years of age and had microscopically confirmed *P. vivax* (> 100 and < 100,000 parasites per microliter) and a corrected QT interval according to Fridericia's formulation (QTcF) of less than 450 msec. Eligible female participants were not lactating, had a negative pregnancy test, and agreed to comply with approved contraception. The participants must have the normal G6PD activity. The G6PD activity was determined on the basis of a quantitative spectrophotometric



phenotype assay (Trinity Biotech). Before starting the trial, each study site established a median value for normal G6PD activity among 36 healthy male volunteers who otherwise were not involved in the trial. Female patients were required to have a G6PD enzyme level that was  $\geq 40\%$  of the site-specific normal value and male patients were required to have G6PD enzyme level that was  $\geq 70\%$  of the site-specific normal value. Patients were required to have a haemoglobin level of at least 7 g per deciliter, while the female patients with moderate G6PD enzyme activity were required to have a haemoglobin level of at least 8g per deciliter. Exclusion criteria were mixed plasmodium species or severe malaria, severe vomiting, an alanine aminotransferase level more than 2 times the upper limit of the normal range, clinically significant concurrent illness, use of an antimalarial drug within the previous 30 days or use of an investigational drug within the previous 30 days or within five half-lives (whichever was longer), use of concomitant drugs that could affect trial results, previous participant in or treatment within the trial, and a history of allergies or contraindications to tafenoquine, primaquine, or chloroquine.

#### **4.2.3 Randomization and treatments**

The study medication were chloroquine phosphate (300-mg tablets [West Ward Pharmaceuticals], tafenoquine (150-mg film-coated tablets [GlaxoSmithKline]), and primaquine phosphate (15-mg over encapsulated tablets [Sanofi]) (dosages are given for the free-base form). All treatments were administered orally with food. All patients received 600 mg of open-label chloroquine on days 1 and 2 and 300 mg on day 3.

Subjects were assigned to study treatment in accordance with the randomization schedule generated prior to the start of the study. Each subject scheduled to receive

medication received a treatment allocation number when randomized. The randomization number indicated which therapy the subject will receive, the treatment allocation ratio was 2:1 (TQ/CQ: PQ/CQ). Once a randomization number was allocated to a subject, it could not be re-assigned to any other subject. Patients were randomly assigned, in a 2:1 ratio, to receive a single 300-mg dose of tafenoquine while hospitalized on day 1 or 2 or 15 mg of primaquine once daily for 14 days starting on day 1 or 2. To ensure that the patients and investigators were unaware of the trial-group assignments, primaquine-matched placebo was administered to patients in the tafenoquine group and tafenoquine-match placebo was administered to patients in the primaquine group on the scheduled treatment days.

**Table 4.1:** Investigational drug schedule

Treatment arm	Day 1	Day 2	Day 3	Days 4 – 15
Tafenoquine	2 x CQ 300mg	2 x CQ 300mg + 2 x TQ 150mg + 1 x PQ placebo	1 x CQ 300mg + 1 x PQ placebo	1 x PQ placebo
Primaquine	2 x CQ 300mg	2 x CQ 300mg + 2 x TQ placebo + 1 x PQ 15mg	1 x CQ 300mg + 1 x PQ 15mg	1 x PQ 15mg

The study medication administration was directly observed during the hospitalization time and the period of outpatient visits by the study staffs or healthcare workers. If the subject vomited within 1 hour following dosing, a repeat dose was given. If a subject sequentially vomited two doses of study medication, he or she was considered intolerant to study medication. These subjects were withdrawn from study medication. Treatment

was discontinued when a patient had a protocol-defined decrease in the haemoglobin level ( $> 3.0$  g per deciliter or  $\geq 30\%$  from baseline or to have level of  $< 6.0$  g per deciliter), clinically significant changes in the liver chemical profile, a prolongation of the QT interval (corrected with the use of Fridericia's formulation) to more than 500 msec, or a grade 4 adverse event. Only in the case of an emergency or in the event of a serious medical condition, the investigators may unblind a subject's treatment assignment if the individual study treatment is required for the appropriate clinical management.

#### **4.2.4 Procedures**

Trial participants were hospitalized on days 1 to 3, and the trial visits took place on days 5, 8, 11, and 15 during the treatment period and on days 22, 29, 60, 90, 120, 150, and 180 during the follow-up period. Baseline demographic data and a medical history were obtained at screening, and a physical examination was performed. Thick and thin blood smears were prepared for identification of parasites, and parasites were enumerated with the use of established methods [39]. Blood samples for parasitological assessment were obtained at screening and twice daily (every 6 to 12 hours) from day 1 until day 3 or until parasite clearance was confirmed; smears were examined on all subsequent follow-up visits and after recurrence of parasitaemia or withdrawal from the trial. G6PD genotyping was performed at a central laboratory for participants with a hemoglobin decline of 3 g or more per deciliter, with the use of standard methods. Safety was assessed at all visits, at the time of recurrence of parasitaemia, and at withdrawal from the trial. Twelve-lead electrocardiography was performed at screening; 12 hours after the first dose of tafenoquine, primaquine, or placebo; and on

day 29. Analyses of hematologic and clinical chemistry values and urinalysis were performed at screening, on day 3, at follow-up visits until day 120, and at the time of recurrence of parasitaemia or withdrawal from the trial. Methaemoglobin levels were determined with the use of pulse oximetry at screening; on days 2, 3, 5, 8, 11, 15, 22, 29, 60, and 120; and at the time of recurrence of parasitaemia or withdrawal from the trial. G6PD phenotype was determined by means of quantitative spectrophotometric analysis (Trinity Biotech). G6PD genotype was determined for all female patients and for male patients who had a protocol-defined decrease in the hemoglobin level during the treatment period.

#### **4.2.5 Trial populations and outcomes**

The safety population included all patients who underwent randomization and received as least one dose of a trial medication in a blinded manner. The intention-to-treat population was a subgroup of patients from the safety population who had microscopically confirmed *P. vivax* parasitaemia. The per-protocol population was a subgroup of patients from the intention-to-treat population who had no major protocol violations.

In this trial, the primary safety outcome was a protocol-defined decrease in the hemoglobin level ( $> 3.0$  g per deciliter or  $\geq 30\%$  from baseline or to a level of  $< 6.0$  g per deciliter). Secondary safety outcomes were the occurrence and severity of adverse events and the occurrence of abnormal clinical laboratory test results, electrocardiograms, vital signs. Secondary efficacy outcomes were freedom from recurrence of *P. vivax* parasitaemia at 4 months and 6 months and other outcomes consistent with those in the other trial and were evaluated in the per-protocol and

intention-to-treat populations. Freedom from the recurrence was defined as initial clearance of parasitaemia (parasite numbers decreased to below the limit of detection in a thick blood smear and remained undetectable in a second smear collected 6 to 12 hours later), without the patient presenting with *P. vivax* asexual stage parasites at any point in the trial, plus a negative *P. vivax* smear within the acceptable time window for the 6-month assessment.

#### **4.2.6 Statistical analysis**

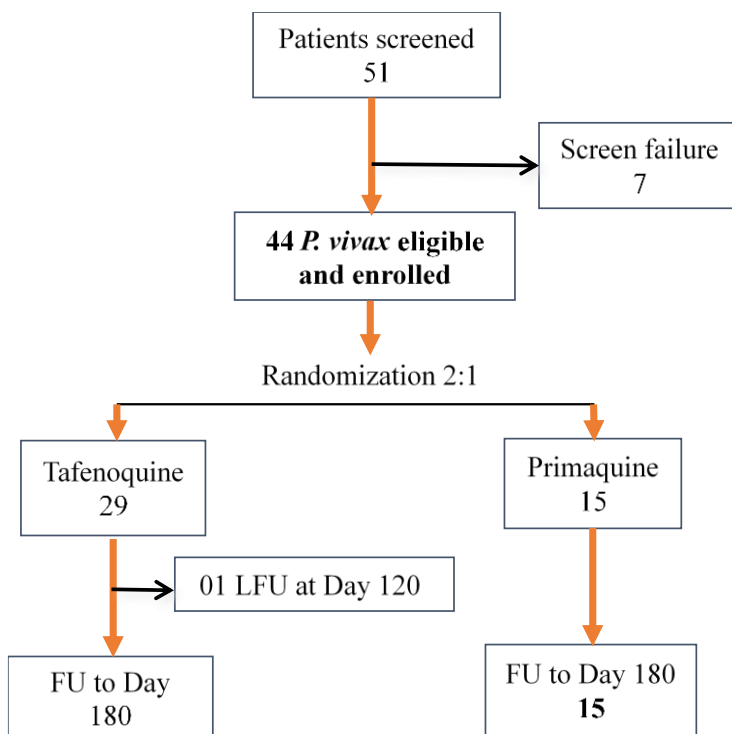
A sample size of 300 patients – 250 male and female patients with normal G6PD enzyme activity and 50 female patients with moderate G6PD enzyme deficiency (> 40 to < 70% of the site-specific normal value) – was planned in this trial. The sample size was determined on the basis of a regulatory requirement to obtain an appropriate overall safety database for tafenoquine (300 mg) in patients with *P. vivax* malaria who had normal or moderately deficient G6PD activity. Analyses were performed with the use of R software, version. All safety data from this trial were summarized with the use of descriptive statistics. For the primary safety outcome, the percentage of patients who had a protocol-defined decrease in the hemoglobin level in each treatment group was reported with 95% confidence intervals, and the absolute difference in this percentage between the treatment groups was reported with 95% confidence intervals.

In this trial, the percentages of patients who were free from recurrence at 4 months and 6 months and the times to clearance of parasites, gametocytes, and fever were estimated with the use of the Kaplan-Meier method.

In the patient-level meta-analysis, non-inferiority of tafenoquine to primaquine with respect to freedom from recurrence at months was assessed in the per-protocol populations. The non-inferiority margins were derived from the study with the use of a 75% preserved effect of primaquine. The non-inferiority margin was a hazard ratio for recurrence of 1.21, or if the assumption of proportional hazards was not met, and odds ratio was to be calculated. The odds ratio comparing chloroquine to primaquine in the phase 2b DETECTIVE trial, with adjustment for region, was 8.66 (95% confidence interval [CI], 2.80 to 26.85). The non-inferiority margin of 1.45 used in this trial was derived from taking the 75% preserved effect of the lower 95% confidence bound of 2.80.

### **4.3 Results**

From October 2015 to May 2016, globally, 251 patients were recruited in this GATHER trial, of whom 74.5% were from South America. Of these 251 patients, 166 were assigned to receive chloroquine plus a single 300-mg dose of tafenoquine while 85 others received chloroquine plus 15 mg of primaquine for 14 days. In Vietnam site, we screened 51 patients positive with *Plasmodium vivax* malaria. Of whom, 44 subjects fulfilled the inclusion and exclusion criteria and were finally enrolled into the study.



**Figure 4.1:** Study population from Vietnam site.

The participants were randomized into either tafenoquine arm (n = 29) or primaquine arm (n = 15). During 6 months of follow-up, 43 (98%) of the subjects completed the study. Only one subject in tafenoquine arm could not continue the study from Day 120 because of his own work in the hometown (Figure 4.1).

The baseline demographic and clinical characteristics of the participants stratified by treatment groups are shown in Table 4.2. Thirty three (7%) of 44 subjects were male. The mean ( $\pm$  SD) age of our participants was  $32 \pm 11$  years. We found that 77% (n = 34) of the patients also had gametocyte in the blood at the time of enrolment. The level of G6PD activity (mean  $\pm$  SD) was  $8.4 \pm 1.9$  IU/gHb, with no significant difference between tafenoquine and primaquine groups. The mean ( $\pm$ SD) haemoglobin concentration at the baseline was  $133 \pm 17.3$  g/L in the primaquine group and  $131 \pm 16.3$  g/L in the tafenoquine group. Demographic and baseline characteristics were

similar between the two arms, except for the temperature of participants at admission time ( $p = 0.03$ ), which did not have effect on the outcome (Table 4.2). During the treatment course of 14 days tafenoquine/placebo or primaquine, all the participants visited the clinics daily to take the investigational drugs.

**Table 4.2:** Baseline demographic and clinical characteristics.

Characteristics	Primaquine N = 15	Tafenoquine N = 29	Total N = 44
Ethnicity (n, %)			
Kinh	7 (46.7%)	11 (37.9%)	18 (40.9%)
Stieng	8 (53.3%)	18 (62.1%)	26 (59.1%)
Sex (n, %)			
Male	11 (73.3%)	22 (75.9%)	33 (75.0%)
Mean age (SD), year	34.9 (12.2)	30.3 (10.2)	31.9 (11.0)
Mean weight (SD), kg	58.8 (7.3)	56.2 (8.4)	57.1 (8.0)
Mean temperature (SD), °C	38.7 (1)	37.9 (0.9)	38.2 (1.0)
Asexual (median, IQR), parasite/uL	10,129 [3,858;16,050]	6,652 [3,120;11,250]	7,532 [3,045;12,904]
Positive gametocyte (n, %)	13 (86.7%)	21 (72.4%)	34 (77.3%)
Mean G6PD activity (SD), IU/gHb	8.09 (2.1)	8.6 (1.7)	8.42 (1.9)
Mean hemoglobin level (SD), g/L	133 (17.3)	131 (16.3)	132 (16.4)

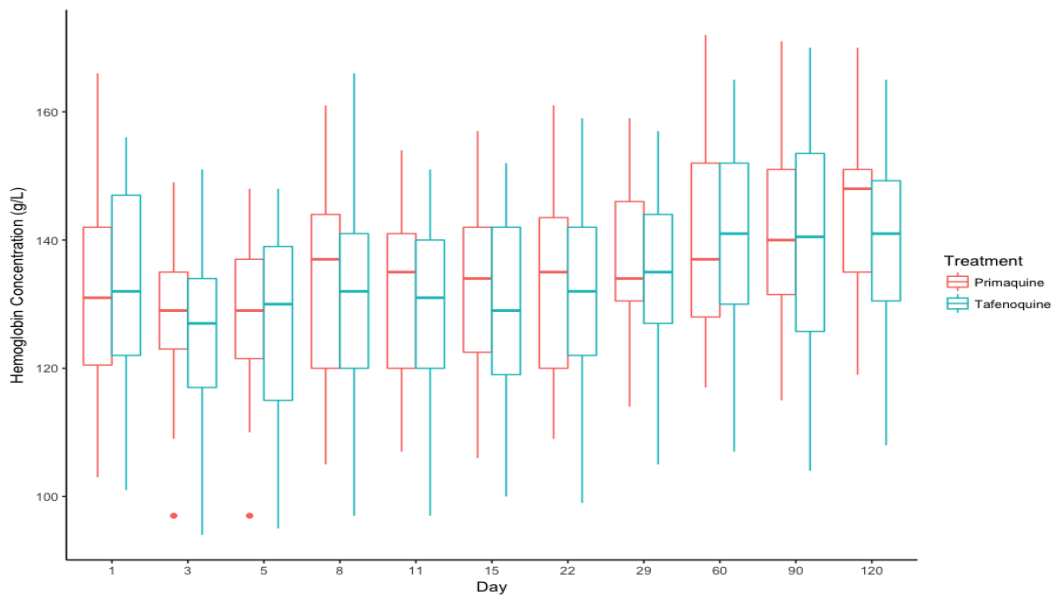
#### 4.3.1 Hematologic safety

The primary outcome was the occurrence of clinically relevant hemolysis in all subjects. The protocol-defined hemoglobin decrease criteria were hemoglobin decrease  $\geq 30\%$  or  $> 30$  g/L from baseline or an overall drop in hemoglobin level below 60 g/L.

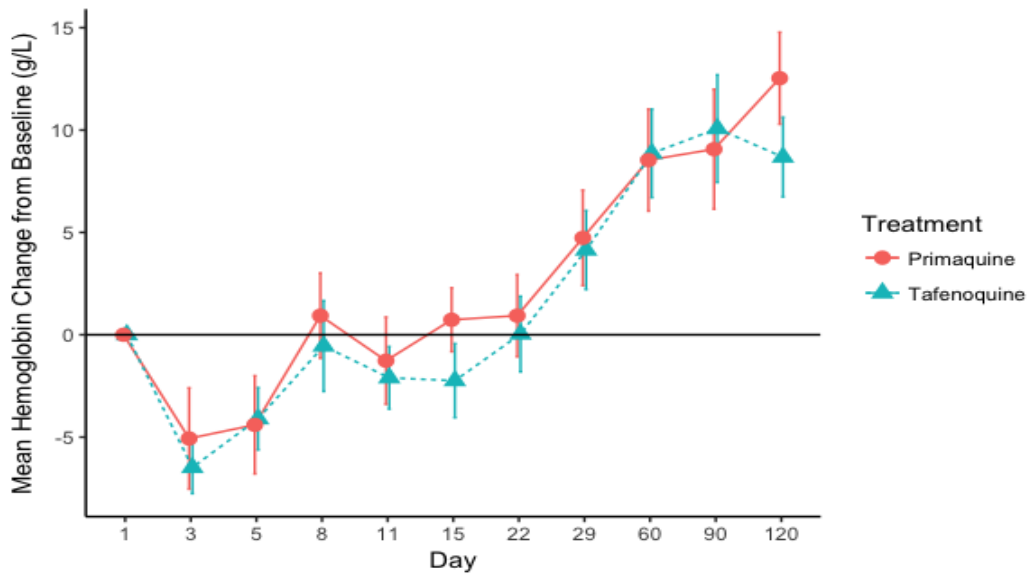
From Vietnam site, no primary-outcome event was reported in both single dose of



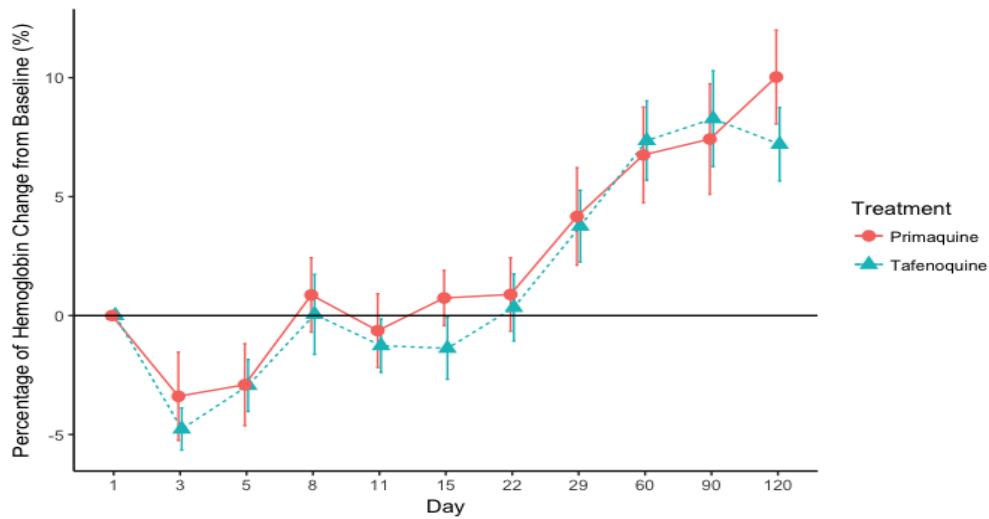
tafenoquine and 14-day course of primaquine. While in multi-site trial, the occurrence of decrease in the haemoglobin level were 2.4% (95% CI: 0.9 to 6.0) in the tafenoquine group and 1.2% (95% CI: 0.2 to 6.4) in the primaquine group. An absolute difference between two groups was estimated as 1.2% (95% CI: -4.2 to 5.0) [89]. All of these events occurred in male patients who had normal G6PD activity and did not require any clinical intervention. In general, patients who received tafenoquine or primaquine therapy shared similar haemoglobin profiles. The range and time course of mean changes in hemoglobin level were similar between treatment arms. After starting the treatment with either tafenoquine or primaquine, the haemoglobin level had a mild decline and decreased to the lowest point on Day 3. In the tafenoquine group, the lowest mean ( $\pm$  SD) level was  $124.6 \pm 14.8$  g/L, and  $127.5 \pm 14.1$  g/dL in the primaquine group. Then, these levels fluctuated within the first month post treatment period. All of the cases with haemoglobin decline, the level recovered spontaneously by day 29 to the baseline value (Figures 4.2 – 4.4).



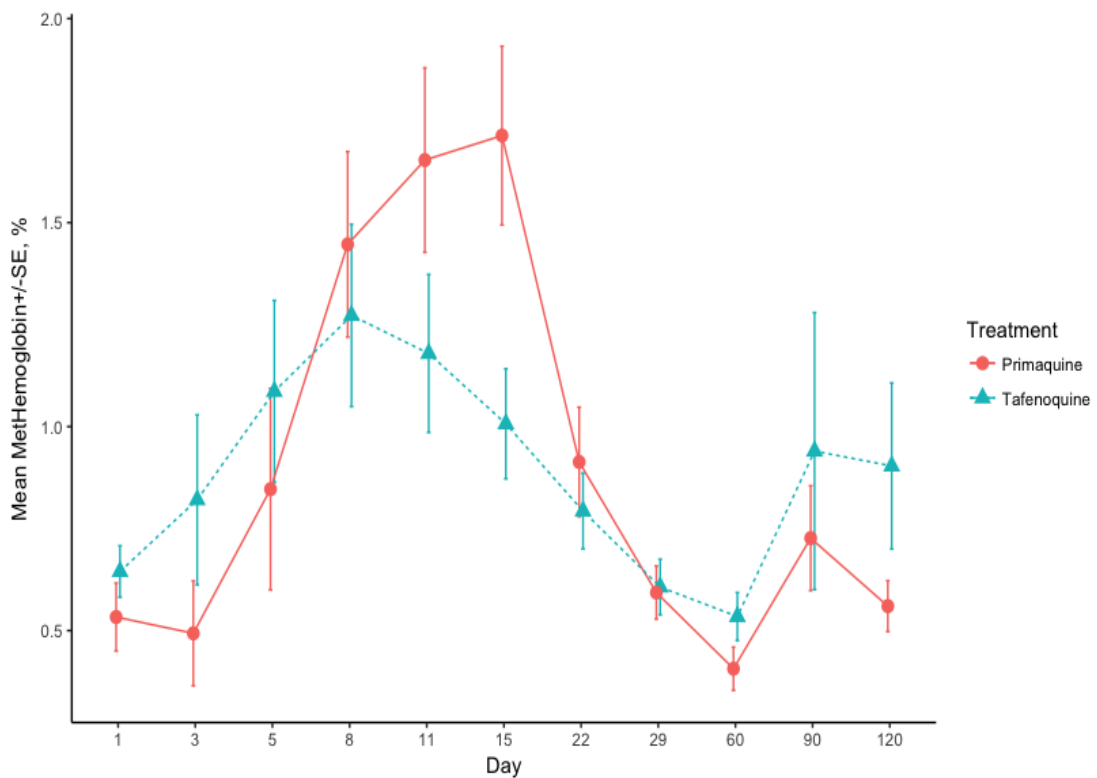
**Figure 4.2:** Distribution of haemoglobin value in *P. vivax* malaria patients treated with CQ plus either single-dose TQ 300 mg or PQ 15 mg for 14 days.



**Figure 4.3:** Mean change in haemoglobin from baseline in *P. vivax* malaria patients treated with CQ plus either single-dose TQ 300 mg or PQ 15mg for 14 days.



**Figure 4.4:** Percentage change in haemoglobin from baseline in *P. vivax* malaria patients treated with CQ plus either single-dose TQ 300 mg or PQ 15 mg for 14 days. The mean level ( $\pm$ SD) of methaemoglobin (MetHb) was 0.53% ( $\pm$  0.32) in the primaquine group and 0.64% ( $\pm$  0.43) in the tafenoquine group. These values were not significantly different. After the treatment with either tafenoquine or primaquine, these values began to increase, with the greater extent in the primaquine group. Then, it reached the highest point on Day 8 in the tafenoquine group and Day 15 in the primaquine group (Figure 4.5). However, this increase of MetHb level following the treatment did not cause any clinical symptoms.



**Figure 4.5:** Mean change in absolute methaemoglobin in *P. vivax* malaria patients treated with CQ plus either single-dose TQ or PQ for 14 days.

In the multi-site trial, two serious adverse events (pyrexia and pneumonia) that belonged to the tafenoquine group were reported. However, neither of these events was associated with a trial medication. From the Vietnam site, no death or serious adverse events occurred in both tafenoquine and primaquine treatment groups during the trial. No patient discontinued the study prematurely due to a safety event. The overall incidence and profile of adverse event seems comparable between the two treatment groups. The percentage of patients with adverse events was similar in the tafenoquine group (86.2% [25 of 29 patients]) and in the primaquine group (73.3% [11 of 15 patients]). Most adverse events were mild to moderate in severity (Table 4.3). Although, the percentage of grade 2 adverse events in the tafenoquine group (27.6%)

were higher compared with those in the primaquine group (13.3%), most of the events were not related to the treatment medication. Laboratory investigations showed a transient increase in creatin phosphokinase and alanine aminotransferase with the severity of grade 1 or 2 in both treatment groups. One patient in the primaquine group was recorded a grade 3 elevation of total bilibrubin concentration. This asymptomatic event occurred during the first acute vivax infection and resolved within 5 days without any intervention. The electrocardiographic evaluation showed no prolongation in the QTc interval.

**Table 4.3:** Frequency of all adverse events of any cause and their severity occurring throughout the study period (day 1 to day 180) in *P. vivax* malaria patients treated with CQ plus either single-dose TQ 300 mg or PQ 15 mg for 14 days.

Event – n (%) patients	Grade*	Tafenoquine (N=29)	Primaquine (N=15)
		<b>Any adverse event</b>	Any
	Grade 1	17 (58.6)	8 (53.3)
	Grade 2	8 (27.6)	2 (13.3)
	Grade 3	0	1 (6.7)
<b>Upper respiratory tract infection</b>	Any	2 (6.9)	3 (20)
	Grade 1	2 (6.9)	3 (20)
	Grade 2	0	0
<b>Urinary tract infection</b>	Any	1 (3.4)	2 (13.3)
	Grade 1	1 (3.4)	2 (13.3)
	Grade 2	0	0
<b>Hookworm infection</b>	Any	7 (24.1)	1 (6.7)
	Grade 1	5 (17.2)	1 (6.7)
	Grade 2	2 (6.9)	0

<b><i>P. falciparum</i> infection</b>	Any	2 (6.9)	0
	Grade 1	1 (3.4)	0
	Grade 2	1 (3.4)	0
<b>Skin infection</b>	Any	1 (3.4)	1 (6.7)
	Grade 1	1 (3.4)	0
	Grade 2	0	1 (6.7)
<b>Gastrointestinal infection</b>	Any	1 (3.4)	0
	Grade 1	1 (3.4)	0
<b>Vomiting</b>	Any	1 (3.4)	0
	Grade 1	1 (3.4)	0
<b>Diarrhea</b>	Any	1 (3.4)	0
	Grade 1	1 (3.4)	0
<b>Dizziness</b>	Any	3 (10.3)	0
	Grade 1	2 (6.9)	0
	Grade 2	1 (3.4)	0
<b>Pruritus</b>	Any	0	1 (6.7)
	Grade 1	0	0
	Grade 2	0	1 (6.7)
<b>Blood CPK increased</b>	Any	3 (10.3)	1 (6.7)
	Grade 1	1 (3.4)	1 (6.7)
	Grade 2	2 (6.9)	0
<b>ALT increased</b>	Any	1 (3.4)	0
	Grade 1	0	0
	Grade 2	1 (3.4)	0
<b>Blood bilirubin increased</b>	Any	0	1 (6.7)
	Grade 3	0	1 (6.7)
<b>ECG QT prolongation</b>	Any	0	0
	Grade 1	0	0
	Grade 3	0	0
	Grade 4	0	0
<b>WBC count increased</b>	Any	1 (3.4)	0
	Grade 1	1 (3.4)	0
<b>Ulna fracture</b>	Any	1 (3.4)	0

	Grade 2	1 (3.4)	0
<b>Acute food poison</b>	Any	0	1 (6.7)
	Grade 1	0	1 (6.7)

\*Common Terminology Criteria for Adverse Events v4.0

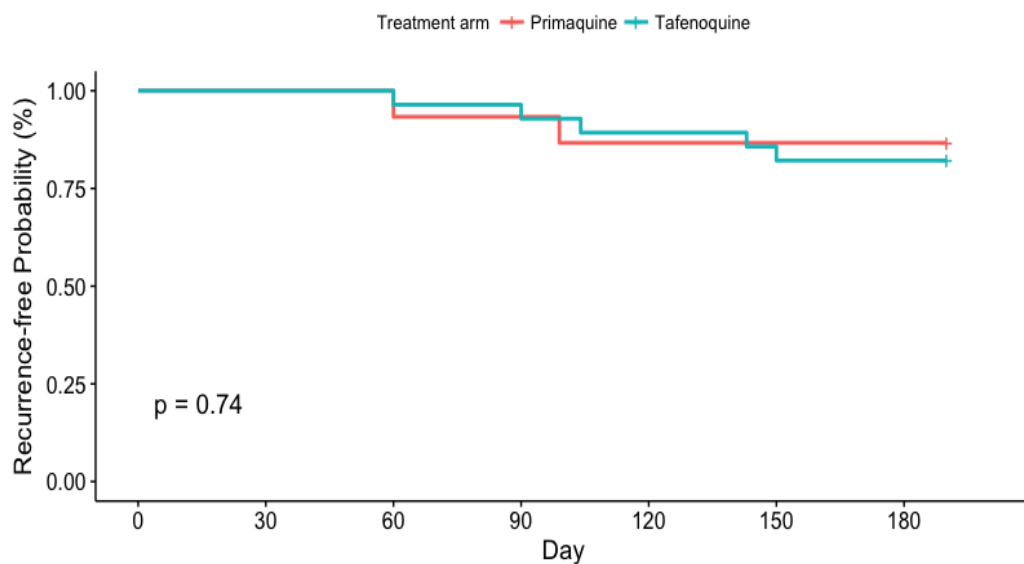
### 4.3.2 Efficacy

Relapse-free efficacy six months post-dosing is the secondary endpoint of this trial. In order to compare the clinical and parasitological response between tafenoquine and primaquine, we assessed the noninferiority of tafenoquine to primaquine in preventing vivax relapse after 6 months of follow-up. The proportion of patients with vivax recurrence per treatment arm was calculated; an odds ratio and 95% confidence interval were estimated. Kaplan-Meier analysis estimated the proportional hazards ratio between the tafenoquine and the primaquine groups.

**Table 4.4:** Logistic regression analysis of recurrence-free efficacy at 6 months in *P. vivax* malaria patients treated with CQ plus either single-dose TQ 300 mg or PQ 15 mg for 14 days.

<b>Outcome</b>	<b>Tafenoquine (N=29)</b>	<b>Primaquine (N=15)</b>
Recurrence-free at month 6, n (%)	23 (79.3)	13 (86.7)
Recurrence, n (%)	5 (17.2)	2 (13.3)
Censored, n (%)	1 (3.5)	0
No assessment available	1 (3.5)	0
Odds ratio of recurrence vs. primaquine (95% CI)	1.41 (0.24, 8.34)	
Kaplan-Meier recurrence-free efficacy rate at month 6, % (95% CI)	82.1 (69.1, 97.6)	86.7 (71.1, 1.0)
Hazard ratio for risk of recurrence vs. primaquine (95% CI)	1.32 (0.26, 6.78)	

The percentage of patients who were free from recurrence over six months of observation was 79.3% (5 of 29 patients) in the tafenoquine group and 86.7% (13 of 15 patients) in the primaquine group. The calculated odds ratio of risk of recurrence comparing tafenoquine to primaquine was 1.41 (95% CI: 0.24 to 8.34). Kaplan-Meier analysis estimated of 6-month recurrence-free efficacy were 82.1% (95% CI 69.1 to 97.6) for tafenoquine and 86.7% (95% CI 71.1 to 1.0) for primaquine (Figure 4.6). The proportional hazards ratio at month 6 was 13.2 (95% CI: 0.26 to 6.78). From odds ratio and hazards ratio comparing tafenoquine and primaquine, tafenoquine non-inferiority to primaquine could not be demonstrated. By 4 months of follow-up, similar analysis could not prove the non-inferiority in efficacy between tafenoquine and primaquine (Table 4.5).



**Figure 4.6:** Kaplan-Meier analysis of 6-month recurrence-free efficacy for TQ versus PQ in *P. vivax* malaria patients treated with CQ plus either single-dose TQ 300 mg or PQ 15 mg for 14 days.



**Table 4.5:** Logistic regression analysis of recurrence-free efficacy at 4 months in *P. vivax* malaria patients treated with CQ plus either single-dose TQ 300 mg or PQ 15 mg for 14 days.

Outcome	Tafenoquine (N=29)	Primaquine (N=15)
Recurrence-free at month 4, n (%)	25 (86.2)	13 (86.7)
Recurrence, n (%)	3 (10.3)	2 (13.3)
Censored, n (%)	1 (3.5)	0
Odds ratio of recurrence vs. primaquine (95% CI)	1.37 (0.19, 9.52)	
Kaplan-Meier recurrence-free efficacy rate at month 4, % (95% CI)	84.6 (56.3, 95.4)	86.7 (71.1, 1.0)
Hazard ratio for risk of recurrence vs. primaquine (95% CI)	1.27 (0.29, 7.83)	

#### 4.4 Discussion

*Plasmodium vivax* malaria and its relapses caused by the activation of hypnozoites could contribute to substantial medical complications and death [175]. Primaquine is the only available drug that can kill the hypnozoite and has been used for over 60 years. In order to achieve the full efficacy, the 14-day course of primaquine is recommended [45]. This is one of the challenges in implementing the radical cure of primaquine in the malaria-endemic regions because most of the patients will not follow such a prolonged course of therapy when the acute symptoms have been subsided. When first developed in 1980s, tafenoquine possessed a long-acting pharmacokinetic property that could solve the potentially important problem of poor adherence by only a single dose course [176]. Nevertheless, like other 8-aminoquinoline, tafenoquine also cause acute haemolysis anemia in subjects with G6PD deficiency. In this trial, the hematologic

safety profile of the single-dose tafenoquine and 14-day primaquine groups was comparable. Both of the treatment groups caused the hemoglobin level among patients with normal G6PD slightly decline and then recover to baseline value within one month. From this multi-site trial, only one female who had moderate G6PD enzyme deficiency was recruited into the primaquine group. Tafenoquine has been known to cause haemolysis in G6PD deficient subjects [94]. In G6PD heterozygous individuals with intermediate activity, the decline in haemoglobin level was similar in the tafenoquine group (300 mg single dose) and in the primaquine group (15 mg/day x 14 days) [174]. Thus, the haemolytic effect of tafenoquine still requires further evaluation in patients with *P. vivax* malaria and have G6PD enzyme activity less than 70% of the site-specific normal value.

In Vietnam site, the recurrence free rate after 6 months of follow-up in the tafenoquine group (82.1%; 95% CI: 69.1 to 97.6) was lower than the rate in the DETECTIVE phase 2b trial (89.2%; 95% CI: 77 to 95) [88]. This number in the primaquine group (86.7%; 95% CI: 71.1 to 1.0) was, however, higher than in phase 2b (77.3%; 95% CI: 63 to 87) and in the multi-site analysis (75.1%; 95% CI: 64.2 to 83.2) [89]. The non-inferiority of tafenoquine to primaquine could not be demonstrated in our study. This could be due to the directly observed therapy has been applied to all subjects participated into the study during the study medication period. The direct supervision of drug administration helps to increase the adherence of the patient and, hence might improve the antirelapse efficacy. Furthermore, the relapse rate of vivax malaria varied across the geographical location, and the higher frequency in East Asia and Oceania than elsewhere was reported [10, 150]. Patients from these areas required higher doses of primaquine to achieve the maximum radical curative efficacy [45]. In phase 2b DETECTIVE trial, a

clear dose-response relationship was shown [88]; therefore a higher dose of tafenoquine should be further evaluated in this region.

Although, primaquine has shown its efficacy in preventing relapse, most patients with *P. vivax* malaria receive only a schizonticide owing to the fear of haemolysis and non-adherence [158]. From our study, the single-dose tafenoquine showed no safety concerns when given in *P. vivax* malaria patients with normal G6PD activity. There was a possibility that primaquine offered a slight efficacy advantage over tafenoquine in the study population with a high compliance to the 14-day course. Nonetheless, extensive resources should be deployed to maintain the adherence of primaquine therapy. Without such interventions, adherence rates as low as 24% have been reported in South East Asia, with a corresponding attenuation of efficacy [68, 161]. In contrast, there are operational advantages to a single-dose medication, such as tafenoquine. The convergence of enhanced G6PD testing technology, and the convenience of single-dose tafenoquine present an opportunity to further examine ways to achieve an effective *P. vivax* radical cure.

A major strength of our study is that all drugs were administered under direct observation from study staff during the treatment period. All of the participants received the full course of antirelapse therapy regardless they were randomized to either single-dose tafenoquine or 14-day primaquine group. This ensures the adherence of the subjects and can fully assess the efficacy of both treatments. However, the multi-site study only recruited one female subject with intermediate G6PD enzyme activity who was randomly assigned to the primaquine group despite an extension of the study recruitment window and study location to Krong Pa Medical Center, we could not

estimate the hemolytic safety profile of tafenoquine among patients with *P. vivax* malaria who have intermediate G6PD activity.

In summary, the haematologic safety profile of single-dose tafenoquine was comparable with the 14-day primaquine in *P. vivax* malaria patients with normal G6PD activity. Although, single 300 mg dose of tafenoquine could not show the noninferiority in efficacy to 14-day primaquine (0.5 mg/kg/day), the between-group difference may be within the range of acceptable clinical variability. With a prolonged half-life, this even shorter course of tafenoquine should significantly improve patient's compliance and thus effectiveness of relapse prevention.

## Chapter 5

### Prevalence and genotype of Glucose-6-phosphate dehydrogenase deficiency in malaria endemic regions in Vietnam

#### 5.1 Introduction

Almost three billion people are at risk for *Plasmodium vivax* (*P. vivax*) infection globally, and outside of Africa it is the predominant cause of malaria [147]. Although vivax malaria was once considered a benign infection, recent evidence suggests a considerable morbidity and mortality, associated mainly with anaemia due to recurrent episodes [48, 177, 178]. The propensity for *P. vivax* to relapse after prolonged periods of asymptomatic carriage undermines the patients and healthcare providers' perceptions of the importance of radical cure, and this in turn confounds malaria control and elimination efforts. Complete achievement of eradication of *P. vivax* will need the systematic treatment of the dormant liver forms (hypnozoites) of the parasite.

The only class of anti-malarial drugs currently available to eliminate the hypnozoites are the 8-aminoquinoline compounds and these cause haemolysis in glucose-6-phosphate dehydrogenase deficiency (G6PDd) individuals [151]. The degree of haemolysis depends on total dose of primaquine (PQ), underlying G6PD variant and age of the red blood cell (RBC) population (with lower G6PD activities in older cells) [76]. Primaquine-based radical cure in G6PD normal patients is usually administered over 14 days at a total dose of either 3.5 or 7 mg/kg bodyweight depending on the assumed susceptibility of dominant local parasite strains [179]. Although either PQ regimen is usually well tolerated in G6PD normal subjects, it is often not prescribed

due to fears of drug-induced haemolysis. Moreover, when the long treatment course of PQ is prescribed the long treatment course, which is normally 14 days, can be associated with poor adherence and thus effectiveness [180]. Tafenoquine (WR 238605), another 8-aminoquinoline, was discovered by scientists at the Walter Reed Army Institute of Research in 1978, and has been approved by the Food and Drug Administration in 2018 [181]. The half-life of Tafenoquine (TQ) is 14 days compared to 4-6 h for PQ, and this allows adequate cure with a single 300mg dose [88]. With the prolonged half-life, this is the alternative intervention for the treatment and relapse prevention of *P. vivax* malaria. A single dose regimen of TQ is likely to improve treatment adherence significantly, however may also raise the potential for sustained haemolysis in G6PDd patients and for this reason TQ is likely to be ready for use only after G6PD normal status has been confirmed.

Glucose-6-phosphate dehydrogenase deficiency (G6PDd) is an X-linked disorder of the red blood cells common in malaria endemic countries [100]. G6PD is an essential enzyme in the pentose phosphate pathway (PPP), the only pathway for human RBC to maintain the cells' redox potential by reducing  $\text{NADP}^+$  to NADPH [97]. Non-synonymous mutations in the gene can decrease enzyme activity or reduce the stability of the enzyme, resulting in different degrees of G6PD deficiency [102]. The loss of G6PD enzyme activity is associated with the reduced response of red blood cells when exposed to oxidative stress such as fava beans ingestion, certain medicines or infections [97]. The prevalence of this enzymopathy is different among regions and populations, commonly seen tropical and sub-tropical areas [101]. Since the gene is located on the X chromosome, males are hemizygous and are either classified as phenotypic G6PD

deficient or normal. Females, on the other hand, can be homozygous deficient (two gene copies with a deleterious mutation), homozygous normal with both gene copies expressing G6PD variants, or heterozygous (one gene copy encoding a normal G6PD variant and one gene copy encoding a deficient G6PD variant) [182]. As a consequence, phenotypic G6PD activities in heterozygous females can manifest in a wide range from deficiency to normal status. This could pose challenges to the accurate diagnosis in current tests to detect G6PD deficiency. More than 400 variants of G6PD have been described [96]. The two most common G6PD variants are the G6PD A- and G6PD Mediterranean variants. G6PD A- is common across the African continent and is categorised as a Class III deficiency, whereas G6PD Mediterranean is common among Italians, Arabs, and Jews, and is categorised as a Class II deficiency. In Southeast Asia, the most common variant in Myanmar and Thailand is G6PD Mahidol (Class III), whereas in Laos and Cambodia, the most common variant is G6PD Viangchan (Class II) [95].

The G6PD deficiency distribution strongly differs from one ethnic group to another, and geographical location. It was suggested that G6PD deficiency confers resistance to falciparum malaria since the highest frequencies were found among populations living in endemic areas [183]. In Viet Nam, different studies have been conducted to investigate the G6PD deficiency status in hemolytic subjects as well as mutations in G6PD gene in many regions [184-187]. The prevalence of G6PD deficiency varies among different regions and different ethnic populations, commonly from 8.7% to 14.0% [186]. These studies have found common causative mutations for G6PD deficiency in some provinces and ethnic populations such as Kinh and other populations

in Lam Dong, Kinh in Ho Chi Minh city, and S'tieng in Binh Phuoc. In 2013, two causative mutations of the G6PD deficiency in Kinh population have been determined including Viangchan (871G>A) and Canton (1376G>T). These two missense mutations have been screened and occurred with high frequencies in the G6PD deficient patients according to the study in Lam Dong province and Ho Chi Minh city. The other mutations G6PD Kaiping (1388G>A), G6PD Union (1360C>T) were also detected [184]. In these studies, the mutation G6PD Mahidol (487G>A) has not been detected [187]. In central and northern Vietnam, G6PD Viangchan was found to be dominant, followed by G6PD Canton [185]. In order to promote the radical treatment in *P. vivax* malaria elimination target, our objective of this study is to assess the prevalence of G6PD deficiency and to identify the most common variants causing G6PD deficiency in populations living in malaria endemic regions in Vietnam.

## **5.2 Methods**

### **5.2.1 Survey design and sample collection**

This was a cross sectional survey conducted in villages in Dak O commune, Binh Phuoc province and in Phuoc Ha commune, Ninh Thuan province in Vietnam [188]. The villages were selected based on parasite prevalence, enthusiasm of villagers to participate, and access. In 2015, in Dak O commune, the annual malaria incidence was 25 *P. falciparum* cases and 17 *P. vivax* cases in 1000 people. While, in Phuoc Thang commune, the values were 24 *P. falciparum* cases and seven *P. vivax* cases in 1000 people. The inhabitants of six villages were invited to participate in baseline and subsequent 3-monthly surveys up to 24 months, which included the collection of venous blood samples to investigate the asymptomatic *P. falciparum* malaria infections in the



regions. These blood samples were analyzed to detect submicroscopic malaria and G6PD testing. G6PD deficiency was determined by FST at recruitment. The G6PD study protocol was reviewed and approved by the Institute of Malariology, Parasitology and Entomology in Ho Chi Minh City (185/HDDD), the Institute of Malariology, Parasitology and Entomology in Quy Nhon, and the Oxford Tropical Research Ethics Committee (1015-13). The written informed consent was obtained from subjects aged more than 16 years and parental or guardian consent was obtained if subject was children less than 16 years.

### **5.2.2 Laboratory methods**

Samples collected during population surveys were analyzed for G6PD phenotype using the FST (R&D Diagnostic, Greece) following manufacturer's instruction [189]. The FST was performed as semi-quantitative test thus allowing intermediate results to be reported. Blood was collected onto filter paper and DNA extracted using DNA Blood mini kits (Qiagen, Germany). Genotyping was performed in method like this. Samples tested with FST and detected as G6PD deficient or G6PD intermediate were analyzed by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) for a panel of four mutations: Viangchan, Union, Canton, Kaiping.

**Table 5.1:** Primers sequences, restriction enzymes and annealing temperatures of PCR-RFLP protocol used.

Assay	Oligonucleotides	T <sub>ann</sub>	Restriction enzyme	Reference
Viangchan	5'-CCTGAGGGCTGCACATCT-3' 5'-GTCGTCCAGGTACCCTTTGGG-3'	64°C	<i>Hpy</i> 188III	-
Union	5'-ACGTGAAGCTCCCTGACGC-3' 5'-GTGAAAATACGCCAGGCCTTA-3'	58°C	<i>Hha</i> I	Huang <i>et al.</i> , 1996
Canton	5'-ACGTGAAGCTCCCTGACGC-3' 5'-GTGAAAATACGCCAGGCCTTA-3'	58°C	<i>Afl</i> II	Huang <i>et al.</i> , 1996
Kaiping	5'-ACGTGAAGCTCCCTGACGC-3' 5'-GTGCAGCAGTGGGGTGAACA-3'	58°C	<i>Nde</i> I	Huang <i>et al.</i> , 1996

### 5.2.3 Statistical analysis

The data was collected and built in Excel database. The variables included sample ID, gender, FST phenotype, genotype (when available), and village/province name.

### 5.3 Results

From December 2013 to January 2016, a total of 1915 blood samples were collected during several population surveillances in 6 villages in Binh Phuoc and Ninh Thuan provinces. Of which, 908 (47.4%) samples were from males. The G6PD deficiency status was identified by FST method at enrollment.

The overall prevalence of G6PD deficiency detected by fluorescent spot test (FST) in samples collected was 6.5% (125 of 1915). When stratified by gender, this prevalence

was 8.6% (78 of 908) in males and 4.7% (47 of 1007) in females. Additionally, 118 (6.2%) samples was detected as “intermediate”, in which 51 (5.6%) were in males and 67 (6.7%) in females (Table 5.2).

**Table 5.2:** Results of the fluorescent spot test (FST) by gender

FST results	Males (%)	Females (%)	Total (%)
Deficient	78 (8.6)	47 (4.7)	125 (6.5)
Intermediate	51 (5.6)	67 (6.7)	118 (6.2)
Normal	779 (85.9)	893 (88.6)	1672 (87.3)
Total	908 (47.4)	1007 (52.6)	1915 (100)

The prevalence of G6PD deficiency detected by either FST or inferred from genotype increased to 8.3% (158 of 1915). By provincial level, this study showed variable frequency among different regions in country. The prevalence of G6PD deficiency were 6.6% and 10.1% in Binh Phuoc and Ninh Thuan province, respectively. This difference was statistically significant ( $p < 0.05$ ). At village level, the prevalence of G6PD deficiency changed at even greater variability. They were 11.7% in Gia, 10% in Tra No, 9.2% in Tan Ha, 8.8% in Bu Bung and 4.3% in Bu Khon. These prevalence was significant different between village Bu Khon and other villages (Gia, Tra No, Tan Ha and Bu Bung). While in La A village, no G6PD deficient sample was recorded in La A (Table 5.3).

**Table 5.3:** The prevalence of G6PD deficiency by village level

Site	Province	Samples analyzed			G6PD deficiency
		Male	Female	Total	
Bu Bung	Binh Phuoc	243	256	499	44 (8.8)
Bu Khon	Binh Phuoc	262	246	508	22 (4.3)
Gia	Ninh Thuan	217	294	511	60 (11.7)
La A	Ninh Thuan	25	27	52	0 (0)
Tan Ha	Ninh Thuan	137	158	295	27 (9.2)
Tra No	Ninh Thuan	24	26	50	5 (10.0)
Total		908	1007	1915	158 (8.3)

The prevalence of G6PD variants was described in Table 5.4. Viangchan variant was found to be the most frequent variant among those analyzed, accounting for 96.4% of all mutations found. Union variant only had only 0.4% of the samples analyzed. Although, Union and Kaiping variants were investigated, no mutation was found.

**Table 5.4:** Distribution of the G6PD variants by gender and genotype

Gender	Genotype	G6PD variants					% carriers
		Total	Viangchan	Union	Canton	Kaiping	
Male	Hemizygote	52	50	2	-	-	5.7
Female	Homozygote	13	13	-	-	-	1.3
	Heterozygote	45	43	2	-	-	4.5

A dash indicates that a mutation was not found

#### 5.4 Discussion

The assessment of the prevalence of G6PD deficiency and identification of the mutation are essential to implement the effective 8-aminoquinoline agents in order to achieve the radical cure of *P. vivax* malaria. In this study, based on the determination of G6PD deficiency by FST criteria, we found that the prevalence of G6PD deficiency was 6.5%

in malaria endemic regions. Our finding demonstrated a high variability of G6PD deficiency prevalence among different regions. The prevalence ranged from 0% in La An village to 11.7% in Gia village. In 2009, a community based study in minority ethnic male in Central and Northern of Vietnam found that the prevalence of G6PD deficiency was 4.0% [185]. Another survey in Lam Dong province showed this prevalence was 2.3% [187]. This variability was also observed in Venezuela [190], in India [191] and in several malaria endemic countries [182].

Although four mutations were studied for G6PD genotype, Viangchan variant was observed at a highest prevalence (96.4%). From previous study, this mutation was known as the most common G6PD deficient variant in the Vietnamese population [184, 187, 192]. Not only in Vietnam, G6PD Viangchan is also considered a dominant variant in Southeast Asia as well [193-195]. Moreover, the hemolytic risk in subjects with G6PD-Viangchan and Mahidol mutation following 8-aminoquinolines treatment has been described [174, 196, 197]. The finding of high frequency of Viangchan mutation accompanied with its hemolytic risk in patients has a number of clinical implications. It is important to determine the G6PD status in patients before taking any radical therapy. Especially, in women with G6PD heterozygote genotype, G6PD testing in the field needs to accurately identify the intermediate G6PD phenotype.

Our findings present several limitations. Firstly, the study was based on population surveys however the analyzed samples came from within two malaria endemic regions. This sampling method could therefore not able to generalize the overall prevalence of G6PD deficiency. Secondly, the results from FST test could not adequately interpret the deficient status in case of heterozygous woman with intermediate G6PD activity.

Thirdly, with high molecular heterogeneity in the G6PD gene in Southeast Asia [95], the other mutation might be missed if only four variants were investigated.

In summary, in two malaria endemic provinces in Vietnam, the prevalence of G6PD deficiency was around 10%, with a high proportion of Viangchan variant. The substantial reliable G6PD testing is a consideration when applying the radical cure of *P. vivax* malaria.

## Chapter 6

### Discussion

#### 6.1 Short course of primaquine and the radical cure of vivax

Since 1990s, Vietnam has made tremendous progress in reducing the mortality and morbidity related to malaria [198]. Thanks to the large scale implementation of control intervention such as insecticide-treated bed nets, indoor residual spraying, and prompt, free-of-charge diagnosis and treatment, the number of cases fell from 130,000 in 2004 to 6,870 in 2018 with one recorded death [11]. The Vietnamese government is aiming to achieve national malaria elimination by 2030 in accordance with the GMS regional malaria elimination strategy [199]. In Vietnam, malaria still persists at low prevalence mainly in remote mountainous and forested areas, inhabited by ethnic minority populations. The ethnic minorities carry a large share of malaria burden related to their remote settlements and the need to make a living with forested activities [200-202]. Our study found that most malaria patients were males who mostly worked outdoors in borderline areas. This increases the risk to acquiring mosquito bites and infection with the *P. vivax* parasite. Forest malaria has a major role on malaria burden and constitute challenge in controlling malaria [200, 202]. As malaria control accelerates, the *P. vivax* proportion in co-endemic areas tends to rise compared to that of *P. falciparum* [151]. In Viet Nam, recently, this ratio *P. vivax* and *P. falciparum* has increased gradually. In 2018, from report of National Malaria Control Programme, the incidence of falciparum and vivax malaria was accounted for 61% and 36% of malaria cases, respectively [11]. The morbidity and mortality of *P. vivax* have been underestimated, with evidence of fatality and other comorbidities like malnutrition, HIV, or coinfection [10, 203]. *P.*

*vivax* infection in childhood or pregnancy is also associated maternal anemia, spontaneous abortion, low birthweight and health development [204, 205]. Possessing distinguishable characteristics, *P. vivax* has the property to develop the liver-stage, hypnozoites, which can cause multiple relapses. This special feature makes its control more difficult to achieve [9]. Geographically, there are high variability in the prevalence of *P. vivax* strains accompanied with its latency, likelihood of relapse and frequency of relapse [150]. Strains in found in Southeast Asia and Oceania have the shortest latency time to relapse [10]. For all of these reasons, the task of vivax relapse prevention needs to be addressed and prioritized in order to eliminate *P. vivax*.

People with *P. vivax* malaria require treatment with an antimalarial drug to treat the blood-stage infection, and a drug to treat the hypnozoite stage to complete the radical cure. Recommendations from WHO include the combination of the chloroquine or an artemisinin-based combination therapy for the blood-stage infection and 0.25 to 0.5 mg/kg/day primaquine for 14 days for the liver stage [45]. Radical cure can only be achieved when the antirelapse therapy is effective. However, the long duration of 14 days can affect the compliance of patients when the acute symptoms have subsided. The determinant of efficacy of primaquine treatment is the total dose rather than the length of the course [159], whereas the adverse reactions are mostly associated with the daily intake [151]. If a higher dose of primaquine could be administered safely over a shorter period of time, it may improve adherence rates. Our findings showed the efficacy of the short 7-day primaquine course was non-inferior to the standard 14-day course in vivax patients with normal G6PD. It is clear that primaquine treatment with total dose of 7 mg/kg demonstrated the substantial clinical benefit by preventing vivax relapse in malaria endemic regions. If no hypnozoitocidal agents were prescribed for



patients with *P. vivax* infection, the relapse rate could be as high as 50%. The major concern of 8-aminoquinoline drugs is the haemolytic risk, especially in G6PD deficiency. This study has shown the haemoglobin levels of G6PD-normal subjects did not differ significantly between two regimens of primaquine during drug administration time. Consequently, it might be safe to prescribe 7-day high dose primaquine for vivax patients. The most observed complaints in short course were related to gastrointestinal disturbance. These symptoms can be relieved if the drugs are co-administered with food. In malaria field settings, with available point of care G6PD testing, the short course of primaquine will offer affordable choice in facilitating the radical cure of *P. vivax* malaria.

The adherence to antimalarial drugs is a major care in malaria endemic areas with low level of health capacity and living standard. Our own experiences with falciparum malaria have shown that the compliance with “more than 3-day regimens” are usually poor. The adherence of short course 7-day of primaquine without direct supervision is still unknown. Further studies are needed to assess this point. Another issue that could be confronted is that the metabolism of primaquine is affected by the cytochrome P450 (CYP) 2D6 enzyme activity [58]. Human CYP2D6 is highly polymorphic, and decreased CYP2D6 enzyme activity has been linked to decreased primaquine antimalarial activity [87]. In any case of series relapses of vivax malaria despite fully administered dosage of primaquine, the testing of CYP2D6 activity should be considered.

## **6.2 Single dose of tafenoquine and the radical cure of vivax**

Tafenoquine, another 8-aminoquinoline derivative, was developed in the 1980s as an alternative to primaquine under the collaboration of Glaxo Smith Kline Pharmaceutical company and Medicines for Malaria Venture [77, 206]. The major advantage of tafenoquine is a long terminal elimination half-life which allows a single dose to be given. The efficacy of its antirelapse property against *P. vivax* hypnozoite has been described recently in large clinical trials [88, 164]. Overall, tafenoquine at a 300 mg single dose was highly efficacious in preventing *P. vivax* recurrences until 6 months after treatment. The relapse free percentage was 63% (95% CI: 55 to 69) for chloroquine plus tafenoquine compared to 28% (95% CI: 20 to 36) for chloroquine alone [88]. In July 2018, the US Food and Drug Administration has approved single dose of 300 mg tafenoquine can be used combined with chloroquine for a radical cure of *Plasmodium vivax* malaria in patient more than 16 years old [181].

The side effect profile of tafenoquine is similar to primaquine. Generally, tafenoquine was generally well tolerated. The most frequent complaint was gastrointestinal abnormalities (nausea and abdominal pain) [91]. The ophthalmic safety of single dose tafenoquine 300 mg has been assessed. Mild vortex keratopathy, corneal deposits was detected in 93% of subjects taking tafenoquine. This disturbance did not associate with any effect on visual acuity and was fully recovered in all subjects by one year [92]. Prolongation of the QT interval is one of the issues in patients treated with antimalarial drugs. Approximately 2% of patients in taking tafenoquine experienced QT prolongation [88] or no case even at a dose of 1,200 mg [207]. The major safety concern with tafenoquine, like primaquine, is drug-induced hemolysis in patients with G6PD enzyme deficiency. The effects of tafenoquine in G6PD deficient heterozygotes were

reported [174]. The level of hemolysis was dose-dependent with greater haemoglobin reductions in patients receiving 300 mg compared to those receiving 200 mg or 100 mg. The hematological safety profile was similar between heterozygote females who received either 300 mg single dose of tafenoquine or 15 mg primaquine for 14 days [174]. These females did not develop clinical symptoms and require any intervention. Primaquine with rapid elimination, can be easily stopped as soon as the first sign of hemolysis is suspected, whereas with longer half-life, tafenoquine can risk any subjects with G6PD deficiency after drug administration.

From this trial, regards to safety concern, 300 mg single-dose of tafenoquine was demonstrated to have the comparable characteristics with those of 14-day primaquine course. Although, the non-inferiority of efficacy between tafenoquine and primaquine could not be proved, the clinical significance of tafenoquine still existed. The convenient practical tafenoquine regimens potentially will improve drug effectiveness due to increased adherence, in contrast to primaquine where adherence is challenged by long period of daily dosing. The implementation of tafenoquine, a newly registered antihypnozoite drug, in the national malaria control programme has a number of considerations: 1) the cost effectiveness balance of tafenoquine treatment in vivax patients; 2) the available and reliable point of care quantitative G6PD diagnostic test. Previously, the cost-effectiveness analyses of tafenoquine have not been made yet. Until recently, the study in Serbia has been conducted to compare the cost-utility of tafenoquine versus primaquine for the radical cure of vivax malaria [208]. From this study, tafenoquine was shown to be cost-effective in comparison to primaquine for the radical cure. However, this study has not evaluated the cost of G6PD testing, a major part in implementing tafenoquine.

### **6.3 G6PD deficiency and radical cure of vivax malaria**

The presence of G6PD deficiency is a major operational obstacle to the implementation of antihypnozoite therapy with 8-aminoquinolines. G6PD deficiency is the most common enzymopathy worldwide affecting approximately 400 million people, the majority of whom are at risk for malaria [95]. As G6PD deficiency is known to be associated with protection against malaria [209], its prevalence in *P. vivax* patients is expected to be lower than in the general population. The prevalence and genetic variants of G6PD deficiency in Asia were found to be heterogeneous [96]. Therefore, the risk-benefit ratio balance should be considered when implementing the 8-aminoquinoline therapy. The overall prevalence of G6PD deficiency allele across malaria-endemic countries is estimated to be 8.0% [182]. The prevalence of G6PD deficiency in several malaria regions in Vietnam changes variously from 0% up to 11% [184, 185, 192]. The distribution of G6PD deficiency phenotypes varies dependent upon the ethnic groups. The malaria burden in Viet Nam is associated with forested areas, where most of the people are various ethnic minorities, e.g., S'tieng, J'rai, Tay, and M'nong. The prevalence of G6PD deficiency in these populations needs to be identified before implementing antirelapse interventions. The risk-benefit ratio balance should be considered what kind of the 8-aminoquinoline therapies could be carried out. In terms of the G6PD genetic mutations, there was a great change in variants and increased diversity across countries. Our study showed G6PD Viangchan were the most common variants in malaria regions, although more specific primers are needed to find out other variants. G6PD Mahidol and Viangchan variants were very common among communities in Myanmar, Thai populations [96]. The dependency of the risk and severity of hemolysis anemia on G6PD variants is highly variable. Mild, limited

hemolysis has been demonstrated in hemizygous males with the African G6PD – variant, the Mahidol variant, and the Viangchan variant [210] and in heterozygous females with the G6PD Mahidol or G6PD Viangchan variant [50, 197] .

#### **6.4 Conclusion**

Over the past 90 years 8-aminoquinolines have been prescribed mostly without testing for G6PD deficiency: initially, because this enzyme defect was unknown; and subsequently because it was largely disregarded. Nowadays there is increasing deployment of semi-quantitative tests, which identify male hemizygotes and female homozygotes, but fail to identify a substantial proportion of female heterozygotes. At the moment, for most malaria-endemic areas where testing is unavailable and primaquine is the only option, radical treatment requires a careful appraisal of risks and benefits, consideration of safer treatment regimens, and education of the patient to stop taking primaquine if adverse effects occur. This risk-benefit assessment requires knowledge of local relapse patterns and of G6PD variants and their severity, and of the availability of medical supervision and access to facilities for blood transfusion. In the future it is hoped that point of care quantitative tests will be developed and deployed in malaria field settings. This will provide accurate assessment of the phenotype of G6PD status, and thus of the potential severity of haemolysis, which is roughly inversely proportional to G6PD activity: the lower the activity, the more severe the haemolysis. These tests will be especially necessary for the safe use of tafenoquine. Currently, some promising point of care quantitative G6PD tests are under investigation [117, 211]. However, no rapid G6PD diagnostic test used in malaria field settings is officially recommended in Vietnam. During the awaiting time until G6PD tests are available and

affordable, both clinicians and patients should be counselled the signs of hemolysis (dark-coloured urine and jaundice) and signs and symptoms of anemia (pallor, fatigue, dyspnea with mild exertion). This careful clinical monitoring can be applied to patients who are prescribed primaquine treatment, but not for single dose tafenoquine, which requires more accurate value of G6PD enzyme activity.

Following the success in national malaria control programme, Viet Nam officially engaged into malaria elimination by 2030. Consequently, the programme has shifted from control to elimination strategies. *Plasmodium vivax* and *Plasmodium falciparum* malaria are the two main species in Viet Nam. Universal treatment that could cure patients with uncomplicated malaria due to either *P. vivax* or *P. falciparum* may offer significant benefits. More feasible options of antirelapse treatment have been demonstrated with comparable efficacy and acceptable safety profile. A strategy of combining an ACT to treat the asexual stages of all *Plasmodium* species and a hypnozoitocidal agent (short course of primaquine or single dose of tafenoquine) to prevent relapse of *P. vivax* could be a plausible approach.

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