Development of vaccines for *Plasmodium vivax* malaria

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A B S T R A C T

*Plasmodium vivax* continues to cause significant morbidity outside Africa with more than 50% of malaria cases in many parts of South and South-east Asia, Pacific islands, Central and South America being attributed to *P. vivax* infections. The unique biology of *P. vivax*, including its ability to form latent hypnozoites that emerge months to years later to cause blood stage infections, early appearance of gametocytes before clinical symptoms are apparent and a shorter development cycle in the vector makes elimination of *P. vivax* using standard control tools difficult. The availability of an effective vaccine that provides protection and prevents transmission would be a valuable tool in efforts to eliminate *P. vivax*. Here, we review the latest developments related to *P. vivax* malaria vaccines and discuss the challenges as well as directions toward the goal of developing highly efficacious vaccines against *P. vivax* malaria.

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1. Introduction – why a vaccine for *Plasmodium vivax* is needed

The last 10 years have seen a dramatic reduction in the burden of malaria, with many countries in the Asia-Pacific and the Americas seeing reduction of >90% in the number of clinical cases [1]. As a consequence, 34 countries are actively attempting to eliminate malaria and the leaders of Central American and East Asian countries have declared their intention to eliminate malaria from their regions by 2020 and 2030, respectively [2,3]. In parallel to this reduction in overall incidence, a pronounced shift in species composition has been observed with *P. vivax* now the predominant *Plasmodium* spp. in vast majority of countries outside Africa [1].

While the reasons for the shift in species composition are not entirely clear, the main reasons are likely to be related to the following aspects of *P. vivax* biology: (i) its ability to relapse from long-lasting, dormant liver stages (the hypnozoites) [4] and (ii) its high transmission potential due to early and continuous production of gametocytes, high infectivity to mosquitoes and shorter development cycle in the vector host compared to other *Plasmodium* spp. [5]. Relapses from emerging hypnozoites may contribute up to 80% of all *P. vivax* blood stage infections [6, Mueller et al., unpublished data] and if not treated appropriately can cause new blood-stage infections months to a few years after the primary infection [7]. As there are no diagnostic tests that can diagnose dormant liver-stage infections, undetectable hypnozoite carriers are an important potential source of re-introduction of *P. vivax*. Unfortunately, the only currently available drug to clear hypnozoites is primaquine, which can cause severe hemolysis in people with glucose 6-phosphate dehydrogenase (G6PD) deficiency [8]. The only other anti-hypnozoite drug in clinical development, Tafenoquine, carries the same risks of hemolysis as primaquine. [9] Given the lack of a cheap, reliable point-of-care G6PD test, many *P. vivax* patients do not currently receive adequate anti-hypnozoite treatment.

At all levels of transmission, asymptomatic and sub-microscopic *P. vivax* infections are very common [10]. Most if not all of these infections produce gametocytes [11] and likely contribute substantially to maintaining *P. vivax* transmission [12]. These infections will not be efficiently cleared unless specifically targeted with active case detection and/or mass drug administration, which is logistically challenging and would again require treating large numbers of individuals with asymptomatic infections or even those without blood stage infections with primaquine to clear hypnozoites.

Despite the high frequency of asymptomatic carriers, numerous clinical studies in diverse geographical locations have now convincingly demonstrated that infection with *P. vivax* can lead to severe disease and death [13–17]. *P. vivax* infections are, thus, not benign as considered previously and new strategies to prevent...
P. vivax malaria are urgently needed. These are particularly important, where weak health systems and/or drug resistance make efficient cases management difficult.

An additional challenge to the elimination of P. vivax lies in the biology of its key vectors [18,19], many of which are outdoor bitting and less anthropophilic than the main African P. falciparum vectors [20]. As a result, traditional vector control measures such as indoor residual spraying and insecticide-treated nets are less effective against P. vivax control in non-African settings. The faster development of P. vivax in the mosquito further impairs the effectiveness of vector control as a larger reduction in mosquito life-span needs to be achieved for a comparable impact on P. vivax transmission.

Vaccines against P. vivax with high efficacy could help address several gaps in our current tool kit and greatly facilitate elimination of P. vivax. A pre-erythrocytic vaccine that prevents infections would not only reduce the number of primary infections but by preventing the establishment of hypnozoites, also reduce the risk of multiple relapses contributing to continued transmission. Given that a single mosquito bite may lead to 1 primary infection and up to 4 relapses (Mueller et al., unpublished data) it is likely that a pre-erythrocytic vaccine would have a larger effect on P. vivax transmission compared to P. falciparum transmission. Similarly, a blood-stage vaccine that blocks parasite growth would not only prevent disease and death but may also dramatically shorten the duration and lower the density of blood-stage parasitemia and associated gametocytemia, thus greatly reducing the transmission potential of P. vivax-infected individuals. Lastly, a transmission blocking vaccine would directly block infection of mosquito vectors and thereby not only accelerate the interruption of transmission but also safeguard against re-emergence of transmission in areas where elimination has been achieved.

2. Feasibility of developing a vaccine for P. vivax vaccine

The epidemiology of P. vivax provides strong indication that the development of P. vivax vaccines should be feasible. Under natural exposure, clinical immunity to P. vivax is acquired significantly more rapidly both in high transmission settings such as Papua New Guinea, where the incidence of P. vivax malaria starts declining in the 2nd year of life [21] and clinical disease is virtually absent in children >5 years while P. falciparum disease remains common in primary school children [22], as well as in lower transmission settings such as Thailand [23], Sri Lanka [24] and Vanuatu [25]. Similarly, in malaria therapy trials effective clinical immunity, even against heterologous challenge, was often observed after as few as 1–5 P. vivax infections [26,27]. Semi-immune individuals generally exhibit very good control of blood-stage parasitemia resulting in significant numbers of sub-microscopic infections even in low transmission settings [10] indicating development of effective blood stage immunity.

There is less evidence for naturally acquired immunity to pre-erythrocytic stages. In limited trials with irradiated sporozoites, protection was achieved but only in subjects who received very high numbers of sporozoites [28]. There is however good evidence for naturally acquired transmission-blocking immunity. Early studies in Sri Lanka showed that sera from P. vivax infected patients were very effective at suppressing infection of the parasites in mosquitoes and that the effect was dependent on antibodies against P. vivax gametocytes and gametes [29]. Similar transmission blocking effects were later confirmed in studies in Thailand and Colombia [30,31]. Together, these observations provide strong evidence that the development of effective P. vivax vaccines should be possible.

3. Target Product Profile (TPP) for a P. vivax vaccine

Table 1 provides a Target Product Profile (TPP) for a possible vaccine for P. vivax malaria. The TPP describes the basic parameters, both ideal as well as minimally acceptable, which the vaccine should have. Industry uses such TPPs to guide product development. A vaccine for P. vivax will likely be needed in settings where transmission rates are low and the entire population is non-immune. As a result, the vaccine will need to target the entire population not just infants or children. Ideally a P. vivax vaccine would both prevent P. vivax malaria with high efficacy (>90%) and also block P. vivax transmission. However, a minimally acceptable target would be a vaccine that protects against P. vivax malaria with high efficacy (at least 80%). The TPP also describes other ideal and minimally acceptable targets for parameters such as product presentation, dosage schedule, co-administration and storage conditions that are relevant for the design and development of a viable vaccine. It would be useful to keep the TPP in mind as one reviews current efforts toward development of vaccines for P. vivax malaria.

4. Leading P. vivax vaccine candidates

Efforts to develop vaccines against different stages of P. vivax have been underway over past two decades. Here, we will review recent progress on leading pre-erythrocytic, blood stage and transmission blocking P. vivax vaccine candidates. Table 2 summarizes key information about the various vaccine candidates discussed in this review. Due to restrictions of space, this review is limited to P. vivax vaccine candidates that have reported progress toward clinical development and does not cover all the P. vivax antigens that are at an early research stage of vaccine development. A more comprehensive survey of malaria vaccine candidates (both P. falciparum and P. vivax) can be found in the ‘Rainbow Tables’ of global malaria vaccine projects [32].

4.1. Pre-erythrocytic stage P. vivax vaccine candidates

4.1.1. P. vivax circumsporozoite protein (PvCSP)

Of the P. falciparum vaccine RTS,S, which is based on the P. falciparum circumsporozoite protein (PfCSP) has consistently demonstrated protection in the range of 30–50% against P. falciparum malaria in Phase II and Phase III trials conducted in children and infants [33,34]. These studies clearly demonstrate that it is possible to elicit partial protection against P. falciparum malaria by immunization with constructs based on CSP. These observations support the development of a vaccine for P. vivax malaria based on the homologous P. vivax CSP (PvCSP).

Yadava et al. have constructed a recombinant PvCSP chimeric antigen (VMP001) that contains the repeat (R) sequences of the two major PvCSP alleles, VK210 (9 repeats) and VK247 (1 repeat) fused to N- and C-terminal conserved regions of PvCSP from the VK210 allele [35]. VMP001 formulated with AS01B was tested in a Phase I/IIa study in malaria naïve healthy volunteers [36]. It was found to be safe and induced high titer antibody responses. Although the vaccine showed an increase in pre-patent period in some immunized volunteers, it did not induce sterile protection against P. vivax sporozoite challenge in any of the volunteers [36].

The immunogenicity of E. coli produced VMP001 was compared with CSV-S, a RTS,S-like vaccine candidate that contains a mixture of free hepatitis B surface antigen (HBsAg) and S. cerevisiae produced fusion protein containing VMP001 sequences fused to HBsAg. CSV-S induces higher levels of PvCSP-specific antibodies than soluble VMP001 when both were formulated with the adjuvant AS01 [37]. Efficacy of VMP001 formulated with CpG 10104 + Montanide ISA720 has also been tested by challenging
Table 1
Target Product Profile for *P. vivax* vaccine.

<table>
<thead>
<tr>
<th>Item</th>
<th>Desired target</th>
<th>Minimally acceptable target</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indication</strong></td>
<td>Prevention of <em>P. vivax</em> malaria and interruption of <em>P. vivax</em> transmission</td>
<td>Prevention of <em>P. vivax</em> malaria</td>
</tr>
<tr>
<td><strong>Target Population</strong></td>
<td>All age groups, pregnant women, immuno-deficient persons</td>
<td>Healthy persons including infants, children and adults</td>
</tr>
<tr>
<td><strong>Route of Administration</strong></td>
<td>Oral or IM or SC</td>
<td>IM or ID or SC</td>
</tr>
<tr>
<td><strong>Product Presentation</strong></td>
<td>Single dose, auto-disposable, pre-filled device</td>
<td>Lyo/liquid formulation. Vials may be accompanied with separate vial containing adjuvant. May require reconstitution</td>
</tr>
<tr>
<td><strong>Dosage Schedule</strong></td>
<td>Single dose, no booster required</td>
<td>2 doses, booster may be required 4–6 months after 2nd dose</td>
</tr>
<tr>
<td><strong>Efficacy</strong></td>
<td>Greater than 90% efficacy against <em>P. vivax</em> malaria and reduces <em>R</em>_efficiency &lt; 1 for all <em>P. vivax</em> strains for at least 2 years</td>
<td>Greater than 80% efficacy against <em>P. vivax</em> malaria for at least 1 year</td>
</tr>
<tr>
<td><strong>Co-administration</strong></td>
<td>Non-inferiority of responses to EPI vaccines when co-administered with other vaccines</td>
<td>Stand-alone product not co-administered with other vaccines</td>
</tr>
<tr>
<td><strong>Shelf-life</strong></td>
<td>36 months</td>
<td>24 months</td>
</tr>
<tr>
<td><strong>Storage</strong></td>
<td>Ambient temperature and withstand freeze–thaw</td>
<td>WHO prequalified, registered with FDA, EMEA</td>
</tr>
<tr>
<td><strong>Product registration</strong></td>
<td>WHO prequalified, registered with FDA, EMEA</td>
<td>WHO prequalified or competent national regulatory agency</td>
</tr>
</tbody>
</table>

IM, intramuscular; SC, subcutaneous; ID, intradermal; *R*_efficiency, effective reproduction rate upon introduction of vaccine; EPI, expanded program of immunization; WHO, World Health Organization; FDA, Federal Drug Administration; EMEA, European Medicines Agency.

Table 2
*P. vivax* vaccine candidates under development.

<table>
<thead>
<tr>
<th>Vaccine candidate</th>
<th>Development Phase</th>
<th>Life cycle stage</th>
<th>Antigen</th>
<th>Delivery system</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VM001</td>
<td>Phase I/II</td>
<td>Liver stage</td>
<td>PvCSP</td>
<td>Rec. protein-AS01B</td>
<td>[35,36]</td>
</tr>
<tr>
<td>CSV-5.S</td>
<td>Pre-clinical</td>
<td>Liver stage</td>
<td>PvCSP</td>
<td>HBsAg fusion-AS01B</td>
<td>[37,38]</td>
</tr>
<tr>
<td>PvCSP-LSP</td>
<td>Phase I</td>
<td>Liver stage</td>
<td>PvCSP</td>
<td>Synthetic peptides- Montanide ISA 720</td>
<td>[41–41]</td>
</tr>
<tr>
<td>ChAd63-PVTRAP/MVA-PVTRAP</td>
<td>Phase I</td>
<td>Liver stage</td>
<td>PvTRAP</td>
<td>Prime-boost, viral vectors</td>
<td>[44]</td>
</tr>
<tr>
<td>PdBPII</td>
<td>Pre-clinical</td>
<td>Blood stage</td>
<td>PdBPI</td>
<td>Rec. protein-GLA-SE</td>
<td>[56,57]</td>
</tr>
<tr>
<td>PdBPII-DED*</td>
<td>Pre-clinical</td>
<td>Blood stage</td>
<td>PdBPI</td>
<td>Rec. protein</td>
<td>[59]</td>
</tr>
<tr>
<td>ChAd63-PVDBPI/MVA-PVDBPI</td>
<td>Pre-clinical</td>
<td>Blood stage</td>
<td>PdBPI</td>
<td>Prime boost, viral vectors</td>
<td>[60]</td>
</tr>
<tr>
<td>PvMSF1p</td>
<td>Pre-clinical</td>
<td>Blood stage</td>
<td>PvMSF1</td>
<td>Rec. protein-Montanide ISA720</td>
<td>[63,64]</td>
</tr>
<tr>
<td>ChAd63-PVAMA1/MVA-PVAMA1</td>
<td>Pre-clinical</td>
<td>Blood stage</td>
<td>PVAMA1</td>
<td>Prime-boost, viral vectors</td>
<td>[68]</td>
</tr>
<tr>
<td>PVAMA1</td>
<td>Pre-clinical</td>
<td>Blood stage</td>
<td>PVAMA1</td>
<td>Rec. protein-adjuvant</td>
<td>[69]</td>
</tr>
<tr>
<td>Pv25S</td>
<td>Phase 1</td>
<td>Trans. stage</td>
<td>Pv25S</td>
<td>Rec. protein-alhydrogel; Rec. protein-Montanide ISA 51</td>
<td>[72,73]</td>
</tr>
<tr>
<td>Pxs28</td>
<td>Pre-clinical</td>
<td>Trans. stage</td>
<td>Pxs28</td>
<td>Rec. protein-adjuvant</td>
<td>[70]</td>
</tr>
<tr>
<td>AnAPN1</td>
<td>Pre-clinical</td>
<td>Mosquito midgut Ag</td>
<td>AnAPN1</td>
<td>Rec. protein-adjuvant</td>
<td>[74]</td>
</tr>
</tbody>
</table>

*Aotus nancymae* monkeys with *P. vivax* sporozoites. Sixty six percent of vaccinated monkeys were completely protected following challenge with *P. vivax* sporozoites. Antibodies against the repeat region of PvCSP showed a statistical association with protection [38].

VMP001 has also been formulated in nanoparticles formed as interlayer crosslinked multimellar lipid vesicles (iCMVs) composed of poly(lactide-co-glycolide acid (PLGA) and a lipid membrane [39,40]. Immunization with the resultant VMP001-iCMVs formulated with monophosphoryl lipid A (MPLA) induced potent humoral responses that lasted for over 1 year in mice at 10-fold lower doses when compared with formulations of soluble VMP001 [39]. Additionally, VMP001-iCMVs enhanced germinal center formation at low doses of antigen where no GC induction occurred with soluble protein [39]. Antibodies raised by VMP001-NP vaccinations also exhibited enhanced avidity and affinity against the PvCSP repeat domains [39].

Based on the identification of B and T cell epitopes in PvCSP, long synthetic peptides (LSPs), N (N-terminal), R (Repeat), and C (carboxyl terminal) have also been developed for assessment as vaccine candidates [41]. The three LSPs have been tested in Phase I clinical trials both individually, formulated with Montanide ISA720 in escalating doses (10 µg, 30 µg, and 100 µg/dose) [42], and as a mixture of 3 LSPs (50 and 100 µg/dose of mixture of peptides) formulated with Montanide ISA 720 and Montanide ISA 51 [43]. Antibodies were elicited against each of the peptides in the mixture and recognized native PfCSP on the surface of sporozoites [43]. The Montanide ISA51 formulation elicited higher antibody titers and interferon production.

4.1.2. *P. vivax* thrombospondin related adhesive protein (PvTRAP)

Bauza et al., have used the chimpanzee adenovirus ChAd63 and modified vaccinia virus Ankara (MVA) to express *P. vivax* thrombospondin-related adhesive protein (PvTRAP) [44]. Priming with recombinant ChAd63 expressing PvTRAP followed by boosting with recombinant MVA expressing PvTRAP induced high antibody titers and T cell responses in mice [44]. With the help of an infection model using transgenic *P. berghei* parasite expressing *P. vivax* TRAP, the authors found that both CD8+ T cells and antibodies mediate protection, suggesting that recombinant ChAd63 and MVA expressing PvTRAP have potential for development as pre-erythrocytic-stage vaccine candidates [44].

4.2. Blood stage *P. vivax* vaccine candidates

4.2.1. Receptor-binding region II of *P. vivax* Duffy binding protein (PvDBPII)

PvDBP is a microneme protein that binds the Duffy blood group antigen on human reticulocytes to mediate invasion by *P. vivax* merozoites [45]. A conserved, 330 amino acid cysteine-rich region, referred to as region II (PvDBPII), within the 130 kDa PvDBP mediates engagement with Duffy antigen [46]. Blocking this critical receptor–ligand interaction with antibodies elicited against PvDBPII offers a potential mechanism to block invasion [47] and prevent *P. vivax* malaria. Recently, it has been reported that *P. vivax* can also infect Duffy-negative individuals suggesting that the parasite has alternate invasion pathways that do not depend on Duffy antigen [48]. However, *P. vivax* ligands and erythrocyte receptors involved in invasion of Duffy-negative reticulocytes remain to be
identified. Also, it remains to be seen how commonly such alternative pathways are used for reticulocyte invasion by *P. vivax* field isolates from different geographical locations. It may be necessary to target these interactions in addition to PvDBPII if such invasion pathways are commonly used.

The amino acid residues within PvDBPII that make contact with the Duffy antigen include conserved hydrophobic and positively charged residues in the central region between cysteines 5 and 7 of PvDBPII that form a relatively flat, surface exposed binding site [49–51]. A glycan masking study demonstrated that N-glycosylation at a site adjacent to the predicted Duffy interaction site abolished PvDBPII binding to Duffy and resulted in weaker binding inhibitory antibody responses when the glyco-sylated PvDBPII was used for immunization [52]. In contrast, addition of N-glycosylation sites distal to the predicted Duffy-binding site did not inhibit binding, and elicited better binding inhibitory antibodies when compared to wild-type PvDBPII [52]. These studies validated the mapping of critical binding residues to the central region of PvDBPII. A ‘receptor-mediated dimerization model’ that predicts additional binding residues in the dimer interface has also been proposed [53]. In this model, interaction with the Duffy antigen leads to dimerization of PvDBP, which may be functionally important [53]. Importantly, studies on sequence polymorphisms in PvDBP have demonstrated that the binding residues identified in PvDBPII, whether in the dimer interface [53] or on the surface [51], are highly conserved in *P. vivax* field isolates from diverse geographical regions [54]. Given that the Duffy recognition site appears to be conserved, inhibitory antibodies raised against PvDBPII should be effective against diverse *P. vivax* strains.

A treatment re-infection study conducted in children in a malaria endemic region of Papua New Guinea has shown that naturally acquired high titer anti-PvDBP binding-inhibitory antibodies are associated with protection against *P. vivax* infection [55]. Further, as predicted by structure-function studies of the PvDBPII-Duffy antigen interaction, the inhibitory activity of anti-PvDBPII binding inhibitory antibodies is strain-transcending [55]. However, natural acquisition of such high blocking antibodies following exposure to *P. vivax* infection is rare [55].

Immunogenicity of *E. coli*-produced recombinant PvDBPII has been extensively evaluated. In rhesus monkeys PvDBPII formulated with human-compatible adjuvants Alhydrogel, Montanide ISA720 and AS02A elicits high titer binding inhibitory antibodies [56]. Immune responses have also been tested in mice with GLA-SE, R848 and their combinations [57]. Formulation with GLA-SE increases the diversity of IgG variable region sequences, and elicits strain-transcending antibodies that efficiently inhibit PvDBPII variants from diverse strains [57]. These observations support development of a vaccine based on PvDBPII.

Recently, Chootong et al., have reported that dominant, surface exposed B cell epitopes on PvDBPII that are polymorphic may drive antibody responses against polymorphic residues while limiting induction of antibodies against critical conserved epitopes [58]. Recombinant DBPII that lacks one such strain-specific immune-dominant variant epitope ‘DEK’ that is normally present on PvDBPII, leaving intact the less immunogenic but conserved neutralizing epitopes, has been constructed [59]. The resultant DBPII-DEKnull protein bound to erythrocytes and elicited functional anti-DPBP antibodies. While the binding inhibitory response elicited by recombinant DBPII-DEKnull was lower than the PvDBPII Sal I, the functional activity of antibodies had a broader coverage of diverse DBPII alleles [59]. Immunization with recombinant ChAd63 and MVA expressing PvDBPII in prime-boost regimens also yields high titer binding inhibitory antibodies [60]. Priming with ChAd63 expressing PvDBPII followed by boosting with recombinant PvDBPII formulated with adjuvants such as Montanide ISA720 or Abisco yields higher ELISA and binding inhibitory titers compared to ChAd63–MVA prime-boost regimens [60].

4.2.2. *P. vivax* merozoite surface proteins

Merozoite Surface Protein 1 (MSP1) is abundantly expressed on the merozoite surface and is associated with acquisition of naturally acquired humoral and cellular immune responses. Proteolytic processing of ~195 kDa precursor protein yields smaller fragments (83, 30, 38 and 42 kDa) during invasion. The 42 kDa fragment is further cleaved into 33 kDa and 19 kDa C terminal fragments. MSP1 contains domains that share homology with epidermal growth factor (EGF). The 19 kDa cysteine-rich fragment remains attached to the surface of released merozites [61] in the form of a protein complex. Studies in *Macaca sinica* monkeys with recombinant *P. cynomolgi* MSP142 and MSP119 showed that both fragments were highly immunogenic and protected against *P. cynomolgi* blood-stage challenge [62]. These studies provide an example for the use of *P. cynomolgi* as a model for *P. vivax* vaccine development. This non-human primate model could be used more widely to evaluate the potential of *P. vivax* vaccine candidates although final proof of concept will have to come from clinical trials against artificial or natural challenge.

Recombinant PvMSP119 fused with two T-helper epitopes from the 33 kDa PvMSP1 fragment (PvMSP13) was tested for immunogenicity in *Callithrix jacchus* monkeys [63]. Recombinant PvMSP119 has also been tested in combination with PvDBPII in mice. Immunization with combination of PvDBPII and PvMSP119 formulated with the Montanide ISA 720 elicited high titer antibody responses against both antigens [64].

Other merozoite surface proteins that have been explored as potential antigens for vaccine development include PvMSP3 and PvMSP9. In a study of children aged 1–3 years residing in a malaria endemic region of Papua New Guinea, Stanisic et al. [65] have shown that naturally acquired antibodies against the C-terminal Block II of PvMSP3α and the N-terminal domain of PvMSP9 are significantly associated with protection from symptomatic *P. vivax* malaria and prospective risk during 16 months of active follow-up. The region that contains Block II of PvMSP3α and the N-terminal region of PvMSP9, are highly conserved across *P. vivax* isolates [64]. In another study, high titer antibodies to interspecies conserved blocks 2–5 of PvMSP1 (PvMSP1 ICB2-5) were also found to be associated with clinical protection against *P. vivax* suggesting that other regions of PvMSP1 could also be considered for vaccine development [66].

4.2.3. *P. vivax* apical merozoite antigen 1 (*PvAMA1*)

*PvAMA1*, a microneme-secreted 66 kDa protein contains 3 domains referred to as DI, DII and DIII with intra-domain disulfide linkages [67]. Recently, Bouillet et al. [68] have studied immunogenicity of *PvAMA1* using adenovirus and recombinant protein in different prime boost regimens. The ‘Protein/Ad’ group (protein formulated with Montanide ISA720 followed by Ad5PvAMA1) elicited long-lasting *PvAMA1*-specific antibody responses, as well as memory T cell responses [68]. *PvAMA-1* has also been expressed as a secreted protein in *Pichia pastoris* [69]. Formulations with saponin Quil A and incomplete Freund’s adjuvant elicited higher antibody titers when compared to monophosphoryl lipid A (MPLA) or Alum plus MPLA. Importantly, *PvAMA-1* elicited invasion inhibitory antibodies against diverse *P. vivax* strains [69].

4.3. Transmission blocking *P. vivax* vaccine candidates

4.3.1. *Pvs25* and *Pvs28*

Parasite proteins expressed on surface of zygote (*Pvs25*) and ookinete (*Pvs28*) stages each contain four cysteine-rich epidermal growth factor-like domains with a C-terminal
glycosylphosphatidylinositol (GPI) anchor. Recombinant Pvs25 and Pvs28 expressed in *Saccharomyces cerevisiae* formulated with Alhydrogel elicits potent antibodies in mice that completely inhibit parasite development in mosquitoes in membrane feeding assays [70]. Recombinant Pvs25 formulated with Montanide ISA720 and Alhydrogel were tested for immunogenicity in rhesus [71]. The Montanide ISA 720 group elicited antibodies with 10-fold higher recognition titers with potent transmission blocking activity compared to Alhydrogel group [71].

Pvs25H, a vaccine formulation of Pvs25 with Alhydrogel was tested in a dose-escalation (5, 20, 80 µg/dose) Phase I trial in healthy volunteers [72]. The formulations were found to be safe and induced high titer antibodies in a dose dependent manner. However, only a 20–30% reduction in the number of infected mosquitoes was observed with sera from immunized individuals in membrane feeding assays. Though the vaccine provided proof of principle for development of a transmission blocking vaccine based on Pvs25, higher levels of transmission blocking activity are needed for an effective deployable vaccine. In a separate trial, Pvs25 formulated with Montanide ISA 51 generated transmission blocking activity, but the formulation was found to be highly reactogenic and the study was terminated [73].

4.3.2. AnAPN1

Alanyl aminopeptidase 1 (AnAPN1) is a highly conserved midgut surface antigen of *Anopheles* mosquitoes that is essential for ookinete invasion and development. Rabbit antibodies against AnAPN1 have shown transmission blocking activity against both *P. vivax* and *P. falciparum* across divergent anopheline species supporting the development of an AnAPN1-based transmission blocking vaccine [74].

5. Challenges and directions for *P. vivax* vaccine development

The unique characteristics of *P. vivax* pose some specific challenges in studying its biology and designing strategies to control it. For example, the lack of a method for continuous culture of *P. vivax* blood stages makes it difficult to perform growth inhibition assays to identify synergistic combinations of blood stage antigens that can be targeted to inhibit *P. vivax* blood stage growth high efficiency. Several groups have recently succeeded in establishing short-term *P. vivax* culture for invasion assays using enriched reticulocytes from cord blood [75]. Such methods for short term *P. vivax* culture are still dependent on access to fresh *P. vivax* isolates from malaria patients limiting the routine use of such assays to endemic regions. Noulon et al. [76] have shown that immature reticulocytes derived from CD34+ hematopoietic stem/progenitor cells (HSPC) collected from umbilical cord blood were invaded efficiently by *P. vivax*. In addition, CD34+–enriched cells could be further expanded in *vitro* to generate more reticulocytes. Such approaches could be used to identify antigen combinations that may be targeted to yield synergy and high rates of inhibition of invasion. They may also lead to development of methods for long-term culture of *P. vivax* blood stages.

In the absence of a *P. vivax* blood-stage culture system, production of infected mosquitoes for sporozoite or transmission stage studies also requires access to *P. vivax* patients in endemic areas. In order to study hepatocyte invasion stages of *P. falciparum* as well as *P. vivax*, attempts have been made to establish *in vitro* liver models [77,78]. In addition, a reliable humanised mouse model for *P. vivax* liver stages is now available [79]. Both approaches will help to identify and test novel drugs and vaccines targeting hepatocyte stages. Studies on expression of parasite proteins in hypnozoites will determine if these latent stages can also be targeted with novel drugs and vaccines.

The development of a safe and reproducible *P. vivax* sporozoite challenge model in malaria-naïve humans can greatly accelerate clinical development of *P. vivax* vaccines. Herrera et al. have demonstrated the robustness and reproducibility of the *P. vivax* sporozoite challenge model [80,81], which should allow evaluation of efficacy of both pre-erythrocytic and blood stage *P. vivax* vaccines. A *P. vivax* blood stage challenge model that establishes experimental blood stage infection in human subjects by transfer of cryopreserved *P. vivax* patient isolates [82] could also be used to evaluate efficacy of blood stage *P. vivax* vaccines. The use of sporozoite and blood stage challenge models can greatly help validate and down select *P. vivax* vaccine candidates for further clinical testing in more time consuming and expensive field trials against natural challenge in endemic areas.

6. Conclusion

Given that a *P. vivax* vaccine will, in most cases, be used in low transmission settings, ideally one would want a vaccine that has high efficacy (more than 90%) and blocks transmission (Table 1). The development of such a vaccine will likely require combination of multiple antigens that provide synergy to achieve high efficacy. For example, achieving high rates of blood stage growth inhibition may require targeting a combination of key blood stage antigens involved in reticulocyte invasion. In addition, combination of blood stage antigens with liver stage antigens may be needed to achieve high efficacy and inclusion of antigens from sexual and mosquito stages will be needed to inhibit of transmission. As seen above, most of the vaccines that are currently under development target individual stages and are based on single antigens. It is necessary to initiate efforts to combine antigens both within and across development stages to achieve synergy and attain the goal of developing a vaccine for *P. vivax* malaria with high efficacy.

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