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# Progress towards the development of a *P. vivax* vaccine

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## Abstract

**Introduction:** *Plasmodium vivax* causes significant public health problems in endemic regions. A vaccine to prevent disease is critical, considering the rapid spread of drug-resistant parasite strains, and the development of hypnozoites in the liver with potential for relapse. A minimally effective vaccine should prevent disease and transmission while an ideal vaccine provides sterile immunity.

**Areas covered:** Despite decades of research, the complex life cycle, technical challenges and a lack of funding have hampered progress of *P. vivax* vaccine development. Here, we review the progress of potential *P. vivax* vaccine candidates from different stages of the parasite life cycle. We also highlight the challenges and important strategies for rational vaccine design. These factors can significantly increase immune effector mechanisms and improve the protective efficacy of these candidates in clinical trials to generate sustained protection over longer periods of time.

**Expert opinion:** A vaccine that presents functionally-conserved epitopes from multiple antigens from various stages of the parasite life cycle is key to induce broadly neutralizing strain-transcending protective immunity to effectively disrupt parasite development and transmission.

### Keywords

clinical trials; heterologous prime/boost immunizations; viral vectors; virus-like particles; vivax vaccines

## 1. Introduction

The development and implementation of a vaccine to eradicate smallpox triggered a renaissance in vaccine research. This resulted in one of the most economical global health

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Author contributions

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Declaration of interest

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interventions that led to a number of licensed vaccines in the 21st century. Despite the successes of vaccines, 20% of children especially from low- and middle-income countries do not complete the scheduled immunizations in their first year [1].

Malaria is a public health problem and a major burden to socioeconomic development in many developing countries of the world. In 2018, the WHO reported an estimated 228 million clinical cases of malaria leading to 405,000 deaths worldwide. Of these cases, 85% occur in sub-Saharan Africa and Southeast Asia. *Plasmodium falciparum* and *Plasmodium vivax* are the main causative parasites for malaria [2]. Although, *P. vivax* malaria is a significant public health burden in many regions, much of the associated mortality is due to *P. falciparum* infections in non-immune persons, especially children under five years and pregnant women of sub-Saharan Africa [2].

*Plasmodium vivax* has the greatest geographic distribution, accounting for 12.4% of malaria in Africa and >70% in Asia and the Americas [3–5]. *P. vivax* causes an estimated 14.3 million malaria episodes each year and is the leading cause of malaria in Asia and Latin America [6,7]. However, there is increasing evidence that the severity of *P. vivax* is underestimated as it can be non-life-threatening and self-limiting [3]. Also, endemic countries often lack broad access to affordable and accessible healthcare, intensifying the impact of this disease among poorer communities. Vivax malaria incapacitates individuals of all ages from repeated febrile episodes and severe anemia, while recurrent infections can lead to life-long impairment and increased risk for pregnant women [8]. As reports of clinical severity and lethal cases of *P. vivax* infections increase [9–14], together with wide-spread drug resistance [15–20] and relapse infections, the development of an effective antimalarial vaccine is considered an essential part of the overall control strategy to preventing disease [21].

A change in the disease pattern in a population often results from an epidemiological shift. Several factors that may govern this shift are age, number of immunizations, number of vaccinated individuals, different disease serotypes and immunizations for at risk individuals [22,23]. In most vivax malaria endemic areas, transmission is intermittent and acquired immunity is short-lived and biased towards being strain-specific [24,25]. Recent reports indicate that this may be further compounded by *P. vivax* infections and disease in Duffy-negative individuals [26–28], previously thought to be resistant. Thus, there is legitimate and increasing concern that *P. vivax* may adapt to or be present in populations previously considered resistant and greatly and the actual global burden is much higher than currently predicted. All these factors emphasize the need for improving public health and therapeutic strategies including the development of a vaccine against this disease.

Like vaccines against other microbial pathogens, an effective vivax vaccine should provide long-term, broadly-neutralizing, strain-transcending immunity, that eliminates clinical disease and disrupts transmission. Failure of a vaccine against an infectious disease to generate such broadly-neutralizing antibodies will result in re-infection and continued disease transmission within the affected population, as occurred in about 10% of vaccinees' that receive the MMR vaccine [29]. Sometimes, despite the adequate levels of protective antibodies generated after vaccination, these antibodies do not persist over a long period of

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time resulting in disease. To circumvent this, booster doses are essential to maintain the level of protective antibodies to prevent vaccine failure.

Development of herd immunity should lead to resistance to the spread of an infectious agent amongst communities and eventually lead to disease elimination. This type of immunity can develop after infection, resulting in potentially significant morbidity and mortality, or at minimal risk in individuals who receive a vaccine such as oral polio vaccine (OPV) [30]. Scientific studies can help in the immunological and disease surveillance to generate epidemiological models for defining the threshold for herd immunity [31]. For example, cross-sectional and longitudinal studies can examine the antibodies and activated T- cells against the infectious agent. Based on the successes of vaccines that have eliminated other infectious diseases, the past decade has brought fresh impetus to the fight against malaria, driven by a growing appreciation of the humanitarian and economic magnitude of the problem and access to new funding sources.

### 1.1 Malaria Life Cycle

*Plasmodium vivax* has a complex life cycle with multiple stages of cellular differentiation and host cell types that require transmission by an anopheline mosquito. Infection begins with inoculation of sporozoites into the host's skin by an infected mosquito during a blood meal, these then travel to the liver. In the liver, sporozoites traverse then invade and develop in hepatocytes into thousands of merozoites, which are released into the blood stream upon hepatocyte rupture. However, some parasites arrest development in hepatocytes to remain temporarily dormant (hypnozoites), enabling multiple sequential clinical relapses, termed relapse infections, and potential transmission from a single infection. Released into the blood, merozoites infect reticulocytes to initiate cyclical asexual development from rings through trophozoites to merozoites within 48 hrs. Upon maturation, the infected reticulocyte ruptures to release into the blood between 8–32 new blood-stage merozoites to re-initiate the asexual cycle [32]. Consequently, toxins released with this cycle of development lead to a tertian pattern of repeating fever, the paroxysm, characteristic of vivax malaria. Importantly, some merozoites differentiate into sexual erythrocytic stages (gametocytes), even in the first asexual generation of *P. vivax* coming out of the liver, leading to immediate mosquito transmission. In the mosquito, the parasite undergoes sexual reproduction to produce sporozoites, which find their way into the mosquito salivary glands where they become infective and subsequently injected into a new host during a blood meal. Given the complex nature of the parasite's life cycle and the inadequate ability to prevent relapse infections and transmission, vaccine development should be an integral part of the overall strategy for malaria control [33–35].

### 1.2 Immunity to *Plasmodium vivax* infection

The development of a robust and persistent clinical immunity in some residents of endemic regions strongly supports the potential of an effective vaccine. Typically, acquired immunity to *P. vivax* blood-stage invasion ligands play a critical role in controlling blood-stage infection and disease. Studies of these ligands have revealed targets of naturally-acquired immunity (NAI) in individuals exposed to vivax infections. This immunity can trigger a robust protective immune response that inhibits sporozoite or merozoite invasion of host

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cells and protects against clinical disease [36]. Such targets are considered potential vaccine targets.

In endemic regions, the capacity of individuals to develop an adaptive immunity against *Plasmodium* infection and disease increases with age, prior exposure and transmission intensity [37]. The quality and longevity of this adaptive response is highly variable, with some individuals acquiring long-term protection following a limited number of exposures, whereas in other cases repeated exposure is needed to generate and sustain protective immunity.

In *vivax* malaria, the development of NAI is achieved by, exposure to both primary blood-stage and relapse infections that increases with age due to the booster effect by repeated infection [38,39]. NAI responses to *P. vivax* target both pre-erythrocytic and blood-stage antigens and include humoral and cellular components, although most studies have focused on very few candidates (Table 1).

Generally, NAI does not lead to sterile immunity, but decreases parasite densities to reduce the frequency and severity of clinical disease. In areas of high transmission intensity, such as Papua New Guinea, NAI to *P. vivax* becomes prevalent early in life with suppressed parasitemia leading to only few or complete absence of clinical disease in older children and adults [40], compared to *P. falciparum* [41]. On the other hand, in areas of low parasite transmission that are more common for vivax malaria, adults experience disease due to lack of development of robust clinical immunity during childhood. In some low transmission regions, (Amazon Basin or South Pacific), it is common to find individuals with asymptomatic parasitemia [42,43], suggesting that NAI can also occur with relatively few infections although, some host genetic factors (e.g. $Fy^a$ allele), may also play a protective role against clinical disease [44,45]. These data support the possibility of a successful *P. vivax* vaccine in areas of little or no parasite diversity.

### 1.3 Limitations to vivax vaccine development

*P. vivax* is classified as a neglected tropical disease, especially in terms of species-specific therapies for vaccine development and anti-relapse therapies. Compared to *P. falciparum*, there has been no coherent vaccine program for *P. vivax*. This process has been hampered by the lack of a continuous culture system for blood-stages and restricted availability of ideal animal models to study parasite biology. Secondly, the preferential infection of reticulocytes, which account for only 1–2% of total RBCs in peripheral blood, severely hinders the potential to support *P. vivax ex vivo* studies. Hence, many studies are limited by available access to fresh parasites from infected patients and facilities to support non-human primate (NHP) infections. Unfortunately, these NHP-adapted lines do not adequately reflect the genetic diversity of human infections [46]. This, has restricted studying the parasite's biology and the identification of new candidates and their evaluation [47,48]. To support vivax research, most studies have relied on surrogate functional assays to study the potential invasion inhibitory effects of antibodies against *P. vivax* invasion ligands [41,49–53], define the structural determinants for receptor recognition [54] and the epitope targets for immune antibody neutralization [25,55].

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Progress towards developing a robust long-term *P. vivax* culture method remains limited [47,50,56] and instead advances in *in vitro* culture of *P. knowlesi* in human RBCs, a closely related zoonotic species that naturally infects macaques, have provided an important support for more advanced laboratory studies [57] [58] opening the door to some experimental functional studies of *P. vivax*.

Altogether these limitations represent a major hurdle for testing vaccine efficacy and hinder the progress of *P. vivax* vaccine development. Controlled human malaria infections (CHMI) have been widely utilized for studying vaccine efficacy during the development of *P. falciparum* vaccine candidates [59–63]. However, CHMIs for *P. vivax* are more recent due to logistical challenges and the possibility of a relapse infection. Different groups in Australia, Colombia, and the USA have been working towards successfully developing blood-stage [64] and sporozoite (including mosquito-bite) *P. vivax* CHMIs [65–69]. McCarthy et al., established a parasite blood bank from an infected volunteer to overcome logistical challenges of a mosquito-bite CHMI [64]. Their team in Australia developed this infected blood-stage malaria (IBSM) inoculum as an alternative strategy to test vaccine efficacy [64,70]. Nonetheless, even as these models provide critical opportunities for testing vaccine immunogenicity and efficacy in experimentally controlled settings for early phase clinical trials prior to more costly larger-scale clinical studies, it is important to note that CHMI immunity may not fully replicate protective responses of NAI.

Genetic diversity is also an important consideration for vivax vaccine development as the genetic diversity observed in *P. vivax* is greater than in *P. falciparum* [71–73]. This may be a challenge if immunity is biased towards immunodominant variant epitopes, leading to strain-specific protective immune responses [24,55,74]. However, our studies have indicated a modified vaccine design can overcome strain immunity by focusing immune responses to conserved functional epitopes, which are otherwise less immunogenic [75]. Generally, identification of new candidates and epitopes follow *P. falciparum* research; however, *P. vivax* research comparatively has limited funds to progress.

### 1.4 *Plasmodium vivax* blood-stage vaccine candidates.

The pathology of *P. vivax* infections depends critically on the parasite's ability to recognize and invade reticulocytes, a complex process dependent upon a series of highly specific, and sequential ligand-receptor interactions between merozoites and the host erythrocyte surface proteins [76–78]. Blood-stage vaccines have so far focused on inducing broadly neutralizing antibodies against parasite invasion ligands to block interactions with host cell receptors, thereby preventing invasion, growth, and clinical disease. In addition, a blood-stage vaccine has the potential to reduce gametocytemia in the host and indirectly reduce transmission. Humoral immune responses to blood-stage antigens are believed to be an important component of NAI to malaria [79,80].

Preclinical studies have characterized *P. vivax* merozoite antigens that might be viable potential vaccine candidates, based on their immunogenicity in animal models, recognition by NAI antibodies from vivax-exposed individuals with NAI and most importantly the ability to elicit parasite invasion-inhibitory antibodies. Leading blood-stage vaccine candidates include the Duffy-binding protein (DBP) [41,81–83], apical membrane antigen-1

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(AMA1) [84], several reticulocyte binding protein (RBPs) [38,85,86], and the major merozoite surface proteins (MSPs), which include MSP1 [87–90], the MSP3 family [91–94], and MSP9 [95–97]. Of these antigens, MSP-1 and the DBP have received the most attention, but only DBP has advanced to Phase Ia clinical trials [98,99] (Table 1).

**1.4.1 *Plasmodium vivax* Duffy binding protein—**The *P. vivax* DBP is an apical organelle protein sequestered in the microneme and released to the merozoite surface during reticulocyte invasion. DBP belongs to the Duffy binding-like erythrocyte-binding protein (DBL-EBP) family, encoded by *erythrocyte binding-like (ebl)* genes [100,101], with homologs in other *Plasmodium* species [102–106]. Members of this family share similar molecular structures and functional characteristics [53,100–103]. In some *Plasmodium* spp. multiple *ebl* genes enable them to readily use alternative receptor pathways for invasion while *P. vivax* appears to be especially dependent upon on a single receptor, the Duffy antigen receptor for chemokines (DARC or Fy) [107–109].

The DBP-DARC interaction is associated with the decisive and irreversible step of junction formation between the merozoite and the host reticulocyte [51,76,78,97,108,110]; although, alternate invasion pathways now appear evident for *P. vivax* as discussed below [111–113]. This parasite's strong preference for the DARC invasion pathway represents a weakness and provided early justification of DBP as a prime target for vaccine-induced immunity against asexual stages of the parasite.

The DBP receptor-binding domain termed Region II (DBPII) [100], contains the critical residues for receptor binding [24,52,114,115]. Structural studies revealed that DBPII dimerizes upon DARC engagement in a step-wise fashion to create a stable heterotetramer [114,115]. Supporting its potential as a vaccine candidate included numerous studies of individuals in endemic regions, demonstrating that naturally-acquired anti-DBPII antibodies with significant quantitative and qualitative serological responses [25,39,83,116] can block DBP-DARC interaction and inhibit invasion [25,41,50,117]. Further studies revealed that the epitope targets of natural-acquired anti-DBPII inhibitory antibodies map to the dimer interface, suggesting that interference with dimerization is a major factor underlying anti-DBP NAI [25,114]. Naturally-acquired antibodies that inhibit this interaction associate with clinical immunity [118,119].

Typically, disease-causing infections are absent in most endemic areas with a high prevalence of DARC negativity [28,120–123]. However, there are increasing reports of *P. vivax* infections occurring in DARC-negative individuals [26–28]. The molecular basis of these infections is yet to be resolved, although it is suggested that these infections in people carrying the null alleles may be viable due to transient expression of DARC in erythroid bone marrow precursors cells of DARC-negative individuals [124]. Alternatively, duplications of the *dbp genes* may allow vivax to evade host anti-DBP humoral immunity by using a secondary invasion pathway [112,125–127].

**1.4.1.1 DBP-based Vaccine:** DBP is so far the leading vaccine candidate for targeting the disease-causing blood stages of *P. vivax* malaria. The development of a DBP-based vaccine candidate based on the Sal-1 allele has progressed through pre-clinical studies

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[54,75,79,128–131] and two recent Phase Ia human clinical trials (Table 1) [98,99]. In preclinical studies, immunogenicity studies in laboratory animals produce anti-DBPII antibodies, which inhibit DBPII-DARC interaction. Despite being a promising vaccine candidate, the presence of immunodominant variant epitopes in DBPII misdirects immune responses that compromises vaccine efficacy in eliciting high titer neutralizing antibody to conserved strain-transcending functional epitopes [25,55,74,75].

Naturally-occurring polymorphisms in DBPII confer significant differences in sensitivity to inhibition by immune antibodies [24,55,132], with evidence of DBPII variant-specific antibody responses that correlate with homologous and not heterologous protection [133]. Even a single amino acid substitution can alter the antigenic character of a parasite antigen thus providing compelling evidence that immune selection is a driving force for allelic variation. Thus, it is critical to have a rational vaccine design and immunization strategy to focus immune responses to conserved functional epitopes that are targets of naturally occurring strain transcending anti-DBP inhibitory antibodies. Similar to approaches used for ligands of other microbial pathogens, several basic approaches have been applied to circumvent the inherent bias of eliciting a strain-specific immunity in a DBPII vaccine, including:

(i) *Combination allele vaccines.* The objective is to create broader specificity by directing the bulk of antibody to common epitopes within the constituent alleles that make up the vaccine. A vaccine made up of antigenically-distinct *dbpII* alleles elicited a higher antibody response and broader specificity to the individual antigens used in vaccine compared to the single alleles, suggesting that multiple DBPII variant alleles may be required in a vaccine for broader coverage [128]. Other studies also showed that single malaria antigens tend to induce protection against the homologous but not heterologous parasite strains [24,84,134,135], while a multiple component/allele vaccine did overcome strain-specific immunity with *P. falciparum* pre-clinical vaccine PfAMA1 [136–138] and the pneumococcal vaccine [139].

(ii) *Immunofocusing.* Epitope specificity is critical for vaccine design against malaria antigens. Dominant B-cell epitopes within DBPII are polymorphic surface-exposed motifs, [25,54], which tend to create an inherent bias towards a strain-specific immune response and limit induction of immune response towards more conserved protective epitopes [140,141]. Some elite responders in endemic regions do produce broadly inhibitory anti-DBPII antibodies [74], an indication that conserved epitope targets of strain-transcending immunity are present in DBP. An engineered DBPII vaccine, termed DEKnull-2, lacking the immunodominant variant surface epitopes, elicited broadly functional anti-DBPII antibodies to shared epitopes on multiple *dbp* alleles and inhibited parasite invasion of reticulocytes *in vitro* [75,81,129,142]. Most importantly, DEKnull-2 was recognized by naturally acquired anti-DBPII inhibitory antibodies [56], indicating that the vaccine contained conserved epitopes associated with natural protective immune response to non-dominant epitopes. This supports the strategy of targeting immune responses to conserved functional epitopes to avoid induction of strain-specific responses to dominant variant epitopes.

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*(iii) Sub-unit DBPII vaccine.* Another practical approach to avoid strain-specific immunity is to identify minimal conserved epitopes within DBPII that elicit protective neutralizing antibodies against the intact native ligand. Stable strain-transcending immunity in elite responders in endemic regions offers the potential to identify such target epitopes to guide vaccine development [25,41,50,83]. These individuals are capable of producing high titers of invasion-inhibitory anti-DBPII antibodies [25,74,133]. By screening DBPII phage libraries or overlapping DBP peptides (mimotopes) with broadly inhibitory antibodies, subunits of the native refolded protein that are associated with protective immune response were identified [25,143]. Similarly, structure-based vaccinology is gaining ground as a new approach to identify functional epitopes for malaria vaccine development.

Structural studies revealed that DARC-binding residues are critical for DBPII-DARC dimerization upon receptor binding [114,144]. Furthermore, co-crystallization of DBP-DARC together with naturally acquired human [25,145] and vaccine-induced [146] inhibitory anti-DBP antibodies enabled identification of epitopes associated with these inhibitory antibodies. These data demonstrate that epitopes of naturally-acquired antibodies bind to the dimer interface and adjacent DARC binding groove thereby interfering with dimerization [25,114,144,145,147], while mAbs derived by vaccination with rDBPII (in mice and humans) have so far bound to epitopes within subdomain 3 (SD3) of DBPII, probably steric hindrance that indirectly hinders dimer formation [81,146].

Each strategy above has the potential to elicit antibodies that favor responses against conserved protective epitopes, with functional inhibition against broader allelic variants and diverse *P. vivax* strains, thus providing critical information on motifs to be included in a DBPII-based vaccine to induce broadly neutralizing and global strain-transcending protection.

*(iv) Viral vectored DBPII vaccine.* In a DBPII vaccine Phase Ia trial, an adenovirus serotype 36 (ChAd63) and a modified vaccinia virus ANKA (MVA) targeting DBPII-Sal1 strain were used as the delivery methods. These viral-vectored vaccines were well tolerated and demonstrated a safety profile in malaria-naïve adults, inducing DBPII specific antibodies including B cell and T cell responses [98]. Similarly, in a related Phase I randomized trial, a rDBPII vaccine formulated in GLA-SE adjuvant was safe and immunogenic in naïve adults [99]. Functional analysis demonstrated that anti-DBPII antibodies induced in both vaccine studies blocked binding of DBPII-DARC interaction *in vitro*. These studies further validate the vaccine potential of DBP and supports targeting parasite invasion ligands for vaccine development. However, further studies are required in *P. vivax* challenge models and/or ability to protect against natural infection in a Phase IIb trial in endemic regions.

**1.4.2 *Plasmodium vivax* EBP2—**A novel homolog of DBP, termed *P. vivax* erythrocyte binding protein 2 (PvEBP2) [71,111,148], was recently identified as a potential alternate invasion pathway ligand [100]. However, the lack of sequence similarity indicates that PvEBP2 is genetically distant from *P. vivax* DBP and other *Plasmodium* DBPs [111]. PvEBP2 is also under strong diversifying selection [149] but with lower SNPs relative to DBP [111], binds exclusively to reticulocytes, with a preference for immature ($CD71^{high}$) and Duffy-positive reticulocytes with only minimal binding to DARC negative reticulocytes

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[112]. Despite lack of direct evidence, its conserved features and epidemiological data suggests PvEBP2 might play a role in a DARC-independent invasion of reticulocytes [112,149]. Evidence for positive diversifying selection within the PvEBP2 ligand domain similar to that on DBPII is an indication of an important biological function including reticulocyte invasion and/or a target of acquired immunity [149]. Furthermore, the role of PvEBP2 as a protein ligand is supported by recent serological analysis [150,151]. PvEBP2 is a target of NAI following natural exposure to *P. vivax* infection and is suggested to be a possible serological marker for detecting recent *P. vivax* infections [152]. In addition, anti-PvEBP2 antibody levels are shown to be positively correlated with age, cumulative exposure and are associated with protection and correlate with reduced risk of clinical disease [119,153,154]. Even though PvEBP2 has emerged as potential vaccine candidate, there is a need for more functional studies to evaluate NAI and design strategies to replicate long-term protective anti-PvEBP2 immunity.

**1.4.3 *Plasmodium vivax* reticulocyte binding proteins—**The *P. vivax* reticulocyte binding proteins (PvRBPs) represent additional ligand families implicated as important in the process of reticulocyte invasion. The restricted preference of *P. vivax* to invade reticulocytes is attributed to the RBPs [86,155,156]. It is believed that PvRBPs target reticulocytes for invasion and then trigger the release of DBP from the micronemes for the final high-affinity binding and irreversible step of junction formation just before invasion [110]. Homologs of these proteins in other *Plasmodium spp.* are implicated in early phase of invasion and regulate different invasion pathways [155,157–159]. In *P. falciparum*, these reticulocyte-binding protein homologs referred to as PfRH ligands are well characterized vaccine candidates [160]. Given their essential role in the invasion process, and the vaccine potential of its homologs in other species, RBPs are considered attractive vaccine targets against asexual blood-stage development.

There are 11 members of the *rbp* gene families reported in *P. vivax:* five full genes (*rbp1a, rbp1b, rbp12a, rbp2, rbp2c*), three partial genes (*rbp1p1, rbp2p1, and rbp2p2*), and three pseudogenes (*rbp2d, rbp2e, rbp3*) [111,155,161,162]. Although functional redundancy is not yet defined for the PvRBPs, similar to its homologs in *P. falciparum* and other species, it is suggested that the multiple PvRBPs may provide *P. vivax* phenotypic variation allowing the plasticity to recognize and use different receptors and pathways for invasion [155,163,164]. Likewise, it is speculated that RBPs might play a role in a DARC-independent invasion pathway for *P. vivax* infections in DARC negative individuals [28,165]. Thus, the generation of effective immunity to the PvRBPs may require targeting conserved functional domains of multiple members of this multi-gene family [166–169].

Despite the essential role played by the PvRBP ligands in the invasion process, only a few (RBP 1a, 2a, 2b and 2c) have received much attention [111,118,153,170–173], with the molecular function of other members currently unknown. Members of the PvRBP1 family characterized so far reveal differential binding specificities for normocytes and /or reticulocytes (reviewed in [174]). The large sizes (250–350kDa) and the limited knowledge of the receptors for members of this family have limited the progress towards vaccine development. However, PvRBP2b is implicated as the primary determinant of reticulocyte

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tropism of *P. vivax* and binds to transferrin receptor 1 (CD71), which is highly expressed on reticulocytes but not on mature erythrocytes [173].

Similar to other asexual stage vaccine candidates, the PvRBPs are genetically diverse [160,175,176], suggesting that these adhesins are under diversifying selection and hence attractive immune targets [175–177]. Similar patterns of immune selection have been observed with other microbial adhesion molecules including PfRh2 [178], DBP [24,179], PfAMA-1 [180,181], which ultimately results in antigenically-distinct variants in the population and a bias towards strain-specific immunity. This diversity is believed to provide the parasite with a host immune escape mechanism favoring its survival.

Naturally-acquired antibodies to PvRBPs are prevalent in residents of endemic regions with different transmission intensities, and similar to DBP, anti-PvRBP serological response correlates with age, cumulative parasite exposure and clinical protection [112,150,170,182–185] and may even last longer than anti-DBP antibodies in the absence of repeated exposure [175]. Similarly, vaccine induced immunity against functional regions of the PvRBPs proteins are associated with inhibition of reticulocyte binding and merozoite invasion of reticulocytes *in vitro* [170,172,173,186] as is the case with PfRH ligands in *P. falciparum* [163,187–190]. These features further strengthen PvRBPs as prime targets for blood-stage vivax malaria.

**1.4.4 *Plasmodium vivax* Apical Membrane Antigen (PvAMA1)—**AMA1 is a unique multi-stage specific vaccine target important in the host cell invasion processes of sporozoites [191–193] and merozoites [192–194]. AMA1 is a highly conserved apicomplexan ligand that is sequestered in the microneme until invasion is initiated [195] and provides a unique opportunity as a multi-stage vaccine target. It is shown to work together with proteins of the rhoptry neck protein (RON) complex. A tight junction between RON2, a rhoptry neck protein, and AMA1 is essential for invasion [196]. Similar to DBP, crystallographic studies of AMA1 have shown that AMA1 polymorphisms flanking a hydrophobic receptor-binding motif that is formed by two PAN domains help evade immune responses [197–199].

Naturally acquired antibodies against PvAMA1 block receptor binding, similar to anti-DBPII immunity. However, polymorphic residues adjacent to the receptor-binding pocket motif for the RON2 receptor are associated with strain-specific immune responses and consequently induction of strain-specific immunity may be a challenge to strain-transcending vaccine efficacy. Although analysis of neutralizing antibody responses to PvAMA1 identified the 1F9 epitope as an attractive antigenic target, it is polymorphic and may be associated with strain-limited immune protection [197].

Similar studies from rodent and non-human primate show that PvAMA1 is a target for protective immune responses [84,200]. A recombinant vaccine based on domain II of PvAMA1 in different adjuvants formulations elicited significant anti-AMA1 antibody titers in mice. Most importantly, these vaccine-induced antibodies were inhibitory against reticulocyte invasion by different Asian *P. vivax* isolates [192,193]. Furthermore, immune-epidemiological studies show naturally-acquired antibodies to PvAMA1 even in cases of

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very limited exposures. Altogether, these data indicate that PvAMA1 can be considered among the most promising blood-stage antigens to be used as a subunit malaria vaccine [201,202].

#### 1.4.5 *Plasmodium vivax* Merozoite surface Protein (PvMSP1) and chimeric vaccine designs—

MSP1 is a large post-translationally processed protein tethered to the merozoite surface by a C-terminal glycosylphosphatidylinositol group on its 42 kDa fragment ($MSP1_{42}$) [203]. During invasion, a parasite-expressed subtilisin-like protease further cleaves this $MSP1_{42}$ fragment resulting in $MSP1_{19}$ and $MSP_{33}$ [204]. Some studies showed that MSP1 is highly immunogenic and naturally-acquired antibodies to the C-terminal fragment disrupted merozoite invasion [194,205,206]. Consequently, MSP1 was a component of a number of previous vaccine studies. Despite the early support as a vaccine target and data that identified $PvMSP1_{19}$ C-terminal fragment as a critical binding domain for erythrocytes, PvMSP1 lacks a clearly defined functional role and is now considered to be a part of the parasite's immune evasion mechanisms.

An important contribution of these earlier vaccine studies was the use of chimeric *P. berghei* that expressed $PvMSP_{19}$ to circumvent the lack of a challenge model which impeded preclinical testing of vivax blood-stage candidates. Mice immunized with a chimeric $PvMSP1_{19}$, generated a strong cytophilic antibody response along with CD4 and CD8 T cell responses against $PvMSP1_{19}$ making these modular chimeric T-cell epitopes as a promising strategy for inducing a protective immune response [194,207].

Other studies contributed development of a heterologous prime-boost strategy involving an adeno virus-vectored vaccine encoding two *P. vivax* blood-stage antigens PvAMA1 and $PvMSP1_{42}$ in *Aotus I. lemurinus* monkeys. Significant protection against blood-stage challenge in *Aotus* monkeys was observed, indicating the antigen delivery approach is safe and immunogenic [208]. While this regimen requires further development, the results emphasize the importance of heterologous/prime boost strategies for increasing the efficacy of blood-stage vivax vaccines.

### 1.5 Pre-erythrocytic vaccine

Pre-erythrocytic (PE) vaccines target the early stages from *Plasmodium* sporozoites infection until completion of liver stage development and breakthrough to blood-stage. This is an important bottleneck of *Plasmodium* life cycle and PE vaccines aim to prevent infection when the parasite burden is at its lowest. Early support for PE vaccines was based on studies with irradiated sporozoite vaccine strategies in the *P. berghei* rodent model that demonstrated sterile protection [209,210]. Subsequent studies in monkeys and in humans supported the potential for PE vaccine development [211,212]. However, potentially significant production, and logistical challenges have limited irradiated (whole) sporozoite vaccine approach, with much research turned to subunit vaccines. Even as PE vaccines have great potential, the requirement to induce sterile protection has long been considered a major weakness. In addition, PE vaccination against *P. vivax* will need to be equally effective against hypnozoite development.

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#### 1.5.1 *Plasmodium vivax* Circumsporozoite protein (PvCSP)—

The CSP is the major surface protein of *Plasmodium* sporozoites and considered promising vaccine targets against PE stages of malaria parasites since they are directly exposed to host immune antibodies as sporozoites migrate to the liver during the early phase of infection (Figure 1). In *P. vivax* NAI and controlled human malaria infections, antibodies to PvCSP correlate with short-term protection [213]. However, unlike the rodent malaria parasites and *P. falciparum*, PvCSP is genetically diverse with two distinct strain types, VK210 and VK247, differing in central repeat region [214].

Despite licensure of a PfCSP vaccine, Mosquirix™ [215], only a few pre-clinical and human clinical trial studies of *P. vivax* PE vaccine candidates primarily evaluating CSP-based vaccines have been reported [216–222]. In addition, advancement has been hindered by the inherent complications related to the nature of the relapsing *P. vivax* infections. The vaccines evaluated so far include synthetic peptides, different types of recombinant proteins, and a chimeric PvCSP as summarized in Table 1 [68,223–226]. Although these *P. vivax* vaccines have been safe, they are poorly immunogenic and failed to elicit protection against infection by sporozoite challenge. Further optimization with different antigens and/or adjuvant formulation is required to improve its efficacy.

A chimeric PE vaccine, vivax malaria protein 1 (VMP001), incorporated the N- and C-terminal regions and truncated repeat regions from both VK210 and VK247 strains of PvCSP. VMP001 induced high titer antibodies in mice, using Montanide ISA 720 [227,228] as well as a strong cellular immune response with synthetic TLR4 (GLA-SE) [229] as an adjuvant. Similar antibody and cellular immune responses were observed in monkeys immunized with VMP001-GLA-SE [230] and VMP001 formulated in TLR9 agonist and protected against challenge infection with *P. vivax* sporozoites [231]. In a related study, VMP001 conjugated to a lipid enveloped polymer poly (lactide-*co*-glycolide) acid nanoparticles (VMP001-NP) adjuvanted in MPLA, elicited a balanced Th1/Th2 humoral response in mice with enhanced avidity and affinity toward the domains within PvCSP implicated in protection and were able to agglutinate live *P. vivax* sporozoites [232].

In a Phase 1/2a clinical trial, VMP001 formulated in the GSK Adjuvant System AS01B (VMP001/AS01B) was well tolerated and immunogenic, with volunteers generating robust humoral and cellular (CD4+ T cell) immune responses to the vaccine antigen (Table 1). Though the vaccine did not induce sterile protection, there was a significant delay in time to parasitemia observed in 59% of vaccinated subjects [68].

Significant progress in VLP vaccine design have led to increased PvCSP immunogenicity in recent years [220,221]. An innovative design Rv21, similar to RTS,S, improved immunogenicity of PvCSP VLP approach even at low doses (5 μg) when combined with Matrix-M adjuvant [220]. Another VLP approach using Qβ-peptides from *E. coli* bacteriophage showed similar outcomes by coupling fragments of PvCSP VK210 formulated in Matrix-M adjuvant [221]. Similar to earlier studies with blood-stage vaccine studies, an important advance in evaluating protective efficacy has been the introduction of transgenic *P. berghei* expressing PvCSP [219–221,233]. Upon challenge with these PvCSP

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transgenic sporozoites, improved protective efficacy was observed in these approaches [220,221].

#### 1.5.2 *Plasmodium vivax* Thrombospondin-related anonymous protein (PvTRAP)—

PvTRAP, also known as sporozoite surface protein-2 (SSP2), is a conserved microneme protein vaccine target involved in sporozoite motility and enables invading sporozoites to bind heparin sulfate molecules on hepatocytes [234,235]. Very few preclinical studies have evaluated the efficacy of a PvTRAP vaccine. Long synthetic peptides within the N-terminal region of the PvTRAP binding motif administered with both Freund and Montanide ISA 720 adjuvants to BALB/c mice and *Aotus* monkeys induced antibodies reactive to *P. vivax* sporozoites [236]. In *Aotus* monkeys, the vaccine formulated in CFA/IFA induced higher titers compared to Montanide ISA 720 and partial protection was observed upon intravenous challenge with $2\times10^4$ *P. vivax* sporozoites. In a related study, a heterologous prime-boost vaccine study with recombinant ChAd63 and MVA both expressing PvTRAP, respectively, induced high titer antibodies and high T cell response in immunized mice, which partially protected against challenged infection with a chimeric *P. berghei* expressing PvTRAP [237]. These results show PvTRAP as a potential vaccine candidate. However, further assessments in different vaccine strategies and animal models is still required.

#### 1.5.3 *Plasmodium vivax* cell-traversal protein for oökinetes and sporozoites (PvCelTOS)—

CelTOS is a conserved microneme protein in all *Plasmodium* spp. and has an essential function in sporozoites and oökinete cell traversal motility. PvCelTOS released from micronemes, targets the inner-leaflet of cell membranes allowing parasites to exit cells in mosquitoes and human hosts [238]. This function is essential for parasite motility and establishing successful infections, making it an ideal multi-stage vaccine candidate to prevent infection and transmission. In addition, PvCelTOS shows very limited genetic diversity among global clinical isolates [239].

Mice immunized with recombinant PvCelTOS induced both humoral and cell-mediated immune responses that reduced infection, while passive transfer of anti-PvCelTOS antibodies conferred protection to mice [240,241]. Efficacy with PvCelTOS vaccines with different strategies to boost immunogenicity have produced mixed results even with induction of high antibody titers [242,243]. Overcoming an important limitation of vivax malaria research efficacy in immunized mice was assessed with challenge infections of PvCelTOS-expressing transgenic *P. berghei* sporozoites. Promising results were provided by viral vectored PvCelTOS leading to sterile protection in 30% of ChAd63- PvCelTOS and ChAd63-VLP groups versus 10% ChAd63-MVA [244]. Further supporting a PvCelTOS vaccine, immunizations of human volunteers with irradiated sporozoites induced sterile immunity [245,246] and a strong anti-PvCelTOS response that correlated with protection [247].

### 1.6 Sexual-stage antigens

The current strategies to control malaria are inadequate to eradicate vivax malaria, especially relapse infections and transmission. Therefore, it is of importance to develop new tools to

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reduce the reproduction rate of the parasite resulting from relapse. Transmission-blocking vaccines (TBVs) are extremely important interventions to address this issue and reduce malaria transmission [248–250]. As discussed above for CelTOS, TBV antibodies against the parasite oökinete or mosquito midgut surface (oökinete receptors) antigens taken up with the bloodmeal can prevent the oökinete invasion of the midgut. Therefore, TBV antibodies can abrogate the cascade of secondary infections resulting in reduced parasitic reproducing rate and further transmission [249–251]. Below TBV candidates other than PvCelTOS are discussed.

The *P. vivax* oökinete surface protein (Pvs25) is a leading malaria transmission-blocking vaccine (TBV) candidate based on its high immunogenicity in animal models, transmission-blocking activity of antibodies elicited in clinical trials and high conservation among *P. vivax* isolates from endemic areas. In a single blinded, dose escalating, controlled Phase I study, Pvs25 expressed in *S. cerevisiae* and formulated in Montanide ISA 51 was terminated due to systemic reactogenicity (Table 1) [252]. However, the same antigen adjuvanted with alhydrogel, showed mild adverse reactions indicating the importance of adjuvant choice in designing clinical trials. Anti-Pvs25 vaccine-induced antibody responses were functional as they showed transmission-blocking activity in mosquito feeding assays that correlated to antibody titers [253].

In a related study in mice, Pvs25 and another TBV, Pvs28, formulated in alum induced antibodies that arrested the growth of *P. vivax* in mosquitoes fed with infected blood meal [254]. A field study showed that naturally acquired antibodies from *P. vivax* infected patients from regions with differential transmission intensities had anti-Pvs230 antibody responses [255]. Therefore, this vivax candidate, Pvs230D1-EPA, an orthologue of the *P. falciparum* candidate Pfs230D1- EPA has been garnering attention for clinical trials [256].

Another leading TBV candidate is the *Anopheline* alanyl aminopeptidase N (*An*APN1), a mosquito midgut surface protein, which mediates *Plasmodium* establishment in the mosquito [251,257–259]. Studies have shown that anti-*An*APN1 antibodies completely inhibit the development of naturally-circulating gametocytemic isolates of *P. falciparum* in Cameroon from infected volunteers in two independent transmission seasons [251]. A related study also evaluated cross-species efficacy of these antibodies against *P. vivax* from infected volunteers in Thailand, with only partial inhibition observed, suggesting a role of polymorphisms in immune response to *An*APN1 across different vectors [251].

The mechanism of action of TBVs in general is restricted solely to the activity of inhibitory antibodies [251,257,259]. Therefore, a successful TBV candidate should induce potent, high-titer antibodies that may be sustainable for one season of transmission.

## 2. Conclusions

*P. vivax* vaccine development has been slowed by the complex nature of the parasite's biology, technical challenges due to the lack of *in vitro* culture, and limited access to experimental models to screen new vaccine candidates. Despite these challenges, vivax vaccine research has slowly progressed in recent years with development of transgenic

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rodent malaria models for some vaccine targets, a humanized mouse model and a new culture platform for liver stage studies, and the development of *P. knowlesi* lines adapted to human blood-stage culture. Recent advances in the use of these new platforms, structural vaccine design and better vaccine delivery platforms, will accelerate candidate identification and screening and enhance vaccine efficacy. With new adjuvants and delivery systems, there is a renewed interest in reestablishing the current vaccine candidates to accelerate them to clinical trials and eventual licensing. Engineered viruses tagged with recombinant proteins and VLPs could ultimately increase immunogenicity, safety and efficacy. These platforms can stimulate the antibody and cell-mediated responses and the same time serve as compatible adjuvants for human use, thus increasing the efficacy of these previously explored candidates. Also, heterologous prime-boost strategies could result in persistent immune response over longer periods of time, while multivalent-multistage vaccines will help prevent disease and transmission. Considering effective *P. vivax* vaccines will be essential for malaria eradication, these newer strategies will be remarkable to progress towards the clinical assessment of these vivax vaccine candidates.

## 3. Expert Opinion

*Plasmodium* invasive stages display an array of surface antigens important for initiating infection, promoting disease and enabling transmission and are, therefore, key targets for vaccine intervention. Epitope specificity is critical for vaccine efficacy. Thus, a rational vaccine design that focuses on the immune response to functionally conserved epitopes is essential to enhance induction of broadly neutralizing strain-transcending protective immunity. Such a vaccine should include functional epitopes of multiple antigens from different stages of the parasite life cycle.

Optimal immunogenicity and safety are critical outcomes of any effective vaccine. Hence, the study design, biological variables, delivery methods and route of administration are key factors to take into consideration.

Equally important are methods for novel candidate identification and validation, especially animal models for *in vivo* studies. Despite the availability of surrogate assays and more advanced *in vitro* parasite culture methods, including the use of chimeric/transgenic parasites for vivax vaccine studies, immunization challenge studies in primates remain the more direct and reliable measure of vaccine efficacy *in vivo*. All these factors are subject to availability of adequate funding, which has been a limiting factor for vivax vaccine studies. A full summary of challenges to *P. vivax* vaccine development and an action plan are summarized in Table 2.

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## Article Highlights

- *Plasmodium vivax* malaria remains an important public health problem, yet there is no vaccine to prevent transmission and disease.
- There is a need for novel candidate identification and validation.
- A multivalent, multi-stage vaccine candidate elicits a stronger and robust immune response that can provide cross-strain protection.
- Immune focusing and structure-based vaccine design can enhance vaccine efficacy.
- Heterologous prime-boost strategy may induce a persistent, long-lasting immune response.
- Different platforms such as viral-vectored subunit vaccine candidates and virus-like particles can be effective tools for vaccine delivery.

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**Table 1:**

(a) An overview of selected promising *Plasmodium vivax* vaccine candidates that have progressed into clinical trials with their correlates of protection and vaccine outcomes

| Candidate Vaccine/ platform | Host (Clinical & preclinical studies) | Correlates of protection | Outcome | Progress |
|---|---|---|---|---|
| **Blood-stage candidates** | | | | |
| PvDBPII | Phase 1a (UK adults) NCT01816113 | Antibodies against PvDBPII | No serious adverse events. Across all vaccinated individuals median polyclonal IgG $EC_{50}$ values were comparable in PvDBPII-DARC *in vitro* binding. | Completed [1] |
| PvDBPII/GLA-SE | Phase 1 (34 Indian males) CTRI/2016/09/007289 | PvDBPII-specific antibodies | No adverse events were observed. 50μg treatment, 82% mean binding-inhibitory activity was observed in PvDBPII-DARC binding assay at day 180 | Completed by PATH/MVI [2] |
| **Pre-erythrocytic candidates** | | | | |
| *P. vivax* irradiated sporozoites | Phase I/IIa (Colombia, adults) NCT01082341 | *Anti- Pv*CSP IgG1 levels correlates with protection | Partial protection observed in 42% of the Fy+ volunteers. | MVDDC, NHLBI/NIH [3] |
| PvCSP- derived long synthetic peptides, Allohydrogel Montanide ISA 720 51 | Phase 1b (Colombia adults) NCT01081847 | Antibody responses | Transient pain at injection site and induration occurred in the Montanide 50 μg group, PBMCs from all groups secreted IL-5 and IFNγ. | Completed by MVDDC [4] |
| VMP001-AS01B | Phase I/IIa (USA, adults) NCT01157897 | Antibody titers | Significant delay in infection patency observed in 59% of vaccinated subjects. | Ongoing studies by WRAIR, MVI, GSK [5] |
| **Sexual-stage candidates** | | | | |
| Pvs25- Recombinant Pvs25, Montanide ISA51 | Phase I (USA, adults) NCT00295581 | Antibody responses correlated with transmission blocking activity | High reactogenicity observed | Completed by DIR/ NIAID, JHSPH [6] |

**PvCSP-** *Plasmodium vivax* circumsporozoite protein

**PvDBPII-** *P. vivax* Duffy Binding protein region II

**PvDBPII/GLA-SE-** recombinant *P. vivax* DBP region II formulated with glucopyranosyl lipid adjuvant-stable emulsion

**DARC-** Duffy antigen receptor for chemokines

**VMP001-** *Vivax* malaria protein 001

**PBMC-** Peripheral blood mononuclear cells

**cPvMSP1-** Chimeric *Plasmodium vivax* Merozoite surface protein 1

**Pvs25-** Ookinete surface protein

**LLPCs-** Long-lived plasma cells

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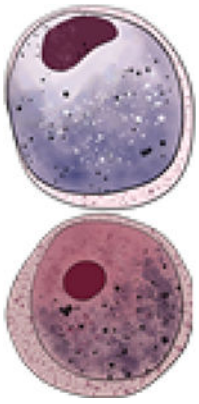
**Table 1:**

(b) Potential *Plasmodium vivax* vaccine candidates targeting different stages of the life cycle.

| Different stages of life cycle | | Potential *P. vivax* vaccine candidates |
|---|---|---|
| Merozoite | | PvDBP [1,2,7–9]<br>PvRBPs [10,11]<br>PvEBP2 [12]<br>PvMSP1[13–18]<br>PvAMA1[14] |
| Sporozoite | | PvCSP [4,5,19–35]<br>PvTRAP [22,35–37]<br>PvCelTOS [34,35,38–40] |
| Macrogametocyte<br>Microgametocyte |  | Pvs25 [6,41,42]<br>Pvs28 [42]<br>Pvs230 [43,44] |

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| Different stages of life cycle | | Potential *P. vivax* vaccine candidates |
|---|---|---|
| Oökinete | | |

**PvDBP-** *P. vivax* Duffy Binding protein region

**PvRBPs-** *P. vivax* Reticulocyte binding proteins

**PvEBP2-** *P. vivax* Erythrocyte binding protein2

**PvMSP1-** *P. vivax* Merozoite surface protein 1

**PvAMA1-** *P. vivax* Apical membrane antigen 1

**PvCSP-** *P. vivax* Circumsporozoite protein

**PvTRAP-** *P. vivax* Thrombospondin-related anonymous protein

**PvCelTOS-** *P. vivax* Cell-traversal protein for oökinetes and sporozoites

**Pvs-** *P. vivax* sexual stage

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**Table 2:**

Overview of *P. vivax* vaccine development: challenges, problems, potential actions, and 5-year plan to overcome these impediments.

| Challenges | Problem | Actions | 5-year plan |
|---|---|---|---|
| **Blood-stage** | | | |
| Clinical infection of Duffy-negative individuals | *P. vivax* blood-stage merozoites use alternative ligands to invade Duffy-negative individuals | Target multiple epitopes essential for merozoite invasion | Identify novel ligands that facilitate merozoite invasion of Duffy-negative individuals |
| Low parasitemia and asymptomatic infections (lack of febrile malaria) | Parasite's preference for Reticulocyte and host factors masks clinical infections | Better surveillance systems *i.e.* detection of sub-patent parasitemia | Develop tools to culture *in vitro* |
| **Pre-erythrocytic stage** | | | |
| Preventing/eliminating dormant hypnozoites | *P. vivax* hypnozoite dormancy and reactivation | Research into hypnozonticidal strategies *i.e* drugs, PE vaccines | Establish *in vitro* liver stage assays to interrogate novel antigens/ targets such as ILSDA |
| **Sexual stage** | | | |
| Targeting early formation of gametocytes | *P. vivax* sexual reproductive efficiency | Identifying blood-stage and gametocyte vaccine targets to prevent transmission | Develop *in vitro* tools to facilitate gametocyte cultures |
| Poor immunity conferred from natural exposure | Multiple vaccine doses within a transmission season could affect vaccine efficacy | Improving on adjuvant formulation, vaccine delivery and antigen selection to develop protective immune responses | Develop a potent single dose TBV candidate |
| **Overall challenges** | | | |
| Lack of *P. vivax* sporozoites or merozoites | Parasite availability has hampered clinical trials and basic science research. | Use of *in vivo* models cultivate parasites and develop CHMI models (sporozoite inoculation, mosquito-bite challenges, and blood-stage inoculation). | Increase access to parasites from endemic regions. Overcome logistical challenges of mosquito-bite challenges by inoculating IBSM to test vaccine efficacy. |
| Overcoming parasite immune escape mechanisms | *P. vivax* genetic diversity and antigenic polymorphism | Identify conserved functional epitopes of neutralization and possibly target parasite using a multiantigen vaccine approach | Generate monoclonal antibodies (mAbs) that can neutralize multiple strain as passive immunization |
| Scarce genetic tool kit | Forward and reverse genetics tools are limited | Use transgenic or chimeric parasites to infer functions of *P. vivax* genes | Develop *in vitro* cultures, Use NHP models |
| Dearth of funding for *P. vivax* research | Inadequate funding for *P. vivax research* compared to *P. falciparum* | Increase funding to novel *P. vivax* vaccine research | |

**mAbs**-Monoclonal antibodies

**PE**-Pre-erythrocytic

**ILSDA-**In vitro Liver-stage Development assay

**PEV-** Pre-erythrocytic vaccine

**TBV**-Transmission blocking vaccine

**CHMI-**Controlled human malaria infections

**IBSM-** Infected blood-stage malaria

**NHP**-Non-human primate