Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite

Ivo Mueller, Mary R Galinski, J Kevin Baird, Jane M Carlton, Dhanpat K Kochar, Pedro L Alonso, Hernando A del Portillo

Plasmodium vivax **is geographically the most widely distributed cause of malaria in people, with up to 2·5 billion people at risk and an estimated 80 million to 300 million clinical cases every year—including severe disease and death. Despite this large burden of disease, *P vivax* is overlooked and left in the shadow of the enormous problem caused by *Plasmodium falciparum* in sub-Saharan Africa. The technological advances enabling the sequencing of the *P vivax* genome and a recent call for worldwide malaria eradication have together placed new emphasis on the importance of addressing *P vivax* as a major public health problem. However, because of this parasite's biology, it is especially difficult to interrupt the transmission of *P vivax*, and experts agree that the available methods for preventing and treating infections with *P vivax* are inadequate. It is thus imperative that the development of new methods and strategies become a priority. Advancing the development of such methods needs renewed emphasis on understanding the biology, pathogenesis, and epidemiology of *P vivax*. This Review critically examines what is known about *P vivax*, focusing on identifying the crucial gaps that create obstacles to the elimination of this parasite in human populations.**

Lancet Infect Dis 2009; 9: 555–66

Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea (I Mueller PhD); **Emory Vaccine Center, Yerkes National Primate Research Center and Department of Medicine, Division of Infectious Diseases, Emory University, Atlanta, GA, USA** (M R Galinski PhD); **Eijkman–Oxford Clinical Research Unit, Jakarta, Indonesia** (J K Baird PhD); **Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, Oxford University, Oxford, UK** (J K Baird); **Department of Medical Parasitology, New York University Langone Medical Center, New York, NY, USA** (J M Carlton PhD); **Kothari Medical and Research Institute and Consultant Malaria Research Projects SP Medical College and AG of Hospitals Bikaner, Rajasthan, India** (D K Kochar MD); **Barcelona Centre for International Health Research (CRESIB), Hospital Clinic/IDIBAPS, Universitat de Barcelona, Barcelona, Spain** (P L Alonso MD, H A del Portillo PhD); **and Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain** (H A del Portillo)

Correspondence to:
Ivo Mueller, PNG Institute of Medical Research, PO Box 60, Goroka, EHP 441, Papua New Guinea
ivomueller@fastmail.fm

Hernando A del Portillo, Barcelona Centre for International Health Research (CRESIB), Hospital Clinic/IDIBAPS, Universitat de Barcelona, Roselló 132, 5a planta, 08036, Barcelona, Spain
hernandoa.delportillo@cresib.cat

Life cycle

Plasmodium vivax has unique biological features that distinguish it as a species. The figure depicts the basic understanding of the plasmodium life cycle, with the inclusion of features that relate to *P vivax*. The most obvious features that distinguish *P vivax* from *Plasmodium falciparum* include the development of dormant hypnozoite forms in the liver that cause subsequent infections in the blood called relapses, the appearance—sometimes before onset of clinical symptoms—of round gametocytes in the peripheral blood (ie, not banana-shaped gametocytes like those produced by *P falciparum*), a predilection (or requirement) of merozoites for reticulocytes as host cells, circulation of all blood-stage developmental forms in the peripheral blood, the absence of electron-dense protrusions (known as knobs in *P falciparum*), and presence of numerous caveolae–vesicle complexes along the surface of infected red blood cells.

Sporozoites, injected through the bite of anopheline mosquitoes, migrate to the liver within minutes, invade hepatocytes, and develop into either an actively dividing schizont or a dormant hypnozoite.[1] The biological determinant that dictates the active or dormant development pathway is entirely unknown. The activation of hypnozoites weeks, months, or even years later causes the reactivation of a blood infection, clinical malaria, and the potential for transmission of the sexual gametocyte forms. The trigger for the activation of hypnozoites is not understood, though stress seems to play a part, and distinct patterns of relapse linked to local mosquito seasonal abundance suggest a darwinian genetic process at work to ensure transmission and the propagation of the species.

Another noteworthy biological feature of *P vivax* is its preferential, if not exclusive, targeting of reticulocytes as its host cells in the blood.[6] The biological basis of this apparent requirement, apart from specific parasite ligands that might allow entry into this host cell,[7] is not understood. This could also be a parasitic adaptation to limit hyperparasitaemias and associated virulence (since reticulocytes comprise only 1–2% of erythrocytes), or the reticulocyte might offer a special microenvironment to support the growth of this species. While *P vivax* develops in this host cell, it produces specific proteins to create large so-called cleft structures in the infected red blood cell membrane and many caveolae–vesicle complexes, which look like a profuse speckling in Giemsa-stained blood smears (known as Schüfnner's dots); these clefts and caveolae–vesicle complexes have also been seen in electron micrographs.[4] The function of these intriguing structures and their potential as targets of intervention remain largely unexplored, though research can now take advantage of proteomics and advanced microscopy technologies to unravel their components and biological roles.

Also of special note, *P vivax* becomes much more amoeboid than *P falciparum* while it grows in the reticulocyte, and it also greatly enlarges the host cell and increases its deformability.[3] All blood-stage forms of *P vivax* are found in the peripheral circulation, like most *Plasmodium* species, and the enhanced deformability might somehow help their safe passage through the spleen. If so, the parasite would not need adhesive knobs for sequestration in deep vascular beds as a way of avoiding passage through the spleen. Lack of sequestration and cytoadherence in *P vivax* however, needs to be critically re-evaluated, because formal hypotheses of adherence to the spleen[8] and the lungs[9] have been suggested. This scenario contrasts with the consistent absence of mature asexual forms in *P falciparum* infections (except in severe cases with very high parasitaemias), because the mature asexual blood-stage forms of *P falciparum* cytoadhere to a variety of receptors and become sequestered in the deep vasculature of various tissues and organs.[10] Red blood cells infected with mature stages of *P falciparum* are rigid and cannot pass safely through the spleen.

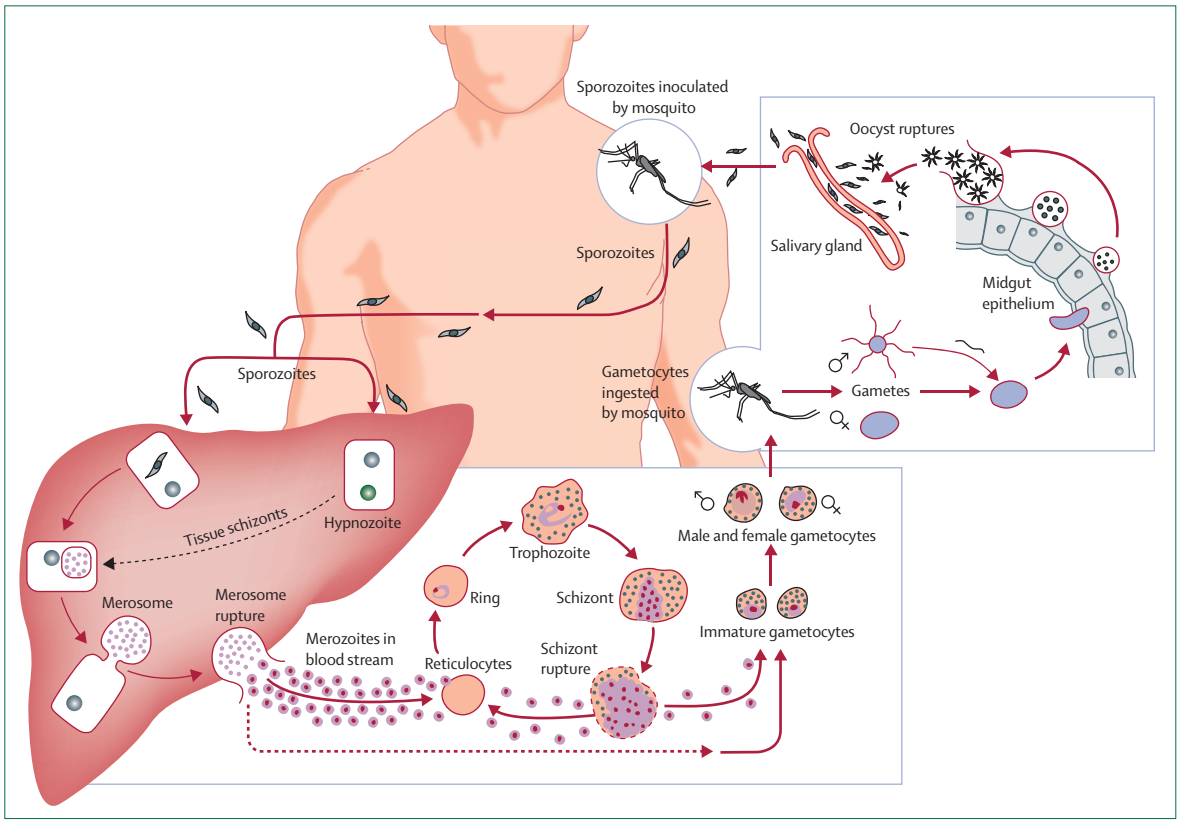

Figure: **Life cycle of the human malaria parasite *Plasmodium vivax***
Once infective sporozoites are inoculated into the skin by female anopheles mosquitoes, they reach the bloodstream and enter hepatocytes initiating the exoerythrocytic stage. Within the liver, *P vivax* can either differentiate into tissue schizonts, which after thousands of mitotic replications in individual hepatocytes release merozoites into the bloodstream, or differentiate into a dormant stage called a hypnozoite that, upon activation after months or years, causes clinical relapse.[1] The merosome featured here has, so far, only been described in malaria in rodents[2] but is predicted to be present in late stage liver infections with *P vivax* and other species. During the erythrocytic stages, *P vivax* merozoites predominantly, if not exclusively, invade reticulocytes,[2] and these cells become enlarged and more deformable.[3] This cyclical developmental process takes about 48 h. In addition, *P vivax* produces specific proteins to create caveola–vesicle complexes that appear as profuse speckling in Giemsa-stained blood smears, known as Schüfnner's dots.[4] Moreover, some *P vivax* parasites can differentiate into mature gametocytes before a clinical infection and illness develops, thus having the advantage of continued transmission to the insect vector before the appearance of clinical symptoms and subsequent treatment.[5] Circulating gametocytes are a rounded shape and on uptake in the blood meal of anopheles mosquitoes begin the sexual cycle, which includes release of the male and female gametes, fertilisation, and formation of a motile ookinete that crosses the midgut epithelium. Differentiation into a new replicative form known as the oocyst, release of sporozoites, migration, and invasion of the salivary glands ends this complex life cycle in which the parasite undergoes more than ten stages of cellular differentiation and invades at least four types of cells within two different hosts.

Like most *Plasmodium* species, the shape of *P vivax* gametocytes within infected red blood cells look circular in Giemsa-stained blood smears, by contrast with *P falciparum* and the chimpanzee parasite *Plasmodium reichenowi* with their characteristic elongated gametocytes.[11] Another characteristic that distinguishes vivax from falciparum malaria, and one of crucial clinical importance, is that *P vivax* gametocytes develop early in the infection and can be seen in the peripheral circulation before or at the beginning of clinical symptoms. Thus, an infected but asymptomatic, and therefore an as yet untreated individual, might serve as a reservoir to successfully transmit the infection to mosquitoes.[5] This transmission before clinical disease and treatment might in part explain why resistance to chloroquine has emerged in *P vivax* parasites more than 30 years after resistance in *P falciparum*. In essence, most gametocytes produced during infections (blood stage) would have experienced less drug pressure, thus reducing the chance for transmission of mutated drug-resistant parasites. However, this accepted view needs to be questioned, since a comprehensive and retrospective analysis of gametocytaemia and fever in patients with neurosyphilis experimentally infected with *P vivax*, showed that gametocyes were never seen in the blood before clinical symptoms began.[12]

Burden and epidemiology

The worldwide burden of malaria caused by *P vivax* has not been reliably estimated. Guerra and colleagues[13] estimated 2·6 billion people living at risk, and Hay[14] and Price[15] put the range of likely annual infections at 132 million to 391 million. The greatest proportion of the worldwide vivax malaria burden almost certainly lies in

south and southeast Asia (probably more than 80% of infections), with perhaps underappreciated numbers of infection in Africa.[13,16] The total burden of malaria in the Americas is low compared with that in Africa, but *P vivax* seems to account for more than 70% of malaria in the Americas and locally causes a substantial clinical and socioeconomic burden. Effort must be invested in mapping the range of and relative risk of infection with *P vivax* on the basis of cartographic modelling of assemblies of reliable cross-sectional data giving quantitative measures of probability, as has been done with *P falciparum*.[17] Special focus should be on establishing the burden in particular risk groups, such as young children or pregnant women. Given that over a large part of its geographical distribution *P vivax* affects all age groups, including the most economically active, it will be important to accurately estimate the socioeconomic costs of *P vivax* across different levels of endemicity. The research needed on the burden, epidemiology, clinical patterns, and host response is summarised in panel 1.

Panel 1: **Advances needed in burden, epidemiology, clinical patterns, and host response research**

- Estimate global burden of vivax malaria and define risk of vivax malaria within the biological spatial limits of transmission
- Define the distribution of inherited blood disorders linked to malaria and determine the association with protection against vivax malaria
- Characterise severe disease syndromes and establish case-definition for severe vivax malaria
- Look at the targets of naturally acquired immunity and find measurable signs of protection against *P vivax* infections and clinical disease
- Find the contribution of critical host immune responses and parasite characteristics with clinical pathology
- Find burden and clinical patterns associated with *P vivax* in pregnancy

The geographical distribution of endemic vivax malaria overlaps with that of endemic falciparum malaria, except in temperate zones, such as the Korean peninsula, where only vivax malaria occurs, and in much of sub-Saharan Africa where Duffy negativity seems to exclude endemic vivax malaria. *P falciparum* tends to be dominant in cross-sectional surveys of parasitaemia prevalence, and it almost uniformly has higher mean parasitaemia compared with *P vivax*. The higher prevalence of *P falciparum* is not entirely the rule however, with Latin American countries reporting upwards of 80% of infections due to *P vivax* and regions of India and China showing predominance of *P vivax*. Some studies suggest vivax malaria might reduce the severity of falciparum malaria, but others suggest that it might increase the risk of severe disease.[18–20] These trends, which are very poorly understood, might be the product of competition between the species, as well as the outcome of immunomodulatory effects resulting from unknown coinfection dynamics.

The seemingly uniform lower prevalence of vivax malaria in endemic areas might be partly because of the tendency of *P vivax* to achieve and maintain lower-density parasitaemia (perhaps because of its strong host-cell preference for reticulocytes), and the inverse relation between diagnostic sensitivity and parasitaemia count. Correct estimates for the burden of *P vivax* will thus require improved diagnostic methods. The detection of low level asymptomatic and mixed species infections with conventional light microscopy is limited. Present rapid diagnostic tests do not include a *P vivax* specific antigen, but diagnose *P vivax* through the detection of pan-plasmodium antigens (currently lactate dehydrogenase or aldolase) that have limited sensitivity at parasitaemias of fewer than 200 parasites per μL.[21] PCR-based assays, although more sensitive and high throughput, are difficult to use in routine diagnostic procedures. New, more sensitive, vivax-specific diagnostic assays are therefore needed. Immunological assays might be attractive since they can be used in worldwide population surveys to detect asymptomatic carriers and people recently exposed to *P vivax*.

The entomological inoculation rate (the product of the vector biting rate times the proportion of mosquitoes infected with sporozoite-stage malaria parasites) or the incidence density of new infections measures the force of infection with falciparum malaria. In the case of vivax malaria, such measures provide only a partial assessment of risk. The activation of hypnozoites to create acute vivax malaria represents a major contributing factor. In essence, the liver serves as a reservoir for new infections in the blood, new episodes of disease, and possible transmission. In any given community, no study reported so far has successfully measured the relative contributions of new mosquito inoculations of *P vivax* sporozoites versus activation of dormant liver-stage hypnozoites to the force of infection of the blood. Appropriate control strategies for *P vivax* depend on addressing this general lack of understanding. In *P vivax* endemic communities, it might be preferable to focus resources on resolving hypnozoite infections, even more so than preventing new mosquito infections, which, relatively speaking, might prove to be a minor contributor to the burden of blood infections.

The inherited lack of Duffy glycoprotein expression on the surface of red blood cells is known to prevent invasion by *P vivax* merozoites,[22] and the dominance of this trait among many Africans (especially in western Africa) explains the near absence of *P vivax* infections in these populations. Notably, this protective trait has recently been questioned because *P vivax* infections were detected in Duffy negative individuals in Africa and Brazil.[23,24] Despite these observations, the Duffy factor represents a rare example of a widely accepted hypothesis linking protection from vivax malaria to an

inherited trait. Although other inherited blood disorders might also have been selected for by *P vivax*, few have been investigated. This lack of study might be the product of both the perception of vivax malaria as being unlikely to exert a survival pressure on human beings, and the relative difficulty of exploring such associations without a *P vivax* in vitro culture system. Genetic traits linked to malaria represent clinically important associations, and such knowledge can support the rational development of control strategies, as well as the practical advantage of protecting clinical trials of interventions from confounding by genetic factors.

Measuring the risk of infection and developing rational control strategies will require a better understanding of the epidemiology of endemic vivax malaria. This will be especially true for estimating and monitoring the worldwide burden of disease, the interactions with falciparum malaria within and among hosts, the contribution of the liver reservoir of infection, the role of asymptomatic infections, and the epidemiology of inherited blood disorders linked to vivax malaria.

Clinical aspects

Almost any textbook of medicine will refer to vivax malaria as relatively benign compared with falciparum malaria and only rarely causing death by rupture of the spleen. Some texts will go on to explain that the non-aggressive course of vivax malaria might be attributed to its inability to adhere to vascular endothelium and its strict preference for invading reticulocytes. This widely accepted view of vivax malaria is now being challenged. Recent studies have pointed to a spectrum of severe disease that essentially resembles that of falciparum malaria: cerebral malaria (including generalised seizure and status epilepticus), hepatic dysfunction with severe jaundice, acute lung injury, acute respiratory distress syndrome, pulmonary oedema, shock, renal failure, splenic rupture, severe thrombocytopenia and haemorrhage, and severe anaemia.[25] In almost any clinic treating patients who present with such syndromes, the diagnosis would likely be severe falciparum malaria. Even where reliable microscopic diagnosis confirms only *P vivax*, *P falciparum* might still be presumed and its adherent properties might be cited as the basis of its apparent absence in peripheral blood smears. Nonetheless, case reports of severe falciparum-like malaria attributed to *P vivax* have been published, including several using PCR diagnostics.[26] Reliable exclusion of *P falciparum* by PCR diagnostics has alerted health-care workers and the malaria research community to the reality of severe and lethal disease caused by *P vivax*. However, exclusion of other infectious causes in such patients has thus far been limited and some caution is therefore warranted. Retrospective and prospective studies of hospital admissions in endemic areas have supported *P vivax* as a cause of falciparum-like syndromes of severe disease.[19,20,27] However, no longitudinal study of hospital admissions has yet offered either the reassurance of PCR diagnostics or the exclusion of other endemic infections such as viral hepatitis, dengue, leptospirosis, and bacterial sepsis among others, with the exception of the recent report from India of severe vivax malaria proven through PCR in 40 patients.[28]

The presentation of severe disease with PCR-confirmed *P vivax* monoinfection seems as clinically challenging as that of *P falciparum*. The risk of death with a diagnosis of severe vivax malaria can be nearly identical to that of patients diagnosed with severe falciparum malaria.[20,26,27] Severe vivax malaria has presented with various clinical syndromes as already detailed. In hospital-based studies, severe anaemia proved the most common sign in one study,[20] whereas respiratory distress[19,29] or liver dysfunction[27,28] were predominant in the other studies. The perception of vivax malaria as relatively benign compared with falciparum malaria might stem in part from early studies in predominantly temperate regions, which generally showed a less aggressive clinical course. Recent studies from Korea tend to support this view.[30] Severe and fatal vivax infections might be largely limited to either tropical strains or to chronic exposure.

If the clinical observations summarised here are supported by more detailed investigation of the pathophysiology of vivax malaria, the basis of severe disease caused by falciparum malaria might require re-evaluation.[31] The hallmark features of *P falciparum* malaria that have been deemed causative factors for severe disease in about 1% of cases—eg, sequestration in deep vasculature and promiscuous invasion of all red blood cells regardless of age—might have long been viewed through a cloudy lens classifying these diseases as malignant versus benign malaria. Moreover, these hallmark causative factors could be different in *P vivax*, since severe disease might be associated with multidrug resistance.[20,29] Clinical investigation of the pathophysiology of severe vivax malaria will thus directly affect the understanding of severe falciparum malaria, and vice versa.

Chemotherapy

First-line therapies for the radical cure of vivax malaria, chloroquine and primaquine, have not changed in 60 years and there is evidence of increased risk of failure due to parasite resistance.[32] Despite millions of doses used each year for treatment of a potentially life-threatening infection, we know little of the mechanisms of activity or toxicity of primaquine. A practical means of diagnosing therapeutic failure, much less understanding the mechanism of parasite resistance, has not been developed. The research needed in treatment, prevention, and control is summarised in panel 2.

High-grade resistance to chloroquine appears entrenched on the island of New Guinea and other

islands of eastern Indonesia.[32] The risk of therapeutic failure drops as you move north across western Indonesia and into southern Burma, suggesting that the distribution of *P vivax* is expanding towards Indochina and the Indian sub-continent. Recent surveys show that Thailand and India have little or no resistance, but it may be rising in South America.[33] Today, *P vivax* resistance to chloroquine is a substantial threat to health on the Malaysian peninsula, the Indonesian archipelago, and in Oceania. This problem will soon challenge health in the rest of south and southeast Asia (the region with the greatest vivax malaria burden), and in South America. However, the public health urgency to detect and measure the progression of chloroquine-resistant vivax malaria has been almost completely ignored.

A method for diagnosing resistance to chloroquine in vivax malaria was described over 10 years ago.[34] This procedure has drawbacks, but has not been improved or standardised. The method does not distinguish between the reappearance of blood stages, relapse from liver stages, or reinfection from biting mosquitoes. Parasites breaking through normally effective doses of drugs and remaining up to 28 days after treatment (ie, about three times the pharmacological half-life of chloroquine)[35] are classified as resistant regardless of origin. Apart from these ambiguities, reliable diagnosis requires measuring drug concentrations and thus imposes severe operational limitations. Moreover, blood concentrations of drugs for sensitivity or resistance classification derive from indirect observations, and the diagnostic criteria apply only to chloroquine therapy. Responding to the threat of chloroquine-resistant vivax malaria urgently requires validated and standardised tests, in vivo and in vitro, adaptable to resource-limited settings and applicable to multiple therapeutic options as well as development of reliable molecular markers for resistance.

Therapies that can be given after chloroquine failure have only been explored to a very limited extent. Evidence of good effectiveness against resistant strains has been described in a few cases for mefloquine and two combination therapies: atovaquone and proguanil, and dihydroartesunate and piperaquine.[36–38] Coping with the growing distribution of chloroquine-resistant vivax malaria needs further evaluation of these and other therapies. Treatment strategies that effectively deal with both falciparum and vivax malarias, including the dormant liver stages of vivax malaria, might require complex well-controlled trials. Despite the use of artemisinin-combination treatments (ACTs) in many countries to treat malaria on the basis of clinical diagnosis for the treatment of mixed *P falciparum* and *P vivax* infections, few ACTs have so far been thoroughly tested for their effectiveness against *P vivax*. Consequently, evidence-based treatment regimens for ACTs against *P vivax* are lacking.

Killing of the dormant liver stages is needed for the successful control of vivax malaria, and this need imposes serious clinical, technical, and operational challenges. Primaquine is the sole therapeutic option available for doing this, and almost nothing is known of its mechanism of action or haemolytic toxicity in patients with an inborn deficiency of glucose-6-phosphate dehydrogenase (G6PD)—a problem exacerbated by this deficiency being most common among populations most in need of therapy. A systematic survey of G6PD variants likely to impose serious risk with primaquine therapy has not been done, and a practical rapid diagnostic for this inherited deficiency has not been developed.

A 14-day dosing regimen is another serious drawback with primaquine. Most health authorities consider this problem crippling, and studies comparing efficacy with supervised versus unsupervised adherence support that view.[39] An exaggerated sense of toxicity and intolerability with primaquine in people considered good candidates to receive it (G6PD-normal and non-pregnant) has limited exploration of higher daily doses over shortened periods. A theoretically safe and tolerable 3-day regimen (60 mg twice-daily) has not been studied.[40] Lastly, because of the difficulty of studying the G6PD status of a fetus, primaquine cannot be given to pregnant women.

Primaquine might be the most effective drug available for the prevention of vivax malaria in travellers, but it is not licensed for this use in any country. Unlike all licensed antimalarial drugs, primaquine prevents the formation of liver stages during acute infection. This property prevents the substantial problem of relapse weeks or months after initial exposure to *P vivax*.[41] The economic burden imposed with obtaining a regulatory change in the labelled indication for a drug with lapsed patent protection has thus far proved insurmountable.

The US Army invented tafenoquine, a drug in the same 8-aminoquinoline family as primaquine and intended for essentially similar clinical use. The Medicines for Malaria Venture is now developing tafenoquine for elimination of hypnozoites caused by *P vivax* and *Plasmodium ovale* infections, which likewise develops

For more on the **Medicines for Malaria Venture** see http://www.mmv.org

***Panel 2:* Advances needed in treatment, prevention, and control**

- Develop blood schizonticidal therapies proven effective against multidrug-resistant falciparum and vivax malaria
- Develop tissue and liver schizonticidal therapies proven effective against vivax malaria
- Develop in vivo and in vitro tools for assessment of both blood and tissue schizonticidal drug resistance in vivax malaria
- Develop a practical and affordable point-of-care diagnostic for G6PD deficiency
- Expand vaccine development effort focusing upon coformulation strategies with vaccines against falciparum malaria
- Measure effect and cost effectiveness of insecticide-treated nets, intermittent preventive treatment, and indoor residual spraying as interventions against endemic vivax malaria in settings typical of the Americas, south Asia, and southeast Asia
- Develop a model of *P vivax*, host and vector relations and apply the model to assess the effect of different interventions on *P vivax* burden and transmission dynamics

Panel 3: The genome of *P vivax*

A major achievement by the vivax research community has been the recent completion and analysis of the first *P vivax* genome sequence.[48] Using a patient isolate from El Salvador adapted to growth in squirrel monkeys, sufficient DNA was procured for a high-coverage sequence to be generated. At about 27 Mb and containing about 5400 genes, the *P vivax* genome has a similar size and gene count as the *P falciparum* genome but differs in the repeat content and nucleotide bias of its 14 chromosomes, which contain isochore-like regions of high guanine and cytosine content interspersed with regions of high adenine and thymine bias mainly at the subtelomeric ends. No substantial differences in metabolic pathways nor membrane transporter proteins were discerned between the species, and indeed the identification of similar pathways in the *P vivax* apicoplast organelle means that these can potentially be targeted by antimalarial drugs being developed for *P falciparum*. A major finding is the presence of expanded *P vivax* gene families involved in red blood cell invasion and immune evasion—for example, classes of genes coding for proteins found on the merozoite surface, although the functional significance of this remains unclear. Through comparison of about 3300 homologous genes in *P vivax* and a closely related monkey species *Plasmodium knowlesi*,[49] regions of the genome that seem to be evolving faster than other regions, and that contain genes coding for exported proteins, were identified. Finally, analysis of the genome sequence revealed more than 150 microsatellites that are already being used by researchers to discern the genetic diversity and population structure of worldwide populations of the parasite,[50,51] an essential step toward development of control measures. Although the *P vivax* genome has provided a snapshot into the biology of the parasite, many questions remain, not least of which is how the switch to the dormant hypnozoite form occurs—this is sadly not illuminated through the genome sequence. However, transcriptome studies[52] coupled with sequencing of more *P vivax* isolates,[53] will ensure that scientists have the genomic resources to continue their studies into such intriguing phenotypes.

this dormant stage in the liver. However, a substantial gap in our understanding of how 8-aminoquinolines achieve this therapeutic effect complicates this effort. During the 1950s two clinical trials suggested primaquine failed to kill liver stages unless given at the same time with either quinine or chloroquine.[32] The importance of this finding was forgotten after the drug was distributed with chloroquine. The developers of tafenoquine must now contend with development of a companion drug that might substantially improve its therapeutic index, a key issue given its apparently shared ability to induce haemolysis in G6PD-deficient patients. The ability to control vivax malaria in the coming decades might well hinge upon the successful development of tafenoquine and other drugs that safely eliminate hypnozoites.

P vivax hypnozoites

Relapses represent a parasitaemia emerging from a dormant liver-stage hypnozoite, which happens despite effective blood schizonticidal therapy of the primary parasitaemia. The distinctions between reinfection, relapse, and reoccurrence (clinical signs of a previously subclinical parasitaemia) carry great importance in understanding the assessment of treatment outcomes. Although relapse has been known since the end of the 19th century, the dual role of the liver in supporting primary and secondary blood-stage infections was not considered until 1948, with the seminal observations by Short and Garnham[42] of liver-stage schizonts in rhesus monkeys infected with *Plasmodium cynomolgi*. Three decades later, Krotoski and colleagues[43] formally showed the presence of a hypnozoite in the liver of a rhesus monkey experimentally infected with *P cynomolgi*, and a chimpanzee infected with *P vivax*. Since then, little progress has been made to characterise this parasite stage. With funding in place research can resume in this area, immediately building upon the investigations of *P cynomolgi* liver-stage infections as done in the past and also capitalising on vivax infections in New World monkeys using specific parasite–host combinations that are known to produce hypnozoites. A challenge for rational experimentation on the hypnozoite forms will be to develop molecular and cellular markers capable of distinguishing the unique biological characteristics of the hypnozoite. From a practical perspective, such methods are also needed to distinguish clinical relapses from either reinfection or reoccurence. As noted, the hypnozoite represents an important reservoir for new infections, which totally escape the conventional means of malaria control—ie, use of insecticides, bednets, diagnostic methods, and almost all chemoprophylactic or chemotherapeutic interventions. Failure to effectively attack the hypnozoite would have the same consequences as failed control measures, and allow the life cycle of *P vivax* to continue in human populations.

It would be a breakthrough to understand these cryptic parasitic life forms. Initial molecular-based studies with the *msp1* and *csp* genes as genetic markers suggested that the parasites causing relapses were clonally identical to the parasites causing the first primary attack.[44,45] More recently, however, two independent groups using microsatellite markers identified from the genome sequence showed that relapse infections often result from activation of heterologous hypnozoites.[46,47] The genome sequence of *P vivax*[48] (panel 3) opens new avenues to advance our scarce knowledge of the biology of hypnozoites and relapses as well as our limited knowledge in other crucial aspects of the life cycle such as merozoite invasion and antigenic variation. The research needed to further understanding of *P vivax* biology is summarised in panel 4.

Merozoite invasion of reticulocytes and the infected red blood cell membrane

Merozoites have evolved sophisticated molecular machinery for the invasion of reticulocytes. Major advances in revealing the generalised cascade of events and species-specific receptor–ligand interactions that occur as merozoites enter red blood cells have come from investigations of *Plasmodium knowlesi*, *P falciparum*, and *P vivax*.[54] In the case of *P vivax*, merozoites invade reticulocytes and use the Duffy blood group antigens expressed on the surface of red blood

cells as a receptor. As noted, human populations lacking the Duffy factor are highly resistant to *P vivax* blood-stage infections.[22] Molecular approaches led to the discovery of reticulocyte binding proteins 1 and 2, which have been implicated in the cell-tropism for reticulocytes,[7] and to the discovery of the Duffy binding protein and a specific region of this protein that adheres to the Duffy antigen receptor for chemokines (DARC) on red blood cells.[55]

The presence of additional *pvrbp* genes and possible *rbp* pseudogenes in the *P vivax* genome[48] has raised the possibility of a more complex role for this protein family in invasion and, perhaps, evasion strategies. Also, two reports of Duffy-negative individuals with *P vivax* infections might counter the dogma that DARC is absolutely essential for *P vivax* merozoite invasion of red blood cells;[23,24] further molecular epidemiological studies should be done to assess the likelihood and frequency of such Duffy independent infections in the context of understanding population dynamics of *P vivax* transmission. As with *P falciparum*, *P vivax* entry into red blood cells is a complex process, which is anticipated to similarly involve other proteins located at the merozoite surface or within its organelles, and likely involves several alternative receptor mechanisms and invasion pathways.[48] In-depth studies on the association of different red blood cell phenotypes (and the genetic traits shaping them) with risk of *P vivax* infection and the development of high level parasitaemia might shed much needed additional light on *P vivax* capacities for invasion.

Antigenic variation, cytoadherence, and sequestration

Antigenic variation is the process by which parasitic microorganisms use built-in mechanisms to switch expression of variant proteins encoded by multigene families. The alternative expression of variant surface antigens can be used for evading host immune responses.[56] Presumptive variant surface antigen proteins in *P vivax* include those that are expressed by a subtelomeric multigene family called *vir*.[57] The *vir* genes were first identified from a *P vivax* isolate and implicated in immune evasion and chronic *P vivax* parasitaemia. However, analysis of the expressed *vir* gene repertoire of *P vivax*-harbouring reticulocytes recovered from naturally infected individuals showed neither allelic exclusion of *vir* gene expression nor clonal expression of Vir proteins in individual parasites.[58] Thus, whereas some features of the Vir proteins are consistent with a role of *vir* genes in immune evasion, available data do not support the existence of a genuine clonal antigenic variation process involving these proteins.[59] These data, however, were obtained from experiments involving a subset of proteins expressed by this multigene family. The completion of the first genome sequence from *P vivax* (Salvador I strain)[48] has allowed the identification of the complete *vir* gene repertoire in this strain and the genome has also revealed eight new gene families, most of which are located in subtelomeric regions. The role of *P vivax* subtelomeric multigene families in antigenic variation can now be revisited on a genome-wide scale.

P falciparum infected red blood cells containing trophozoites and schizonts sequester by adhering to the endothelial cells of postcapillary venules via variant surface antigens encoded by the *var* subtelomeric multigene family.[60] As a result, these forms rarely appear in the peripheral circulation. By contrast, *P vivax* parasitaemias typically have all blood-stage forms in the peripheral circulation. The cytoadhesive properties of *P falciparum*-infected red blood cells have been proposed to provide the selective advantage of preventing their clearance in the spleen.[10] The influence of the spleen in somehow controlling expression of variant proteins in malaria was clearly shown in *P knowlesi*, where expression of the schizont-infected cell agglutination antigens (homologs of the *P falciparum* variant antigens[61] known as erythrocyte membrane protein 1, expressed by *var genes*) on the surface of the infected red blood cells was lost upon passage in splenectomised rhesus monkeys and regained upon passage in intact rhesus monkeys.[62] Supporting observations showing a role for the spleen in variant antigen expression were also reported for *P falciparum*, *Plasmodium fragile*, and *Plasmodium chabaudi* infections.[63–66] Moreover, in patients that have been splenectomised and were infected naturally with *P falciparum*, tissue sequestration is impaired and mature stages are seen in the peripheral blood circulation.[67] These data agree with the accepted view that the spleen plays an important part in controlling the expression of variant proteins in malaria infections. How *P vivax*-harbouring reticulocytes move through the spleen, whether expression of *P vivax* variant multigene families are spleen-dependent, and whether *P vivax* cytoadheres, remain crucial research questions to be investigated.

***Panel 4:* Advances needed in parasite biology**

- Investigate key steps in hypnozoite formation, metabolism, and reactivation
- Find important processes in the invasion of reticulocytes with a particular focus on ligand–receptor interactions
- Study *P vivax* and reticulocyte blood-stage biochemistry and the infected red blood cell membrane structures
- Re-examine cytoadherent properties of *P vivax* and role of the spleen and variant proteins
- Develop continuous in vitro culture systems for blood-stage parasites
- Expand use of *P cynomolgi* non-human primate models to investigate hypnozoite and blood-stage parasite biology and pathogenesis
- Discover targets for future vaccine and drug intervention on the basis of the biological understanding of the parasite

P vivax in vitro culture systems

A continuous in vitro culture system for *P vivax*, which could generate unrestricted numbers of blood-stage parasites, is among the most important technologies that could be developed to advance research on *P vivax*. However, this goal faces major inherent biological hurdles, especially because of the need for reticulocyte host cells; whether a continuous in vitro culture will be attainable remains uncertain. A straightforward method was reported in 1989 that enabled the maturation of *P vivax* parasite blood samples from patients in short-term cultures, and these ex vivo systems enabled limited but valuable experimentation with these parasites from the field.[68] Also 20 years ago, efforts were made to establish a continuous culture system for *P vivax* with aotus monkey reticulocytes to cycle *P vivax* under shaking conditions.[69] In the past 10 years, improvement of this method has taken advantage of human reticulocytes, modified medium, and culture conditions combining static and shaking periods.[70] Recently, the use of haemopoietic stem cells and addition of specific factors (that drove their differentiation into reticulocytes), represented an improvement because this approach provided a constant source of reticulocytes, albeit in low percentages, needed to develop a continuous culture system.[71] This system is technically demanding, not highly reproducible (as several isolates failed to grow), and does not support the exponential growth of *P vivax* to obtain large parasite yields. Nevertheless, this research is another step among many unpublished attempts to establish a continuous *P vivax* culture system, and it is probably too early to give up hope that creative insights and technologies will one day be applied to achieve this goal.

In addition to developing a continuous in vitro culture for blood-stage parasites, in vitro systems for liver stages would be invaluable to further our knowledge on hypnozoites.[72]

Animal model systems

Animal model systems have substantially advanced biomedical research for many health concerns, and malaria is no exception. From non-human primate experimental infections with malaria parasites of monkeys, apes, and human beings, to small animal model experimental infections with malaria parasites of rodents, studies have helped to shed light on infection dynamics, parasite biology, and pathogenesis.[73] Especially given our lack of knowledge about hypnozoites and the lack of a continuous culture system to study *P vivax* blood-stage parasites, non-human primates have been and continue to be especially important for *P vivax* research. Monkey model systems have the advantages of being able to control the timing of infections and reinfections, the choice of parasite (species, strain, isolate, or clone) and host (*Saimiri*, *Aotus*, or *Macaca* species of monkey) combinations, and the elimination of confounding factors such as malnutrition and other diseases. Moreover, by use of non-human primates it is possible to justify delayed or suboptimal treatments and to devise and implement important sampling schemes (eg, multiple blood draws and liver or bone marrow biopsies). *Aotus* spp monkeys have been reliable for some *P vivax* vaccine trials;[74] however, supplies of these small New World monkeys have been a limiting factor in the scope and breadth of possible research. Published research also shows how *P vivax* infections of *Saimiri boliviensis* monkeys have been crucial for cultivating the parasite for the identification of vaccine candidate antigens and addressing challenging biological questions,[4,7,54] as well as for attaining genomic DNA and chromosomal material for supporting the *P vivax* genome project.[48]

Because of the limitations of using small New World monkeys, with their small blood volumes, ape and macaque malaria models have proven a valuable alternative. Experimental infection of macaque monkeys with *P cynomolgi* will be an especially important model to study hypnozoite biology and severe malaria pathogenesis and pregnancy complications, alongside possible New World monkey models using *P vivax* where feasible. *P knowlesi* and *P cynomolgi* parasites will also serve as outstanding models for helping to answer questions on the invasion of erythrocytes and the biology of the blood-stage forms.

Immunology

As with falciparum malaria, people having chronic exposure to vivax malaria tend to develop some acquired immunity. Studies involving patients with neurosyphilis who were experimentally infected with *P vivax* as a therapeutic measure showed onset of clinical immunity relatively rapidly compared with challenge with falciparum malaria.[75] Field studies in Papua New Guinea recently showed age-dependent onset of protective immunity, with clinical illness caused by vivax malaria being largely limited to children younger than 5 years.[76] Onset of immunity to vivax malaria appeared to occur earlier in life than with falciparum malaria. Studies in Vanuatu, Thailand, and Sri Lanka support that view.[77–79] Those studies could not separate the effects of age and cumulative exposure, and among non-immune migrants to Indonesian New Guinea suddenly exposed to heavy transmission, children and adults seemed equally susceptible to vivax malaria even after onset of age-dependent clinical immunity to falciparum malaria.[80] Reconciling these disparate findings will need in-depth studies into distinct mechanisms of immune acquisition driven by cumulative exposure to and acquisition of an increasingly diverse memory of the antigenic repertoire of the two parasites and by intrinsic factors related to age and recent exposure. To clarify the nature of immune acquisition to *P vivax* (and its difference to that of *P falciparum*) prospective longitudinal studies in different

age groups and in areas with differing intensity of transmission might prove essential. So far only two such studies, both showing clinical protection by IgG antibodies against merozoite surface antigens have been reported.[81,82] Although *P vivax* is known to cause fever with even very low levels of infection associated with very high levels of proinflammatory cytokines,[83,84] little is known about effector cells and parasite triggers, and the role of such host immune responses in the acute and perhaps severe pathology of *P vivax* infections.

Irrespective of the mechanisms at work, studies in heavily endemic areas show onset of protection from severe disease syndromes after about 5 years of age. As with *P falciparum* in holoendemic Africa,[85] interventions aimed at reducing transmission must take into account the consequences to the majority protected against severe disease and the threshold of exposure that maintains that protection.

Vaccines

The search for vaccines against *P vivax* remains a formidable challenge. However, unlike *P falciparum*, very few candidates have been studied. At present there are only two *P vivax* subunit vaccine candidates in clinical trials and a modest number of other candidates being tested in preclinical trials[86,87] compared with *P falciparum* with more than 70 different vaccine formulations available and 23 in clinical trials.[88,89]

P vivax circumsporozoite protein (PvCSP) synthetic long peptides were found to be safe and immunogenic in people,[90] and a clinical trial (phase II) to assess the vaccine's protective efficacy is being planned. Also, recombinant chimeric circumsporozoite proteins encompassing repeats from the two major alleles, VK210 and VK247, are in preclinical development.[91] The N-terminal, cysteine-rich region II of *P vivax* Duffy binding protein (PvRII), alone or in combination with the 19 kDa C-terminal region of merozoite surface protein 1 (PvMSP1-19), was immunogenic in preclinical studies with antibodies elicited against PvRII having a substantial inhibitory effect in in vitro binding assays.[92,93] PvRII also showed limited efficacy in preclinical testing in *Aotus grisiemembra* monkeys[94] as did a recombinant fragment of merozoite surface protein 1 (N-terminal region; Pv200L).[95] Together with the protection offered by naturally acquired antibodies in longitudinal studies,[81,82] these results support a move towards clinical development of these blood-stage antigens. Additionally, immune responses induced by a *P vivax* mosquito stage antigen (Pvs25) based transmission-blocking vaccine were associated with transmission-blocking activity in aotus monkeys[96] and people.[97] However, adverse reactions observed with the Montanide ISA 51 formulations[97] suggest that a better tolerated formulation will be required. Several other *P vivax* vaccine candidate antigens have been under preclinical development, but they are not yet scheduled for clinical testing. Efforts drawing on new data from the *P vivax* genome database,[48] and capitalising on expertise in functional genomics, computational analysis, protein production, functional assays, and animal studies are urgently needed to accelerate the preclinical and clinical testing of alternative *P vivax* vaccine candidates. New creative directions must also be explored to identify new vaccine candidates among the elusive liver-stage antigens.[98]

In addition to increasing the conspicuously neglected effort with vaccines against vivax malaria, vaccine developers should acknowledge the overlapping distribution of falciparum and vivax malaria and create development strategies yielding a single formulation for vaccination against both of these species.

Prevention and control

The almost singular focus of the malaria research community on falciparum malaria, especially in holoendemic Africa, has resulted in the emphasis on so-called global control strategies uniquely well-suited to that setting—eg, insecticide-treated nets and intermittent preventive therapy for infants and pregnant women. Use of insecticide treated nets has had limited effect in southeast Asia,[99,100] and intermittent preventive therapy strategies have not been evaluated outside of Africa. Likewise, strategies uniquely well-suited to less endemic settings, such as indoor residual spraying of insecticides receives little attention from researchers and almost none from implementing aid agencies.

Countries such as Brazil and Thailand where both *P falciparum* and *P vivax* coexist best draw attention to the challenges of control and even more the eventual elimination of *P vivax*. Sustained vector control and good access to effective treatment has meant that malaria transmission in these countries was reduced over the past 30–40 years. In Brazil, *P vivax* cases now account for more than 70% of the burden of disease.[101] In Thailand the rate of decrease in incidence of *P vivax*, but not *P falciparum*, has slowed in recent years resulting in now roughly equal numbers of cases with either infection.[102] These experiences show that *P vivax* cannot be easily eliminated with available methods. If elimination of malaria is to be done in *P vivax* endemic areas, a better knowledge of *P vivax* and a set of new controls specifically targeted against *P vivax*, are urgently needed.

As noted, a crucial weakness in controlling endemic vivax malaria remains the limitations imposed by the sole drug (primaquine) to attack the reservoir of infection in the livers of countless people. Strategies aiming for the elimination of malaria from any given area must devise a way to attack this reservoir. Our almost complete lack of understanding of the biology (and vulnerabilities) of hypnozoites, combined with the serious operational and clinical obstacles confronted with primaquine, foreshadow poor prospects for the practicality of an effective assault on this parasite in the near future.

Search strategy and selection criteria

We searched PubMed for papers written in English and containing the term "*Plasmodium vivax*". There were no date limits included in our search.

Control of *P vivax* will also rely upon vector control, so a comprehensive understanding of the ecology of vector species that transmit *P vivax* is essential. Over the large geographical range of *P vivax*, over 25 anopheline vector species are known to transmit the disease.[103] Many of them tend to have outdoor biting habits and are less anthropophilic than the main vectors of *P falciparum* in Africa. In addition, there is evidence of intimate coevolution between *P vivax* and its vectors.[104] Unfortunately, the published work on many of these vectors is patchy and in many areas in-depth vector ecology studies are lacking. New, in-depth entomology studies are therefore urgently needed.

Control of endemic vivax malaria will require focus upon interventions suited to environments with lower transmission intensity than holoendemic Africa, and upon strategies for elimination of hypnozoites. The possible effect of these strategies on acquired immunity among older children and adult populations where heavy exposure occurs must also be considered.

The complex biology of *P vivax* makes it more difficult to predict the effect of a given intervention on *P vivax* burden and transmission levels. Detailed mathematical models based on the best knowledge of this parasite, and its host and vector relations will thus be required to improve our ability to predict the effect of different interventions (singly or combined) on *P vivax* at the population level.

Conclusions

The search for effective interventions against *P vivax* remains a formidable challenge. Given the substantial difference in the biology, genetics, pathogenesis, and epidemiology of *P vivax* outlined, it cannot simply be assumed that interventions developed and tested for the control of *P falciparum* in sub-Saharan Africa will be similarly successful when used in *P vivax* endemic areas. Unfortunately, the relative neglect of *P vivax* research in the past means that we lack sufficient indepth understanding of the biology and epidemiology of the parasite, and have only started to understand host immune responses and find measurable signs of this protection to support rational development of new interventions. This situation is further complicated by the lack of or limited access to crucial research methods such as in vitro culture, animal models, or modern genomics and proteomic methods.

To address the crucial gaps in our knowledge on *P vivax* and accelerate the development of useful methods of control, a substantial increase in investment in *P vivax* research (ranging from diagnostics, therapeutics, basic biology, epidemiology, clinical studies to mathematical modelling) is urgently needed. Given how complex and interconnected the priority research areas are, a coordinated interdisciplinary approach is essential. Only by bringing together expertise in the different specialties within *P vivax* research will it be possible to bridge the gaps between disciplines to create intellectual and operational synergies. The *P vivax* research community, long labouring in relative obscurity and with severely limited resources, has a history of collaborative work reaching across continents and disciplines. Adequate funding for the many necessary efforts sketched in this Review would no doubt energise *P vivax* research and stimulate the expansion of and rapid progress in this community. The ability of people to control vivax malaria today, and eventually eradicate it, very much hinges upon such investment. In hindsight, the neglect of vivax malaria now seems especially egregious. We must consider further neglect unacceptable and immediately relegate this to history.

Contributors

All authors contributed equally to the preparation of this Review.

Conflicts of interest

We declare that we have no conflicts of interest.

References

1 Krotoski WA. Discovery of the hypnozoite and a new theory of malarial relapse. *Trans R Soc Trop Med Hyg* 1985; **79:** 1–11.

2 Sturm A, Amino R, van de Sand C, et al. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* 2006; **313:** 1287–90.

3 Suwanarusk R, Cooke BM, Dondorp AM, et al. The deformability of red blood cells parasitized by *Plasmodium falciparum* and *P vivax*. *J Infect Dis* 2004; **189:** 190–94.

4 Barnwell JW, Ingravallo P, Galinski MR, Matsumoto Y, Aikawa M. *Plasmodium vivax*: malarial proteins associated with the membrane-bound caveola-vesicle complexes and cytoplasmic cleft structures of infected erythrocytes. *Exp Parasitol* 1990; **70:** 85–99.

5 Boyd MF, Kitchen SF. On the infectiousness of patients infected with *Plasmodium vivax* and *Plasmodium falciparum*. *Am J Trop Med Hyg* 1937; **17:** 253–62.

6 Kitchen SK. The infection of reticulocytes by *Plasmodium vivax*. *Am J Trop Med Hyg* 1938; **18:** 347.

7 Galinski MR, Medina CC, Ingravallo P, Barnwell JW. A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. *Cell* 1992; **69:** 1213–26.

8 del Portillo HA, Lanzer M, Rodriguez-Malaga S, Zavala F, Fernandez-Becerra C. Variant genes and the spleen in *Plasmodium vivax* malaria. *Int J Parasitol*; **34:** 1547–54.

9 Anstey NM, Handojo T, Pain MC, et al. Lung injury in vivax malaria: pathophysiological evidence for pulmonary vascular sequestration and posttreatment alveolar-capillary inflammation. *J Infect Dis* 2007; **195:** 589–96.

10 Miller LH, Good MF, Milon G. Malaria pathogenesis. *Science*; **264:** 1878–83.

11 Coatney R, Collins WE, Warren M, Contacos PG. The Primate Malarias. Washington, DC: US Government Printing Office, 1971.

12 McKenzie FE, Jeffery GM, Collins WE. Gametocytemia and fever in human malaria infections. *J Parasitol* 2007; **93:** 627–33.

13 Guerra CA, Snow RW, Hay SI. Mapping the global extent of malaria in 2005. *Trends Parasitol* 2006; **22:** 353–58.

14 Hay SI, Guerra CA, Tatem AJ, Noor AM, Snow RW. The global distribution and population at risk of malaria: past, present, and future. *Lancet Infect Dis* 2004; **4:** 327–36.

15 Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, Anstey NM. Vivax malaria: neglected and not benign. *Am J Trop Med Hyg* 2007; **77** (6 suppl): 79–87.

16 Rosenberg R. *Plasmodium vivax* in Africa: hidden in plain sight? *Trends Parasitol* 2007; **23:** 193–96.

17 Guerra CA, Gikandi PW, Tatem AJ, et al. The limits and intensity of *Plasmodium falciparum* transmission: implications for malaria control and elimination worldwide. *PLoS Med* 2008; **5:** e38.

18 Snounou G, White NJ. The co-existence of plasmodium: sidelights from falciparum and vivax malaria in Thailand. *Trends Parasitol* 2004; **20:** 333–39.

19 Genton B, D'Acremont V, Rare L, et al. *Plasmodium vivax* and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. *PLoS Med* 2008; **5:** e127.

20 Tjitra E, Anstey NM, Sugiarto P, et al. Multidrug-resistant *Plasmodium vivax* associated with severe and fatal malaria: a prospective study in Papua, Indonesia. *PLoS Med* 2008; **5:** e128.

21 WHO. Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs: round 1 (2008). Geneva: World Health Organization, 2009. https://apps.who.int/tdr/publications/tdr-research-publications/rdt-performance/pdf/full-report-malaria-RDTs.pdf (accessed July 15, 2009).

22 Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to *Plasmodium vivax* in blacks: the Duffy-blood-group genotype, FyFy. *N Engl J Med* 1976; **295:** 302–04.

23 Ryan JR, Stoute JA, Amon J, et al. Evidence for transmission of *Plasmodium vivax* among a duffy antigen negative population in Western Kenya. *Am J Trop Med Hyg* 2006; **75:** 575–81.

24 Cavasini CE, Mattos LC, Couto AA, et al. *Plasmodium vivax* infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception? *Trans R Soc Trop Med Hyg* 2007; **101:** 1042–44.

25 Baird JK. Neglect of *Plasmodium vivax* malaria. *Trends Parasitol* 2007; **23:** 533–39.

26 Kochar DK, Saxena V, Singh N, Kochar SK, Kumar SV, Das A. *Plasmodium vivax* malaria. *Emerg Infect Dis* 2005; **11:** 132–34.

27 Barcus MJ, Basri H, Picarima H, et al. Demographic risk factors for severe and fatal vivax and falciparum malaria among hospital admissions in northeastern Indonesian Papua. *Am J Trop Med Hyg* 2007; **77:** 984–91.

28 Kochar DK, Das A, Kochar SK, et al. Severe *Plasmodium vivax* malaria: a report on serial cases from Bikaner in northwestern India. *Am J Trop Med Hyg* 2009; **80:** 194–98.

29 Fernandez-Becerra C, Pinazo MJ, Gonzalez A, Alonso PL, del Portillo HA, Gascon J. Increased expression levels of the pvcrt-o and pvmdr1 genes in a patient with severe *Plasmodium vivax* malaria. *Malar J* 2009; **8:** 55.

30 Song HH, O SO, Kim SH, et al. Clinical features of *Plasmodium vivax* malaria. *Korean J Intern Med* 2003; **18:** 220–24.

31 Anstey NM, Russell B, Yeo TW, Price RN. The pathophysiology of vivax malaria. *Trends Parasitol* 2009; **25:** 220–27.

32 Baird JK. Chloroquine resistance in *Plasmodium vivax*. *Antimicrob Agents Chemother* 2004; **48:** 4075–83.

33 de Santana Filho FS, Arcanjo AR, Chehuan YM, et al. Chloroquine-resistant *Plasmodium vivax*, Brazilian Amazon. *Emerg Infect Dis* 2007; **13:** 1125–26.

34 Baird JK, Leksana B, Masbar S, et al. Diagnosis of resistance to chloroquine by *Plasmodium vivax*: timing of recurrence and whole blood chloroquine levels. *Am J Trop Med Hyg* 1997; **56:** 621–26.

35 Karunajeewa HA, Ilett KF, Mueller I, et al. Pharmacokinetics and efficacy of piperaquine and chloroquine in Melanesian children with uncomplicated malaria. *Antimicrob Agents Chemother* 2008; **52:** 237–43.

36 Maguire JD, Krisin, Marwoto H, Richie TL, Fryauff DJ, Baird JK. Mefloquine is highly efficacious against chloroquine-resistant *Plasmodium vivax* malaria and *Plasmodium falciparum* malaria in Papua, Indonesia. *Clin Infect Dis* 2006; **42:** 1067–72.

37 Lacy MD, Maguire JD, Barcus MJ, et al. Atovaquone/proguanil therapy for *Plasmodium falciparum* and *Plasmodium vivax* malaria in Indonesians who lack clinical immunity. *Clin Infect Dis* 2002; **35:** e92–95.

38 Ratcliff A, Siswantoro H, Kenangalem E, et al. Two fixed-dose artemisinin combinations for drug-resistant falciparum and vivax malaria in Papua, Indonesia: an open-label randomised comparison. *Lancet* 2007; **369:** 757–65.

39 Baird JK, Hoffman SL. Primaquine therapy for malaria. *Clin Infect Dis* 2004; **39:** 1336–45.

40 Baird JK, Rieckmann KH. Can primaquine therapy for vivax malaria be improved? *Trends Parasitol* 2003; **19:** 115–20.

41 Schwartz E, Parise M, Kozarsky P, Cetron M. Delayed onset of malaria—implications for chemoprophylaxis in travelers. *N Engl J Med* 2003; **349:** 1510–16.

42 Shortt HE, Garnham PC. Demonstration of a persisting exo-erythrocytic cycle in *Plasmodium cynomolgi* and its bearing on the production of relapses, 1948. *Bull World Health Organ* 2000; **78:** 1447–49.

43 Krotoski WA, Garnham PC, Cogswell FB, et al. Observations on early and late post-sporozoite tissue stages in primate malaria: IV, pre-erythrocytic schizonts and/or hypnozoites of Chesson and North Korean strains of *Plasmodium vivax* in the chimpanzee. *Am J Trop Med Hyg* 1986; **35:** 263–74.

44 Craig AA, Kain KC. Molecular analysis of strains of *Plasmodium vivax* from paired primary and relapse infections. *J Infect Dis* 1996; **174:** 373–79.

45 Kirchgatter K, del Portillo HA. Molecular analysis of *Plasmodium vivax* relapses using the MSP1 molecule as a genetic marker. *J Infect Dis* 1998; **177:** 511–15.

46 Imwong M, Snounou G, Pukrittayakamee S, et al. Relapses of *Plasmodium vivax* infection usually result from activation of heterologous hypnozoites. *J Infect Dis* 2007; **195:** 927–33.

47 Chen N, Auliff A, Rieckmann K, Gatton M, Cheng Q. Relapses of *Plasmodium vivax* infection result from clonal hypnozoites activated at predetermined intervals. *J Infect Dis* 2007; **195:** 934–41.

48 Carlton JM, Adams JH, Silva JC, et al. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* 2008; **455:** 757–63.

49 Pain A, Bohme U, Berry AE, et al. The genome of the simian and human malaria parasite *Plasmodium knowlesi*. *Nature* 2008; **455:** 799–803.

50 Imwong M, Nair S, Pukrittayakamee S, et al. Contrasting genetic structure in *Plasmodium vivax* populations from Asia and South America. *Int J Parasitol* 2007; **37:** 1013–22.

51 Karunaweera ND, Ferreira MU, Munasinghe A, et al. Extensive microsatellite diversity in the human malaria parasite *Plasmodium vivax*. *Gene* 2008; **410:** 105–12.

52 Bozdech Z, Mok S, Hu G, et al. The transcriptome of *Plasmodium vivax* reveals divergence and diversity of transcriptional regulation in malaria parasites. *Proc Natl Acad Sci USA* 2008; **105:** 16290–95.

53 Carlton JM, Escalante AA, Neafsey D, Volkman SK. Comparative evolutionary genomics of human malaria parasites. *Trends Parasitol* 2008; **24:** 545–50.

54 Galinski MR, Dluzewski AR, Barnwell JW. A mechanistic approach to merozoite invasion of red blood cells: merozoite biogenesis, rupture, and invasion of erythrocytes. In: Sherman IW, ed. Molecular Approaches to Malaria. New York: ASM Press, 2005: 113–63.

55 Chitnis CE, Miller LH. Identification of the erythrocyte binding domains of *Plasmodium vivax* and *Plasmodium knowlesi* proteins involved in erythrocyte invasion. *J Exp Med* 1994; **180:** 497–506.

56 Borst P, Rudenko G. Antigenic variation in African trypanosomes. *Science* 1994; **264:** 1872–73.

57 del Portillo HA, Fernandez-Becerra C, Bowman S, et al. A superfamily of variant genes encoded in the subtelomeric region of *Plasmodium vivax*. *Nature* 2001; **410:** 839–42.

58 Fernandez-Becerra C, Pein O, de Oliveira TR, et al. Variant proteins of *Plasmodium vivax* are not clonally expressed in natural infections. *Mol Microbiol* 2005; **58:** 648–58.

59 Fernandez-Becerra C, Yamamoto MM, Vencio RZ, Lacerda M, Rosanas-Urgell A, del Portillo HA. *Plasmodium vivax* and the importance of the subtelomeric multigene vir superfamily. *Trends Parasitol* 2009; **25:** 44–51.

60 Scherf A, Lopez-Rubio JJ, Riviere L. Antigenic variation in *Plasmodium falciparum*. *Annu Rev Microbiol* 2008; **62:** 445–70.

61 Korir CC, Galinski MR. Proteomic studies of *Plasmodium knowlesi* SICA variant antigens demonstrate their relationship with *P. falciparum* EMP1. *Infect Genet Evol* 2006; **6:** 75–79.

62 Barnwell JW, Howard RJ, Coon HG, Miller LH. Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in cloned *Plasmodium knowlesi* malaria. *Infect Immun* 1983; **40:** 985–94.

63 David PH, Hommel M, Miller LH, Udeinya IJ, Oligino LD. Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. *Proc Natl Acad Sci USA* 1983; **80:** 5075–79.

64 Hommel M, David PH, Oligino LD. Surface alterations of erythrocytes in *Plasmodium falciparum* malaria: antigenic variation, antigenic diversity, and the role of the spleen. *J Exp Med* 1983; **157:** 1137–48.

65 Handunnetti SM, Mendis KN, David PH. Antigenic variation of cloned *Plasmodium fragile* in its natural host *Macaca sinica*: sequential appearance of successive variant antigenic types. *J Exp Med* 1987; **165:** 1269–83.

66 Gilks CF, Walliker D, Newbold CI. Relationships between sequestration, antigenic variation and chronic parasitism in *Plasmodium chabaudi chabaudi*—a rodent malaria model. *Parasite Immunol* 1990; **12:** 45–64.

67 Demar M, Legrand E, Hommel D, Esterre P, Carme B. *Plasmodium falciparum* malaria in splenectomized patients: two case reports in French Guiana and a literature review. *Am J Trop Med Hyg* 2004; **71:** 290–93.

68 Barnwell JW, Nichols ME, Rubinstein P. In vitro evaluation of the role of the Duffy blood group in erythrocyte invasion by *Plasmodium vivax*. *J Exp Med* 1989; **169:** 1795–802.

69 Mons B, Collins WE, Skinner JC, van der Star W, Croon JJ, van der Kaay HJ. *Plasmodium vivax*: in vitro growth and reinvasion in red blood cells of *Aotus nancymai*. *Exp Parasitol* 1988; **66:** 183–88.

70 Golenda CF, Li J, Rosenberg R. Continuous in vitro propagation of the malaria parasite *Plasmodium vivax*. *Proc Natl Acad Sci USA* 1997; **94:** 6786–91.

71 Udomsangpetch R, Kaneko O, Chotivanich K, Sattabongkot J. Cultivation of *Plasmodium vivax*. *Trends Parasitol* 2008; **24:** 85–88.

72 Sattabongkot J, Yimamnuaychoke N, Leelaudomlipi S, Rasameesoraj M, Cui L, Brewer TG. Establishment of a human hepatocyte line that supports in vitro development of the exo-erythrocytic stages of the malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. *Am J Trop Med Hyg* 2006; **74:** 708–15.

73 Sherman IW. Malaria: parasite biology, pathogenesis, and protection. Washington, DC: ASM Press, 1998.

74 Herrera S, Perlaza BL, Bonelo A, Arevalo-Herrera M. Aotus monkeys: their great value for anti-malaria vaccines and drug testing. *Int J Parasitol* 2002; **32:** 1625–35.

75 Ciuca M BL, Chelarescu-Vieru M. Immunity in malaria. *Trans R Soc Trop Med Hyg* 1934; **6:** 619–22.

76 Michon P, Cole-Tobian JL, Dabod E, et al. The risk of malarial infections and disease in Papua New Guinean children. *Am J Trop Med Hyg* 2007; **76:** 997–1008.

77 Luxemburger C, Thwai KL, White NJ, et al. The epidemiology of malaria in a Karen population on the western border of Thailand. *Trans R Soc Trop Med Hyg* 1996; **90:** 105–11.

78 Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of *Plasmodium vivax* malaria. *Am J Trop Med Hyg* 2001; **64** (suppl 1–2)**:** 97–106.

79 Maitland K, Williams TN, Bennett S, et al. The interaction between *Plasmodium falciparum* and *P. vivax* in children on Espiritu Santo island, Vanuatu. *Trans R Soc Trop Med Hyg* 1996; **90:** 614–20.

80 Baird JK. Host age as a determinant of naturally acquired immunity to *Plasmodium falciparum*. *Parasitol Today* 1995; **11:** 105–11.

81 Nogueira PA, Alves FP, Fernandez-Becerra C, et al. A reduced risk of infection with *Plasmodium vivax* and clinical protection against malaria are associated with antibodies against the N terminus but not the C terminus of merozoite surface protein 1. *Infect Immun* 2006; **74:** 2726–33.

82 King CL, Michon P, Shakri AR, et al. Naturally acquired Duffy-binding protein-specific binding inhibitory antibodies confer protection from blood-stage *Plasmodium vivax* infection. *Proc Natl Acad Sci USA* 2008; **105:** 8363–68.

83 Karunaweera ND, Grau GE, Gamage P, Carter R, Mendis KN. Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in *Plasmodium vivax* malaria. *Proc Natl Acad Sci USA* 1992; **89:** 3200–03.

84 Hemmer CJ, Holst FG, Kern P, Chiwakata CB, Dietrich M, Reisinger EC. Stronger host response per parasitized erythrocyte in *Plasmodium vivax* or *ovale* than in *Plasmodium falciparum* malaria. *Trop Med Int Health* 2006; **11:** 817–23.

85 Snow RW, Marsh K. The consequences of reducing transmission of *Plasmodium falciparum* in Africa. *Adv Parasitol* 2002; **52:** 235–64.

86 Herrera S, Corradin G, Arevalo-Herrera M. An update on the search for a *Plasmodium vivax* vaccine. *Trends Parasitol* 2007; **23:** 122–28.

87 Galinski MR, Barnwell JW. *Plasmodium vivax*: who cares? *Malar J* 2008; **7** (suppl 1)**:** S9.

88 Malkin E, Dubovsky F, Moree M. Progress towards the development of malaria vaccines. *Trends Parasitol* 2006; **22:** 292–95.

89 WHO. Portfolio of candidate malaria vaccines currently in development, March 2005. Geneva: World Health Organization, 2005. http://www.who.int/vaccine_research/documents/en/malaria_table.pdf (accessed July 15, 2009).

90 Herrera S, Bonelo A, Perlaza BL, et al. Safety and elicitation of humoral and cellular responses in colombian malaria-naive volunteers by a *Plasmodium vivax* circumsporozoite protein-derived synthetic vaccine. *Am J Trop Med Hyg* 2005; **73** (suppl 5)**:** 3–9.

91 Yadava A, Sattabongkot J, Washington MA, et al. A novel chimeric *Plasmodium vivax* circumsporozoite protein induces biologically functional antibodies that recognize both VK210 and VK247 sporozoites. *Infect Immun* 2007; **75:** 1177–85.

92 Devi YS, Mukherjee P, Yazdani SS, et al. Immunogenicity of *Plasmodium vivax* combination subunit vaccine formulated with human compatible adjuvants in mice. *Vaccine* 2007; **25:** 5166–74.

93 Moreno A, Caro-Aguilar I, Yazdani SS, et al. Preclinical assessment of the receptor-binding domain of *Plasmodium vivax* Duffy-binding protein as a vaccine candidate in rhesus macaques. *Vaccine* 2008; **26:** 4338–44.

94 Arevalo-Herrera M, Castellanos A, Yazdani SS, et al. Immunogenicity and protective efficacy of recombinant vaccine based on the receptor-binding domain of the *Plasmodium vivax* Duffy binding protein in aotus monkeys. *Am J Trop Med Hyg* 2005; **73** (suppl 5)**:** 25–31.

95 Valderrama-Aguirre A, Quintero G, Gomez A, et al. Antigenicity, immunogenicity, and protective efficacy of *Plasmodium vivax* MSP1 PV200l: a potential malaria vaccine subunit. *Am J Trop Med Hyg* 2005; **73** (suppl 5)**:** 16–24.

96 Arevalo-Herrera M, Solarte Y, Yasnot MF, et al. Induction of transmission-blocking immunity in aotus monkeys by vaccination with a *Plasmodium vivax* clinical grade PVS25 recombinant protein. *Am J Trop Med Hyg* 2005; **73** (suppl 5)**:** 32–37.

97 Wu Y, Ellis RD, Shaffer D, et al. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. *PLoS ONE* 2008; **3:** e2636.

98 Wang R, Arevalo-Herrera M, Gardner MJ, et al. Immune responses to *Plasmodium vivax* pre-erythrocytic stage antigens in naturally exposed Duffy-negative humans: a potential model for identification of liver-stage antigens. *Eur J Immunol* 2005; **35:** 1859–68.

99 Bockarie MJ, Dagoro H. Are insecticide-treated bednets more protective against *Plasmodium falciparum* than *Plasmodium vivax*-infected mosquitoes? *Malar J* 2006; **5:** 15.

100 Luxemburger C, Perea WA, Delmas G, Pruja C, Pecoul B, Moren A. Permethrin-impregnated bed nets for the prevention of malaria in schoolchildren on the Thai-Burmese border. *Trans R Soc Trop Med Hyg* 1994; **88:** 155–59.

101 Coura JR, Suarez-Mutis M, Ladeia-Andrade S. A new challenge for malaria control in Brazil: asymptomatic plasmodium infection—a review. *Mem Inst Oswaldo Cruz* 2006; **101:** 229–37.

102 Childs DZ, Cattadori IM, Suwonkerd W, Prajakwong S, Boots M. Spatiotemporal patterns of malaria incidence in northern Thailand. *Trans R Socp Trop Med Hyg* 2006; **100:** 623–31.

103 Kiszewski A, Mellinger A, Spielman A, Malaney P, Sachs SE, Sachs J. A global index representing the stability of malaria transmission. *Am J Trop Med Hyg* 2004; **70:** 486–98.

104 Joy DA, Gonzalez-Ceron L, Carlton JM, et al. Local adaptation and vector-mediated population structure in *Plasmodium vivax* malaria. *Mol Biol Evol* 2008; **25:** 1245–52.