EXPERT REVIEWS

Vaccines against *Plasmodium vivax*: a research challenge

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Malaria caused by *Plasmodium vivax* continues being a public health problem in tropical and subtropical areas throughout the whole world. In spite of this species' epidemiological importance, its biological complexity has hampered advances being made in the field of vaccine development. Few antigens have been described and analyzed to date in preclinical and clinical studies, thereby highlighting the great challenge facing groups currently working on this parasite species. This review summarizes the most representative work done during the last few years and discusses the approaches adopted in making progress towards an anti-*Plasmodium vivax* vaccine.

Keywords: antigens • malaria • *Plasmodium vivax* • preclinical and clinical studies • tropical and subtropical regions • vaccine

In spite of the progress made by government initiatives and the World Fund for the Control and Prevention of Malaria, this disease continues to be a public health problem all around the world. Approximately 216 million cases and an estimated 665,000 deaths occurred in 2011, mainly in children <5 years old, according to the latest data released by the WHO [1].

Human malaria can be caused by five parasite species from the *Plasmodium* genera (*Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi*). *P. vivax* is predominantly distributed on the Asian and American continents and is responsible for 25–40% of the global malaria burden, causing between 132- and 391-million cases annually [2]. The search for an effective vaccine against *P. vivax* has become a great challenge given this species' biological complexity, its preference for invading reticulocytes, genetic variability mechanisms and the generation of latent forms (hypnozoites).

Although few *P. vivax* antigens have been identified and functionally characterized using traditional molecular biology, immunology and biochemistry approaches, most of the vaccine candidates being tested were found using the aforementioned methodologies. The currently available transcriptome [3], proteome [4] and comparative genomic analysis data [5] for *P. vivax* could be extremely useful in the future to find new stage-specific proteins, similar to those described for other parasite species, which could be essential in developing a vaccine.

The progress of an anti-*P. vivax* vaccine

Given that malaria represents one of the main public health problems around the world, several research groups have made great efforts to develop an effective vaccine against this parasitosis. Advances made to date with regard to the knowledge gained concerning the biology of *P. vivax* have not yet reached the same level as those regarding *P. falciparum*. However, research into the *Plasmodium* life-cycle and its mechanisms for invading red blood cells (RBCs) have led to the establishment of the most appropriate points of intervention for blocking the parasite's development, such as pre-erythrocyte, blood and sexual stages (Figure 1).

Immunization with irradiated sporozoites, recombinant expression of parasite proteins and production of antigens by peptide synthesis are among the methodologies used by research groups orientated towards developing an antimalarial vaccine. *P. vivax* studies to date have dealt with the limited number of candidates that have been identified, characterized (Figure 2) and evaluated in preclinical and clinical studies, compared with *P. falciparum*.

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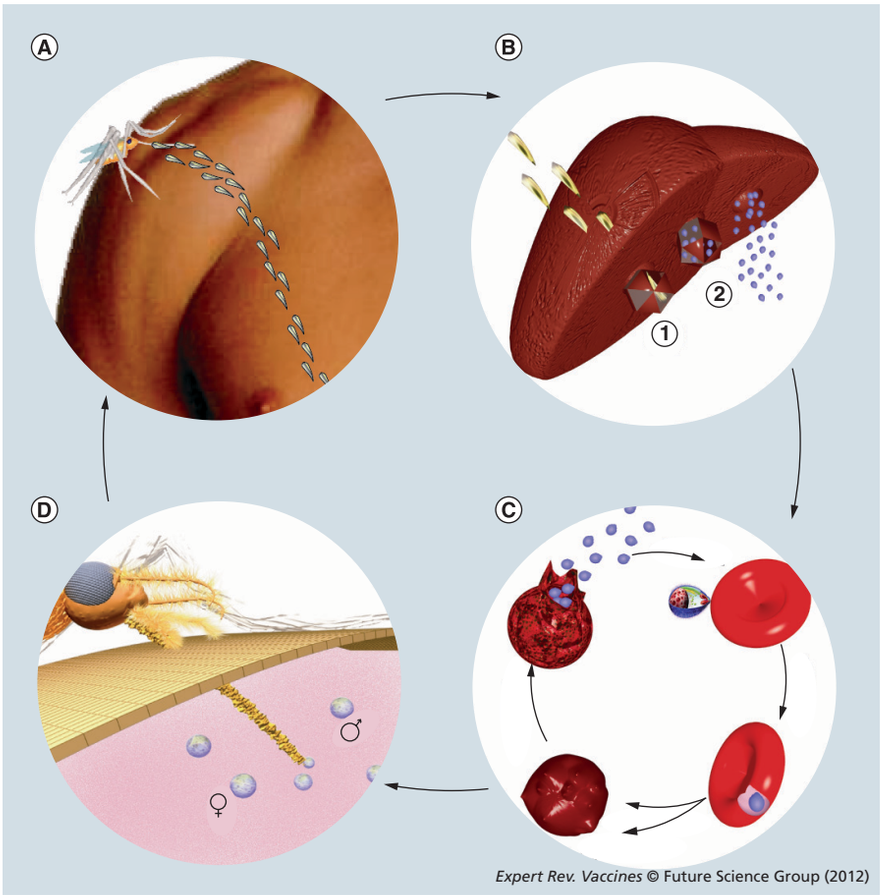

Figure 1. *Plasmodium vivax* life-cycle and intervention points for developing vaccines against several development stages. (A) Infection: sporozoite inoculation via female *Anopheles* mosquito bite. **(B)** Pre-erythrocyte stage: invasion of hepatic cells and generation of (1) hypnozoites or (2) hepatic schizonts. **(C)** Blood stage: beginning of the parasite's asexual development in reticulocytes. **(D)** Sexual stage: differentiation to gametocytes that are ingested by the mosquito.

Vaccine candidates for which the greatest advances have been made to date

Pre-erythrocyte vaccine candidates

The main objective for vaccines directed against this stage is to prevent sporozoite invasion of hepatocytes, or impede the parasite's development within hepatic cells so as to avoid its proliferation in the blood stream. Early studies by Ronald Ross (1899) showed that infected mosquitoes transmitted avian malaria. Later, Sergent *et al.*, in 1910 described that canaries immunized with irradiated *Plasmodium relictum* sporozoites developed partial immunity against exposure to the native parasite. In 1967, Nussenzweig *et al.* showed that immunizing rodents with 5,000–75,000 radiation-attenuated sporozoites induced total protection against challenge in studies orientated towards evaluating the immunological response to infection in preclinical assays, thereby leading to the concept of sterile protection [6]. Many years later, clinical trials reported by Clyde *et al.* in 1975 revealed vaccine safety and immunogenicity using *P. vivax* attenuated sporozoites in humans [7]. Individuals immunized with more than ~1,000 bites from irradiated mosquitoes in such studies achieved short-term protection against the disease. *Aotus* monkeys have been intravenously inoculated with 2, 5 and 10 doses of 100,000 radiation-attenuated sporozoites in recent work; despite the low immune response, the primates became partially protected, as shown by the reduction in parasitemia [8].

Although this pre-erythrocyte vaccine system continues to be considered the gold-standard, no new clinical studies have evaluated safety, reproducibility and protective efficacy in humans. This has been mainly due to technical difficulties presented when advancing *P. vivax* studies, such as not having an infective gametocyte-rich source that could be used as totally viable sporozoite precursors. In addition, it is difficult to estimate the required amount of irradiated sporozoites for creating a protective response in humans and the procedure involving using mosquito bites for immunizing humans is impractical (>1,000 mosquito bites). Using this methodology might be ineffective in individuals having a background of malaria given that even though invasion has been blocked in this first line of defence they could develop the infection as the result of relapses caused by hypnozoites (discussed later). This highlights the need for a multistage vaccine against this parasitosis.

Bearing the aforementioned in mind, efforts were initially centred on developing a subunit-based vaccine. The main candidates have been proteins present on parasite surface or in apical organelles (Figure 2), namely the circumsporozoite protein (CSP) and the thrombospondin-related anonymous protein (TRAP).

CSP has been one of the most studied candidates in various human malaria species. Current preclinical trials with CSP have involved using chimerical recombinant proteins [9], multiple antigen constructs [10] or multiple antigen peptides [11]. These methods have led to the discovery that including CSP protein B- and T-cell epitopes in a vaccine induces high immunogenicity in rodents and primates. Yadava *et al.*, have immunized mice with the VMP001 chimeric antigen developed in 2007 [9] emulsified with Montanide ISA 720 [12]; this recombinant includes the chimeric repeat region representing the two major CSP alleles in *P. vivax* (VK210 and VK247), flanked by the N- and C-terminal regions. The VMP001 vaccine induced a cross-species immune response against *P. falciparum* and *Plasmodium berghei* and generated partial protection in the murine model as evidenced by the lower parasitemia developed in vaccinated mice compared with the control group, as well as the lower parasite burden in the liver

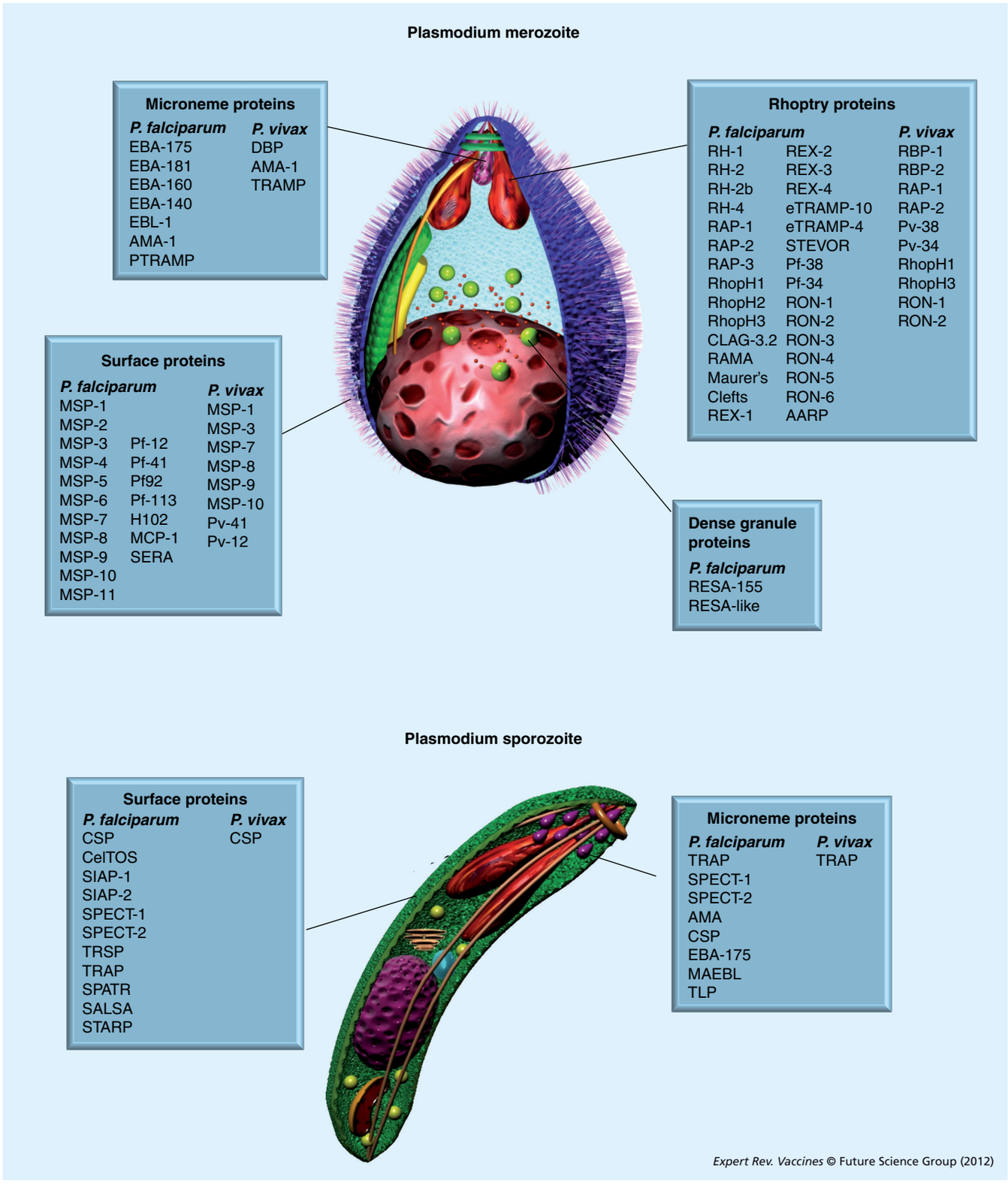

Figure 2. Actual state of proteins identified in merozoite and sporozoite stages in *Plasmodium vivax* and *Plasmodium falciparum*, respectively. Shows the proteins described to date with their respective localization in the parasite (surface, rhoptries, micronemes and dense granules).
P. falciparum: *Plasmodium falciparum*; *P. vivax*: *Plasmodium vivax*.

as evaluated by reverse transcription-PCR. Experiments in *Aotus* monkeys using N- and C-terminal region-derived long synthetic peptides (LSPs) and another based on CSP repeats combined with a tetanus toxin T-cell epitope, both emulsified in Montanide ISA 720 or Freund's complete adjuvant, have shown strong antibody responses that recognize the native protein [13]. Based on this, a Phase I clinical trial was carried out; this described that a vaccine based on individual LSPs or a mixture of these with Montanide ISA 720 was safe, tolerable and immunogenic [14]. A total of 40 individuals immunized with LSP formulated in Montanide ISA 720 or Montanide ISA 51 have recently shown seroconversion; however, LSPs formulated in Montanide ISA 51 produced greater antibody titers and IFN-γ than those emulsified in Montanide ISA 720 [15]. The results suggested that a particular adjuvant could boost a vaccine's effect and this should be considered in future clinical trials.

A study showing the safety and reproducibility of infection caused by 3–9 bites by infected *Anopheles albimanus* mosquitoes in 18 malaria-naive humans [16], in addition to the aforementioned study, evaluating patients' immune response to LSPs [15], have been carried out to promote Phase II trials. However, no reports describing Phase II trial results have been published to date.

Recent studies have focused on searching for a potent adjuvant able to trigger strong, long-lasting humoral immune responses when combined with the *P. vivax* CSP molecule. In particular, mice vaccinated with VMP001 emulsified with synthetic TLR4 (glucopyranosyl lipid adjuvant) in a stable emulsion [17] or conjugated to the lipid-enveloped polymeric PLGA nanoparticles (VMP001-NPs) with monophosphoryl lipid A [18] have shown significantly higher antibody titers than control groups, thus providing support for the use of these formulations in further clinical trials.

Parasite invasion is complex, involving many adhesion molecules [19]. Even though CSP is the major antigen, it is not the only one responsible for target cell invasion. A *P. vivax* study has led to the identification and characterization of a microneme protein called TRAP. Once it was demonstrated that TRAP induced high antibody levels in the Balb/c experimental model, its efficacy in the *Aotus* experimental model was evaluated; inoculating an LSP localized in TRAP's N-terminal region (containing the liver cell-binding domain, known as region 2) induced a good immune response. Nevertheless, protection results were not statistically significant when compared with the control group using this scheme [20].

Intra-erythrocyte stage candidates

Considering that the clinical manifestations and severity of malaria infection coincide with the parasite's development within erythrocytes, antigens expressed during this stage represent a set of important vaccine candidates for blocking invasion. Even though studies have mainly been centred on the Duffy-binding protein (DBP) [21], merozoite surface protein-1 (MSP-1) [22] and apical merozoite antigen-1 (AMA-1) [23], other molecules such as reticulocyte-binding protein-1 (RBP-1) [24], rhoptry-associated protein-2 [25], MSP-9 [26] and -10 [27] have been identified, characterized and evaluated as vaccine candidates.

DBP is a microneme molecule participating in reticulocyte–merozoite tight junction formation. It has been established that this protein forms part of the obligatory reticulocyte invasion route involving interaction with the Duffy antigen receptor for chemokines (DARC), thus leading to wide-ranging research aimed at evaluating this antigen's function and potential as vaccine candidate.

Studies carried out by the Fundación Instituto de Inmunología de Colombia (FIDIC) have shown that DBP has 10 reticulocyte high-activity binding peptides (HABPs), which are mainly located in the protein's region 2 (RII) (**Figure 3**) [28]. Two HABPs able to bind to different HLA-DR molecules (called 1635 and 1638) have been found recently (**Figure 3**). These peptides induced a Th1/Th2 recall response (mainly IFN-γ and IL-6) in 35 individuals who had suffered prior episodes of *P. vivax* malaria as shown by peripheral blood mononuclear cell (PBMC) lymphoproliferation assays, thus highlighting their potential use in a synthetic, subunit-based vaccine [29,30]. Future studies aimed at establishing these sequences' protection-inducing ability have been planned in the *Aotus* experimental model.

Preclinical trials with rodents and *Macaca* monkeys using recombinant PvDBP region 2 (PvDBP-RII) formulated with three adjuvants approved for human use (anhydrogel, Montanide ISA 720 and ASO2A) have shown a correlation between the level of antibodies produced and the potential for inhibiting domain binding to the DARC *in vitro* [21,31]; however, preclinical trials in the experimental *Aotus* model using the same region formulated in Montanide ISA 720 adjuvant have shown no protection [32]. Despite this, this antigen continues to be studied as it has been considered an important component in the obligatory route for invasion of reticulocytes, it has specific binding sequences, antibodies directed against PvDBP-RII are able to inhibit adhesion to target cells, it is highly antigenic and it has universal epitopes.

MSP-1 has been the most widely studied MSP identified to date. It has been suggested that PvMSP-1 probably undergoes a proteolytic processing similar to that of its *P. falciparum* ortholog, thus generating 83, 30, 38 and 42 kDa fragments, the latter being hydrolyzed again in 33 and 19 kDa products.

Several formulations have been evaluated in *P. vivax*. A study by the CDC (Atlanta, GA, USA) used a yeast-expressed recombinant consisting of the MSP-1 19 kDa C-terminal fragment ($MSP\text{-}1_{19}$) and two tetanus toxoid T-helper epitopes for immunizing *Saimiri boliviensis* monkeys with aluminium hydroxide and block copolymer P1005 [33]. The group of monkeys immunized with $MSP\text{-}1_{19}$ emulsified in block copolymer P1005 had partial protection against challenge (three out of five monkeys) with a *P. vivax* Sal-1 homologous strain and generated a greater antibody response than the group immunized with $MSP\text{-}1_{19}$ only or in aluminium hydroxide. Immunization experiments were carried out on New World monkeys (*Callithrix jacchus*) vaccinated with $MSP\text{-}1_{19}$ plus a promiscuous T-cell epitope present in the 33 kDa region and a synthetic universal Pan allelic DR *epitope* emulsified in different adjuvants (Freund's, Quil A, CpG oligodeoxynucleotide (ODN) 2006 or MPL/trehalose dicorynomycolate). The results showed a better response when the recombinant was

administered with Freund's complete adjuvant and Quil A [34], suggesting that immunogenicity depended on the adjuvant used.

Other studies in the *Aotus* experimental model have been carried out by FIDIC; these included immunizing with two recombinant fragments (MSP-1_{14} and MSP-1_{20}) encompassing most of the MSP-1_{33} proteolytic cleavage product. These recombinants are localized in a region presenting low variability and displayed high reticulocyte-binding ability [35], probably mediated by the HABPs comprised in them (Figure 3). A total of 50% [36] to 80% [22] of the primates immunized with the mixture of MSP-1_{14} and MSP-1_{20} recombinant proteins emulsified in Freund's adjuvant were partially protected, showing a relationship between antibody titers, IFN-γ production levels and protection-inducing immunity.

Additional studies have tested recombinant fusion proteins such as PvDBP-RII and MSP-1_{19} obtaining greater immunogenicity in mice when using Montanide ISA 720 adjuvant as an immunobooster [31].

AMA-1 has also been considered to be one of the most important candidates given its participation in merozoites' apical reorientation and subsequent tight junction formation. It has also been found that the protein has an effect on dendritic cell maturation by upregulating CD1a and HLA-DR molecules [37]. The efficiency of Balb/c mice's humoral response has been recently proved regarding immunization with the AMA-1 recombinant protein (amino acids 43-487) formulated in Freund's complete adjuvant, aluminium hydroxide, Quil A, QS-21 saponin, CpG ODN 1826 or TiterMax® (CytRx, CA, USA). A strong immune response with any one of the adjuvants used was produced and thus it is planned to continue assays in nonhuman primates [23].

RBP-1 is a member of a cell-binding protein family identified in *P. vivax*; in the Sal-I strain, this family has 10 *rbp* genes including three partial genes (one *rbp-1* [PVX_125738] and two *rbp-2* [PVX_090330; PVX_101590]), two pseudogenes (*rbp-2d* [PVX_101585] and *rbp-3* [PVX_101495]) and five full-length genes (*rbp-1a* [PVX_098585], *rbp-1b* [PVX_098582], *rbp-2a* [PVX_121920], *rbp-2b* [PVX_094255] and *rbp-2c* [PVX_090325]) [5]. Mapping the whole of *P. vivax* Belem strain rbp-1 (encoded by the *rbp-1a* gene) has led to the definition of four reticulocyte-binding regions (RI-RIV) housing several highly conserved high-binding peptides (Figure 3) [38]. A further study has shown that RBP-1 region I, recombinantly expressed in *Spodoptera frugiperda* (Sf9) insect cell line, intervenes in merozoites' specific binding to reticulocytes [39].

The only formulation that has been evaluated included the protein's region III (containing peptides with a greater binding ability) (Figure 3) recombinantly expressed in *Escherichia coli* and emulsified with Freund's adjuvant; although a significant increase in antibody production and T-lymphocyte stimulation have been observed, no protection was achieved when *Aotus* monkeys previously immunized with the recombinant were challenged with the *P. vivax* VCG-1 strain [24]. Therefore, plans have been made to carry out preclinical assays with protein regions I, II and IV for evaluating the formulation's immunogenic and protection-inducing ability.

Several rhoptry proteins play an important role during invasion of RBC, some of them being classified within the high molecular weight or the low molecular weight complexes. The latter consists of three rhoptry-associated proteins: RAP-1, -2 and -3, of which the first two have been described in *P. vivax* [40,41]. A study using the *P. vivax* RAP-2 recombinant protein formulated in Freund's adjuvant has shown partial protection in *Aotus* monkeys, given that significantly lower parasitemias in the immunized group were observed with respect to the control group [25].

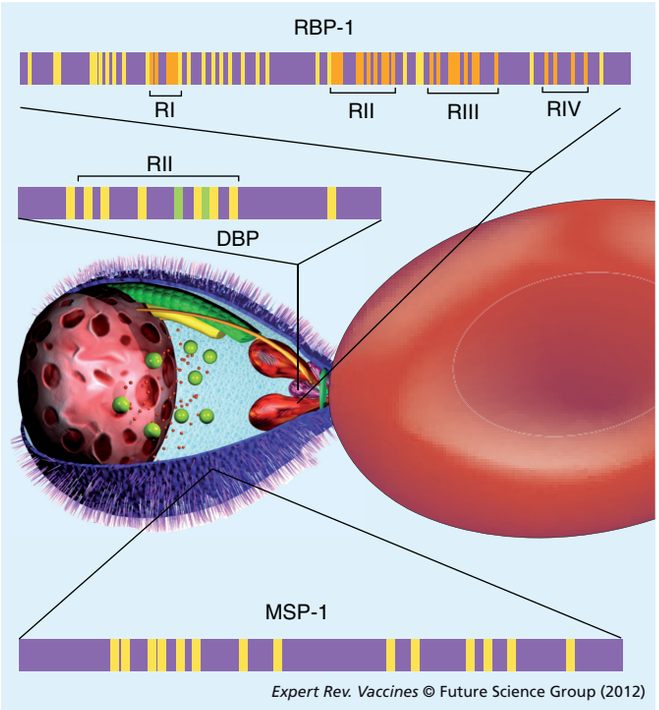

Figure 3. High-activity binding peptides to reticulocytes identified in *Plasmodium vivax* MSP-1, RBP-1 and DBP proteins. HABPs are shown in yellow. Green bars represent the universal peptides found in DBP protein region II (HABPs #1635 and 1638). Orange bars indicate regions where RBP-1 binds to reticulocytes (RI-RIV), which encompass those peptides showing greater binding ability.
HABP: High-activity binding peptide.

Regarding MSP-9, two recombinants have been evaluated in rodents, one covering the N-terminal region (PvMSP-9-Nt) and another covering a block of tandem repeats in the protein (MSP-9-RepII). Both formulations were seen to be highly immunogenic and the N-terminal region stimulated IFN-γ and IL-5 production in the PBMCs of immunized mice [26]. Regarding MSP-10, this protein was initially identified and characterized in 2005 by Pérez-Leal *et al.* [42], and has recently been shown to be antigenic during natural *P. vivax* malaria infection in humans. Its immunogenicity has also been proved in the experimental *Aotus* model using three types of adjuvants: Freund's, Montanide ISA720 or aluminium hydroxide. Two out of the three formulations triggered a strong antibody response, recognizing the protein on parasite surface (demonstrated by immunofluorescence assay); nevertheless, immunized monkeys did not become protected against challenge with the *P. vivax* VCG-1 strain [27].

Transmission-blocking vaccine candidates

The antigens used by the parasite during fertilization and development within the mosquito vector have been used as a strategy for controlling malarial transmission. Pvs25 and Pvs28 are the main surface proteins presenting high expression levels during the ookinete stage; their characterization has led to antigenic and immunological evaluation in experimental models. Rodents immunized with recombinants produced in yeast and absorbed with aluminium hydroxide have been shown to generate a potent immune response capable of inhibiting oocyte development in mosquitoes [43]. In spite of this, more attention has been paid to Pvs25, since a greater yield is obtained when expressed in yeast, its polymorphism is limited and the immune response exerted does not seem to be genetically restricted in the experimental model used. Two clinical trials have been carried out by the Algerian Research Institute and the US National Institute of Allergy and Infectious Diseases (MD, USA). Individuals vaccinated with the Pvs25H recombinant protein formulated in alhydrogel tolerated the formulation and produced antibodies that were functionally active as significant transmission blocking was observed [44]. The other trial was made with the same recombinant but emulsified in Montanide ISA 51; this proved reactogenic in two patients who developed a severe local reaction and the study was thus stopped [45].

A new protein localized on the gamete surface has been identified (Pvs230). Immunizing mice with recombinant Pvs230 region I–IV emulsified in Vaxfectin® (Vical, CA, USA) led to the development of antibodies, and significantly reduced the number of oocysts formed within the mosquito's intestine and the vector infection rate, thereby suggesting that this candidate could be a good target for evaluation in preclinical assays [46].

Promising candidates

While advances made in identifying *P. vivax* antigens have been limited, studying the transcriptome [3], the proteome's partial characterization [4] and immunoproteomic profile [47], as well as studies focused on comparing transcription profiles between different parasite species [5,48], have been of great benefit in obtaining information about proteins that may be important for vaccine development. Different vaccine candidates localized on the surface or apical organelles (rhoptries and micronemes) (Figure 4) have been identified by molecular biology methodologies.

It has been found that some *Plasmodium* proteins are associated with detergent-resistant membranes through glycosylphosphatidylinositol (GPI) anchors; six of them have been identified in *P. vivax* by means of bioinformatics tools and evaluated by molecular biology assays: PvMSP-8 [49], -10 [42], Pv12 [50], Pv34 [51], Pv38 [52] and apical sushi protein (PvASP) [53]. The first three antigens are localized on merozoite surface while Pv34, Pv38 and PvASP are located in the rhoptries. All of them have characteristics relevant to proteins considered good vaccine candidates, such as having a signal peptide, being anchored to the membrane through transmembrane helices or GPI anchors, having binding domains and being able to generate an immune response in natural and/or experimental infections. It has been particularly interesting that Pv34 induced a proliferative response of PBMCs isolated from individuals having a background of *P. vivax* infection, thereby highlighting the importance of carrying out preclinical studies in experimental models. Other surface/rhoptry molecules have been described, such as PvMSP-7 [54], Pv41 [55], PvTRAMP [56], PvClag7 [57], PvRON2 [58] and PvRhopH3 [59]. Some of them have been shown to be immune response targets during a natural *P. vivax* infection (PvTRAMP, PvClag7) thereby supporting the idea of continuing with studies to evaluate their immunogenic activity in the *Aotus* experimental model.

Two new alanine- and tryptophan-rich proteins have been characterized (PvTARAg55 [60] and PvATRAg74 [61]); a proliferative response is induced by these antigens in PBMCs from infected individuals as well as a predominant Th2 response with high levels of IL-4 and IL-10. Another study characterizing and mapping antibody response to MSP-3α has shown at least 15 antigenic determinants within this protein and antibody titers observed correlate with the time of exposure to infections [62].

Protein array or mass spectrometry techniques have been used for identifying new candidates; the first technique has led to 18 highly immunoreactive molecules being recognized by sera from *P. vivax*-infected patients [47] while the second allowed identifcation of four additional ones strongly recognized by immune sera [4]. Some of these match previously described antigens, suggesting that the approach used represents a good option for identifying new candidates.

Problems & limitations regarding *P. vivax* research

Several obstacles inherent in the parasite's biology related to its cell tropism towards reticulocytes, high genetic variability, developing latent hepatic forms and its adaptation in a suitable experimental model have impeded advances being made in the search for a strategy for controlling the disease, such as vaccine development.

In vitro *culture & functional assays*

Tremendous efforts have been made during the last 30 years for standardizing a methodology allowing a *P. vivax in vitro* continuous culture to be maintained. However, the preference of *P. vivax* for invading reticulocytes (representing approximately 0.5-1.5% of the total of RBCs circulating in adults) and the difficulty involved in obtaining a significant percentage of them are the main limitations regarding progress in this research area [63].

The first *in vitro* trials with *P. vivax* were reported during the 1980s; several groups managed to establish a preliminary *in vitro* culture from blood infected with early trophozoites and ring forms, using reticulocyte-rich samples obtained from cord blood. These assays led to DBP's functional characterization, demonstrated by inhibiting invasion by an antibody directed against the erythrocyte Duffy antigen binding region.

Several studies during the last few years have led to a significant advance in the area. The first of them used reticulocytes obtained by differentiating stem cells with specific factors [64]; nevertheless, it has still not been widely used given its complexity and high costs. The second assay managed to standardize a *P. vivax ex vivo* invasion assay protocol, which uses trypsin-treated enriched reticulocytes and a sample with *P. vivax* schizonts from different

clinical isolates; this methodology has been shown to be practical, reliable and reproducible [65] and is currently being evaluated for propagaton of the VCG-1 strain.

Based on the strength of FIDIC's studies involving the molecular characterization of 50 proteins implicated in *P. falciparum* invasion, advances have been made in screening three *P. vivax* molecules. These antigens' regions for specific binding to target cells have been determined by using enriched reticulocytes from samples from a patient suffering from β-thalassemia and synthetic peptides; DBP [28], RBP-1 [38] and MSP-1 [66] have thus been fully mapped (Figure 3). Such advances have provided valuable knowledge for developing novel strategies and methodologies providing in-depth knowledge about the importance of candidates and their inclusion in an epitope-based vaccine. However, technical difficulties associated with obtaining enough reticulocytes for binding studies are reflected in the number of candidates mapped for *P. vivax* compared with those from *P. falciparum* (three vs 50).

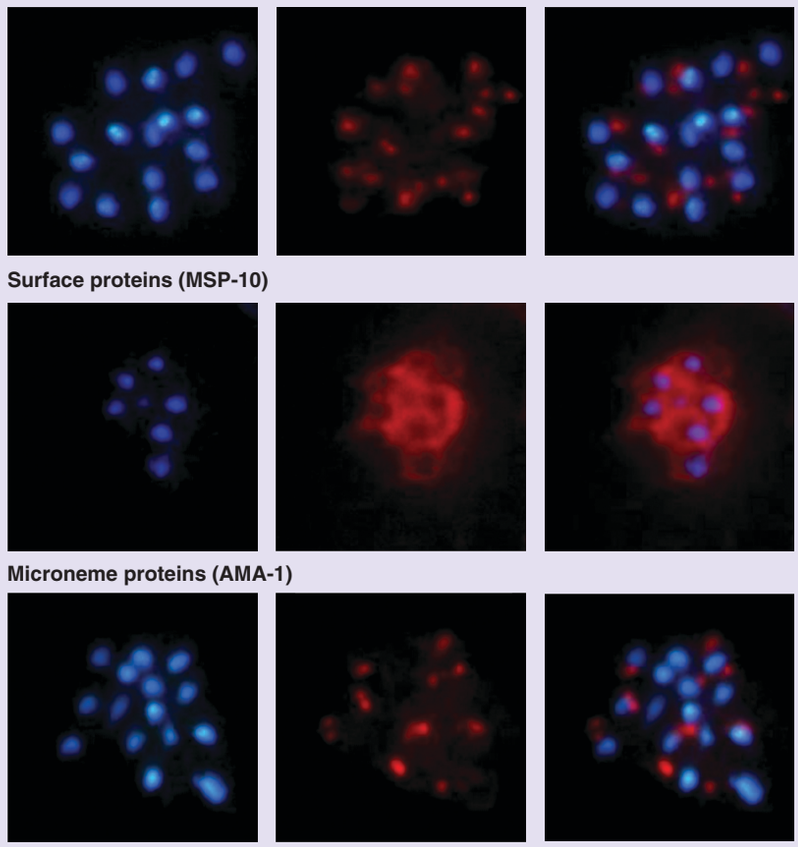

Figure 4. Localization patterns for *Plasmodium vivax* rhoptry (RhopH3), surface (MSP-10) and microneme (AMA-1) proteins. The merozoite nucleus is shown in blue (left panels); RhopH3, AMA-1 and MSP-10 localization is shown in red (central panels), the dotted pattern is characteristic of microneme and rhoptry proteins, whereas the 'bunch of grapes' pattern is characteristic of MSPs; right panels show the overlapping of the aforementioned images.

The parasite's genetic variability

The expression of *P. vivax* proteins with a high degree of polymorphism and the strain-specific immune responses induced by them represent formidable obstacles for developing an antimalarial vaccine. Several studies have shown great genetic variability in proteins such as DBP [67], MSP-1 [68], MSP-3α [69], MSP-5 [70] and AMA-1 [71], which has mainly been attributed to selective pressure exercised by the host's immune system, thereby leading to the fixation of allele variants in the parasite population [71].

It has recently been shown that strong immune responses are directed against variable sequences that do not bind to target cells, whereas highly conserved sequences implicated in interaction with RBC molecules are not immunogenic [19,72]. PvDBP-RII contains both conserved as well as highly polymorphic residues; antibodies generated against the polymorphic ones are short-lived and strain-specific. Mutagenesis assays directed towards variable regions have shown that these are not functionally active for binding to erythrocytes [73]. A recent study involving the modification of a PvDBP-RII peptide (DEK^{null}) in polar polymorphic residues (replaced by small hydrophobic residues), has shown that high immunogenicity is generated and induces the production of antibodies inhibiting binding to erythrocytes [74], thereby highlighting the fact that modifying polymorphic residues could lead to the production of more specific antibodies.

It is particularly interesting that the naturally infected *P. vivax* individuals have high specific antibody titers against DBP and MSP-1 variants, generating a selective effect in immune response regarding different strains [75,76]. These studies suggested that using conserved regions or the most frequently occurring alleles in a population must be born in mind when designing a vaccine.

P. vivax *pathology*

The parasite is characterized by its ability to generate a latent stage within a host's liver, called the hypnozoite (Figure 1), which is being characteristic of some species that infect primates (*P. simiovale, P. fieldi, P. cynomolgi* and *P. schwetzi*) and humans (*P. ovale* and *P. vivax*). This stage is generated after sporozoite invasion of liver cells. It has been shown that activating such latent forms propitiates a new wave of blood parasites that can occur in 3–5 months (short intervals) or 5–10 months (long intervals) [77,78]. Such patterns vary according to a vector's geographical area of origin since individuals from tropical areas have a pattern of frequent relapses within short intervals, whereas those from temperate areas tend to

have relapses in long intervals [77]. This parasite behavior hinders the undertaking of proper therapeutic measures, in particular when people suffer relapses when no longer living in malaria-endemic areas, which leads to delayed diagnosis and treatment.

Furthermore, studies by Chen *et al.* [79] and Imwong *et al.* 2012 [80] have shown that parasite strains found in patients suffering *P. vivax* relapses are genetically different from those found during the primary infection, thus suggesting that an effective pre-erythrocyte vaccine should include the different genotypes to completely block parasite development within the liver and prevent the generation of blood-stage parasites.

Many questions remain regarding hypnozoite activation mechanisms; further molecular studies dealing with the immune recognition of this particular stage should be undertaken considering that robust host–parasite model systems such as *P. cynomolgi*- and *P. simiovale*-infected *Rhesus* monkeys are now available [81].

Another feature of *P. vivax* is that this species can generate a rapid disease transmission, even before individuals become symptomatic. Different to *P. falciparum* behavior, *P. vivax* rapidly develops gametocytes, which can then be ingested by a mosquito vector thereby leading to perpetuation of the parasite's life-cycle even before the infected person is aware of having the disease [82].

Experimental model

Several studies have focused on the search for an ideal animal model for malaria that could mimic the immune response induced in infected humans. The WHO recommended using *Aotus spp.* monkeys in 1988 as an appropriate experimental model for studying human malarial infections [83].

These primates have been widely used in research as their immune system molecules share a high degree of identity with their human counterparts (MHC, immunoglobulins, T-cell receptors and cytokines) [84]. Even though *Aotus spp.* monkeys do not naturally become infected by *Plasmodium* species causing malaria in humans, they have been shown to be highly susceptible to experimental infection. Research was thus advanced leading to adapting *P. vivax* strains (mainly Sal-1 [85], VCG-1 [86] and Chesson [87]) in New World primates such as *Aotus*, *Saimiri* and *Callithrix*, allowing the effectiveness of vaccine candidate antigens to be evaluated in preclinical studies.

The FIDIC Malaria Molecular Biology group adapted the *P. vivax* VCG-1 strain in *Aotus spp.* monkeys; this proved to be highly infective after 22 successive passes, reaching 7.88% parasitemia, as determined by Giemsa, acridine orange staining and real-time PCR [86]. This strain has been used in experimental infection studies involving *Aotus spp.* monkeys previously immunized with some of the previously described proteins (MSP-1 [35], RBP-1 [24], RAP-2 [25] and MSP-10 [27]) in which the immunogenicity, safety and protective efficacy of each of them has been evaluated. It has been found that unvaccinated nonsplenectomized monkeys were not reliably infected, suggesting that the strain cannot generate reproducible courses of the infection in animals with their spleen intact. These results agreed with previous studies using the Sal-1 [88] and Chesson strains [87]. In light of this, the search for new strains able to cause infection in the experimental model without the need for surgical intervention is necessary for continuing to progress in such assays.

Expert commentary & five-year view

Although few preclinical and clinical studies have been carried out during the last few years, there is no denying that a representative progress has been made in characterizing new antigens that are relevant for developing a vaccine against *P. vivax*. The aforementioned inherent difficulties when working with *P. vivax*, as well as the limited number of monkeys that can be used for experimentation (considering that they require to be splenectomized), have highlighted a bioinformatics approach as an interesting alternative for candidate screening. Comparative analysis of the transcriptional profile for malaria-causing parasite species has led to the identification of *P. vivax* genes having a counterpart in other *Plasmodium* species; in the *P. vivax* transcriptome analysis, Bozdech *et al.*, identified 3,566 *P. vivax* open reading frames showing shifts in mRNA abundance across the intra-erythrocyte stage, of which 2,923 have orthologs in *P. falciparum* [3]. *Plasmodium* parasite species share some invasion routes involving genes that are transcriptionally regulated and expressed. Recently, an *in silico* analysis using probabilistic profile hidden Markov models trained with proteins from other *Plasmodium* species for which the role in invasion and other biological parameters had been experimentally determined, allowing the identification of 45 *P. vivax* open reading frames with a potential role in invasion; 13 of them had already been described as vaccine candidates, thus validating the approach [89]. In a complementary study, Frech *et al*. analyzed the chromosome-internal regions of six published *Plasmodium* genomes including *P. vivax* [48]. In this study, 173 genes were identified as *P. vivax* species-specific with an unannotated function; interestingly, a nonsynthetic cluster of eight genes was found on chromosome 6 that might have a similar function in target cell invasion to that of the *P. falciparum* cluster [48]. These results suggest that studying *P. vivax* species-specific genes could lead to a better understanding of this species' unique biological mechanisms, in particular the preference for invading reticulocytes and the development of latent hepatic forms. It is thus expected in the near future that studies leading to the functional characterization as well as determining the antigenic properties of the protein products encoded by the aforementioned genes are undertaken.

The classical approach that has been followed by most groups working in malaria for selecting candidates for a subunit vaccine involves screening for those proteins that had been shown to be more antigenic. Unfortunately, the most antigenic regions usually tend to be the most polymorphic ones, thus making it difficult to avoid strain-specific immune responses. A different approach, reviewed in a recently published paper, has been followed by our group for establishing a logical and rational methodology for designing a vaccine against *P. falciparum* [90]. Rather than targetting the antigenic regions, this approach focused first on identifying conserved HABPs to the respective target cells (in this case RBCs). Taking into account that these conserved HABPs displayed low antigenicity and immunogenicity, they were then

modified to allow a better fit into MHC Class II, thus inducing strong immunogenicity and protection-inducing ability in the *Aotus* experimental model.

Given the urgent need to move forward in the search for a *P. vivax* vaccine, following a similar approach to that undertaken for *P. falciparum* could be a promising strategy to control the malaria burden caused by this parasite species. Some novel advances might allow us to overcome the barriers that exist when working with *P. vivax*; the identification and selection of molecules functionally relevant in target cell binding could be partially solved by comparative genomics studies. Also, reticulocyte purification from different sources enriched in this type of cells will facilitate identifying new HABPs to target cells and their role in invasion inhibition. HABPs need to be modified to allow a better fit into the MHC Class II binding groove, which requires us to carry out 3D-structural analyses for both native and modified peptides by nuclear magnetic resonance. Although strain-specific immune responses can be avoided by selecting HABPs conserved between the different parasite isolates only, the MHC Class II allelic diversity displayed by the host cannot be ruled out; different modified HABPs with a preferential binding to the various HLA-DR families are thus required to provide proper protective coverage to the vaccinated population.

So far, several binding regions have been identified in three *P. vivax* merozoite proteins: DBP, MSP-1 and RBP. HABPs found in such molecules have been synthesized in native and modified forms following the aforementioned methodology and *in vitro* MHC Class II binding assays, as well as immunization experiments in the *Aotus* monkey model are underway. These and novel molecules will continue to be analyzed with the goal of designing a potentially effective multiantigen and multiepitope vaccine against *P. vivax*.

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Key issues

- *Plasmodium vivax* malaria is a prevalent public health problem in tropical and subtropical regions that requires immediate attention.
- The search for a vaccine against *P. vivax* has become an ongoing challenge given the species' biological complexity regarding its cellular tropism, its high genetic variability and the formation of latent forms.
- Immunization with irradiated *P. vivax* sporozoites was the first representative advance providing evidence of blocking the parasite's infection of host hepatocytes.
- Advances made during the last few years evaluating the transcriptome, proteome and *in silico* comparative analysis of different parasite species have been essential to identify antigens that could be included in a vaccine.
- Immune response mechanisms associated with protection have been determined in preclinical studies carried out when immunizing with the attenuated parasite or recombinant proteins.
- Identifying specific reticulocyte-binding sequences opens up a new research field for developing a chemically made, multiepitope and multiantigen vaccine.
- The development of chemically synthesized vaccines represents one of the most promising rationale and logical strategies to be followed for producing a first-generation vaccine against the second most prevalent malarial parasite species around the world: *P. vivax*.

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