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Desenvolvimento de vacinas para *Plasmodium vivax* e descoberta de novos antígenos usando o modelo *P. berghei.*

Tese apresentada ao programa de Pós-graduação em Biologia da Relação Patógeno Hospedeiro do Departamento de Parasitologia do Instituto de Ciências Biomédicas da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Área de concentração: Biologia da Relação Patógeno-Hospedeiro

Orientador: Prof. Dr. Daniel Youssef Bargieri

Versão original

São Paulo

2019

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Plasmodium vivax vaccine development and antigen discovery using *P. berghei* malaria models.

Ph.D. Thesis presented to the Post-graduation program Biology of Host Pathogen Interaction at the Institute of Biomedical Sciences of the University of São Paulo, in order to obtain the degree of Doctor in Sciences

Area: Biology of Host-Pathogen Interactions

Supervisor: Prof. Dr. Daniel Youssef Bargieri

Original version

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2019

ABSTRACT

DOBRESCU, I. ***Plasmodium vivax*** **vaccine development and antigen discovery using** ***P. berghei*** **malaria models.** 2019. 117 p. Ph.D. Thesis (Parasitology) – Institute of Biomedical Sciences, University of São Paulo, São Paulo, 2019.

Malaria is a preventable, diagnosable and treatable disease. Yet, the disease causes thousands of deaths every year, and millions of people are still endangered. Almost all malaria cases worldwide are due to infection with *Plasmodium vivax* or *P. falciparum*. There is an urgent need to eliminate malaria, since drug resistance is reappearing, and new strategies, like efficient vaccines, will be required. Developing a malaria vaccine is one of the greatest challenges in biomedical sciences. Vaccine development against *P. vivax* is even more challenging, because the parasite cannot be continuously cultured in laboratories. The lack of cultures has been an obstacle slowing pre-clinical tests of vaccine formulations against *P. vivax* based on known antigens and makes new antigen discovery particularly difficult. This project had two major proposals: *i)* use a malaria murine model, *P. berghei*, to construct hybrid transgenic parasites expressing a *P. vivax* blood stage antigen, $MSP1_{19}$, and use the hybrid parasite to challenge mice vaccinated with available PvMSP1 based vaccines, testing the efficacy of these formulations; *ii)* use *P. berghei* to search for *Plasmodium* functional homologs of the *P. falciparum*-specific invasion molecule PfRh5, a promising vaccine antigen candidate that has recently been shown to bind to basigin on the surface of host cells and to be essential for invasion. We found that the replacement of *P. berghei* $MSP1_{19}$ by the one of *P. vivax* is possible in both ANKA and NK65 strains. Mutant hybrid parasites infected hosts similarly to the respective wild type. Mice immunization with $PvMSP1_{19}$ vaccine formulations induced high specific IgG titers. The model of BALB/c mice immunization and NK65 hybrid challenge may be a valuable model for testing $PvMSP1_{19}$ vaccine formulations. The formulation FliC-$PvMSP1_{19}$-PADRE + poly (I:C) was the most protective in this model, showing a delay in mortality without control of parasitemia. In addition, *P. berghei* RIPR is essential for parasite erythrocytic replication as it is for *P.* falciparum, localizes at micronemes and its interactome was described.

Keywords: *Plasmodium vivax*, *Plasmodium berghei*, immunization, $MSP1_{19}$, Rh5/RIPR.

RESUMO

DOBRESCU, I. **Desenvolvimento de vacinas para *Plasmodium vivax* e descoberta de novos antígenos usando o modelo *P. berghei.*** 2019. 117 f. Tese (Doutorado em Parasitologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2019.

A malária é uma doença evitável, diagnosticável e tratável. No entanto, a doença causa milhares de mortes a cada ano e milhões de pessoas ainda estão em risco. Quase todos os casos de malária no mundo são devidos à infecção por *Plasmodium vivax* ou *P. falciparum*. Há uma necessidade urgente de eliminar a malária, uma vez que a resistência aos medicamentos está reaparecendo, e novas estratégias, como vacinas eficientes, serão necessárias. O desenvolvimento de uma vacina contra a malária é um dos maiores desafios das ciências biomédicas. O desenvolvimento de vacinas contra o *P. vivax* é ainda mais desafiador, pois o parasita não pode ser continuamente cultivado em laboratórios. A falta de culturas tem sido um obstáculo para testes pré-clínicos de formulações vacinais contra o *P. vivax* com base em antígenos conhecidos e torna a descoberta de novos antígenos particularmente difícil. Este projeto teve duas propostas principais: i) usar o modelo murino da malária, *P. berghei*, para construir parasitas híbridos expressando um antígeno da fase sanguínea de *P. vivax*, $MSP1_{19}$, e usar o parasita híbrido para desafiar camundongos vacinados com formulações baseadas na PvMSP1 e assim testar a eficácia dessas formulações; ii) usar o *P. berghei* para procurar homólogos funcionais da proteína PfRH5 de *P. falciparum*, um antígeno vacinal promissor que recentemente se mostrou ligar-se à basigina na superfície das hemácias e ser essencial para a invasão. Demonstramos que a substituição da proteína $MSP1_{19}$ de *P. berghei* pela proteína de *P. vivax* é possível, tanto nas linhagens ANKA como NK65. Os parasitas híbridos tiveram um padrão de infecção similar às cepas selvagens. A imunização de camundongos com formulações vacinais baseadas na $PvMSP1_{19}$ induziu altos títulos de IgG específicos. O modelo de imunização com camundongos BALB/c e o desafio com o parasita híbrido NK65 pode ser um modelo interessante para testes de formulações vacinais de $PvMSP1_{19}$. A formulação contendo a proteína recombinante FliC-$PvMSP1_{19}$-PADRE + poly (I: C) foi a mais protetora neste modelo, mostrando um atraso na mortalidade dos animais imunizados, apesar de não haver controle de parasitemia. Além disso, a proteína RIPR de *P. berghei* foi mostrada essencial para a replicação eritrocítica do parasita, assim como em *P. falciparum*. A PbRIPR também se localiza nas micronemas e seu interactoma foi descrito.

Palavra-chave: *Plasmodium vivax*, *Plasmodium berghei*, imunização, $MSP1_{19}$, Rh5/RIPR.

Note: This project has two parts (vaccine development and antigen discovery). A general introduction for both parts will be followed by specific introductions for each part. The results and discussion sections are also split in two parts.

1. GENERAL INTRODUCTION

1.1. Epidemiology of malaria

1.1.1. Brief history of malaria

Malaria, from Italian "mala aria", which translates "bad air", describes an ancient disease that in the mid-18th century was believed to be transmitted by fumes in the swamps. Hippocrates, 400 years Before Common Era (BCE), described unhealthy air responsible for diseases characterized by fever. Until the end of the 19th century, the disease was thought to be acquired from inhalation of the bad, heavy, air in swamp regions (Cox, 2010).

In 1880, Charles Louis Alphonse Laveran (Nobel Prize in Physiology or Medicine 1907), a military doctor working in Algeria, observed microscopic microbes in the blood of malaria patients (Laveran, 1880), and was the first to relate the disease to a microbial infection. How these microbes were transmitted to humans was, however, still elusive. Meanwhile, Patrick Manson discovered that filarial worms could be transmitted by mosquitoes and postulated that the same could happen in the case of malaria (Manson, 1878). Few years later, convinced by Manson, Ronald Ross (Nobel Prize in Physiology or Medicine 1902) identified parasitic forms in the gut of a female anopheline mosquito (Ross, 1897) and, working with *Plasmodium relictum* (infecting birds), he found parasite stages in culicine mosquitoes, elucidating the transmission cycle (Ross, 1898). A year later, the Italian malariologists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava showed that human *Plasmodium* spp are transmitted by anopheline mosquitoes (Grassi, 1900).

In the early 1900s, Fritz Schaudinn described that the sporozoite parasite forms present in the mosquito salivary glands could infect red blood cells (Schaudinn, 1903). Thus, for almost fifty years it was thought that blood infection occurred immediately after sporozoite injection during a mosquito bite. It was only in 1947 that Henry Shortt and Cyril Garnham, working in London, showed that a division phase occurs in the liver of monkeys 6-7 days after infection with *P. cynomolgi*, preceding the blood development of parasites (Shortt *et al*., 1948). Thirty years

later, Wojciech Krotoski and Garnham's team showed that *P. vivax* produces dormant forms that can persist for several years in the liver of infected animals (Krotoski *et al*., 1982).

1.1.2. Plasmodium life cycle

Malaria disease is caused by protozoan parasites of the genus *Plasmodium*, that belongs to the phylum Apicomplexa. Many parasitic eukaryotes possessing secretory organelles in the apical complex and forming a parasitophorous vacuole (PV) are members of this group. The phylum comprises important parasites that are causative agents of human and animal diseases, such as *Toxoplasma, Eimeria* and *Cryptosporidium* (Beck *et al*., 2009). The genus *Plasmodium* englobes more than 200 species (Fig. 1), among them six can infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi*, and *P. cynomolgi* (not represented in Fig. 1 as a human parasite). The last two are non-human primate natural parasites but recent studies found submicroscopic and asymptomatic infections in humans (Fornace *et al*., 2016; Imwong *et al*., 2019). *P. knowlesi* infections are, when symptomatic, potentially lethal.

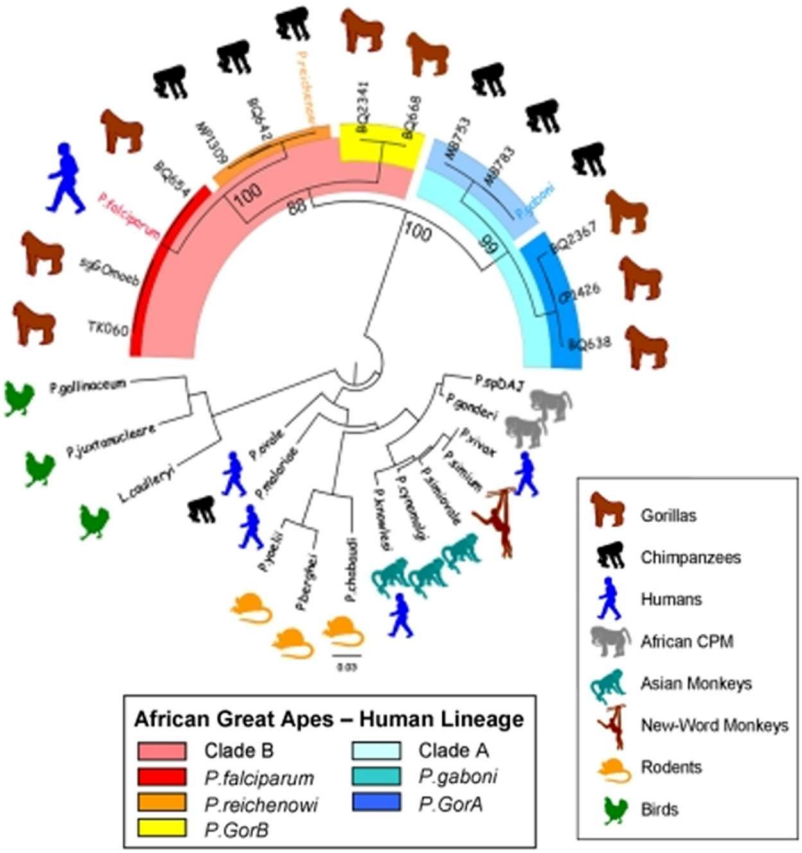

Figure 1 | Phylogenetic relationship of Plasmodium species. (Prugnolle *et al*., 2010).

Malaria spreads from an infected human to another through the transmission by the invertebrate

mosquito vector of the genus *Anopheles* (Fig. 2). Female mosquitos need essential nutrients and iron present in the vertebrate blood to produce and complete the development of their eggs. During a mosquito bite, while the hematophagous insect is searching for the blood vessel piercing the skin with its proboscis, hundreds of motile sporozoites are released into the skin of the vertebrate host, coming from the anopheles' salivary glands (Sidjanski *et al*., 1997). The unfortunate ones end up in the closest lymph node via lymphatic vessels where they die after partial development (Amino *et al*., 2006). Another part of the parasites can partially develop into the skin cells (Gueirard *et al*., 2010). The others migrate through the cutaneous tissues, enter the blood stream and migrate passively to reach the liver.

Sporozoites cross the liver sinusoidal barrier lined by endothelial cells and Kupffer cells to gain the hepatocytes layer. *Plasmodium* sporozoites can traverse hosts cells, including hepatocytes (Amino *et al*., 2008; Mota *et al*., 2001), which rend them competent for the final invasion (Mota *et al*., 2002) and impede sporozoite clearance by Kupffer cells during locomotion inside the sinusoid lumen (Tavares *et al*., 2013). When invading the hepatocytes, the sporozoite forms a vacuole (Mota *et al*., 2001) and starts to develop into a hepatic schizont containing merozoites. In the case of *P. vivax*, *P. cynomolgi* and *P. ovale*, the parasite can remain dormant in the hepatocyte for several years in a form called hypnozoite (Krotoski, 1985). Seven to twenty days after exo-erythrocytic schizogony, the mature schizonts are released in the form of merosomes full of merozoites directly into the bloodstream (Sturm *et al*., 2006).

Merozoites are the parasite forms invading erythrocytes, a process that takes less than two minutes (Gilson *et al*., 2009). In these host cells they undergo asexual replication, during which the parasite develops from rings to trophozoites and multiply to form erythrocytic schizonts containing ten to thirty merozoites. Red blood cell (RBC) membrane rupture, or egress, leads to the release of free merozoites in the blood stream, able to reinvade new erythrocytes. The synchronous rupture of erythrocytic-stage schizonts is responsible for the cyclic fever symptoms due to liberation of toxic components. The repeated invasion and egress of erythrocytes reflects the periodicity of the fever: tertian (48h, *P. falciparum*, *P. vivax*, *P. ovale* and *P. cynomolgi*) or quartan (72h, *P. malariae*). *P. knowlesi* has a 24h cycle.

Along the parasite blood journey, depending on specific signals present in the human serum, a sub-population of intracellular parasites transform into gametocytes (Brancucci *et al*., 2017). Male and female gametocytes are the sexual stages of the parasite, quiescent in the blood circulation. Taken by the anopheline mosquito, gametocytes activate due to external signals

like temperature change (drop of around 5°C), presence of xanthurenic acid, a metabolic intermediate of the tryptophan catabolism in the mosquito midgut, and extracellular pH increase (from 7.2 to 8) (Billker *et al*., 1998, 1997; Garcia *et al*., 1998; Kawamoto *et al*., 1991; Sologub *et al*., 2011). Male gametocyte exflagellates and form eight microgametes capable of fertilizing the macrogamete (female gamete) within sixty minutes after blood ingestion.

Fertilization of the macrogamete by the microgamete gives birth to a zygote that differentiates to become elongated and motile, the ookinete. The ookinete can cross the midgut epithelial barrier to reach the midgut basal lamina where it transforms into an oocyst. Several multiplications occur inside the oocyst to form the sporozoites, which are released after ten to twelve days into the hemolymph and reach the salivary glands of the mosquito, where they wait to be injected into another vertebrate host during a new mosquito blood feeding.

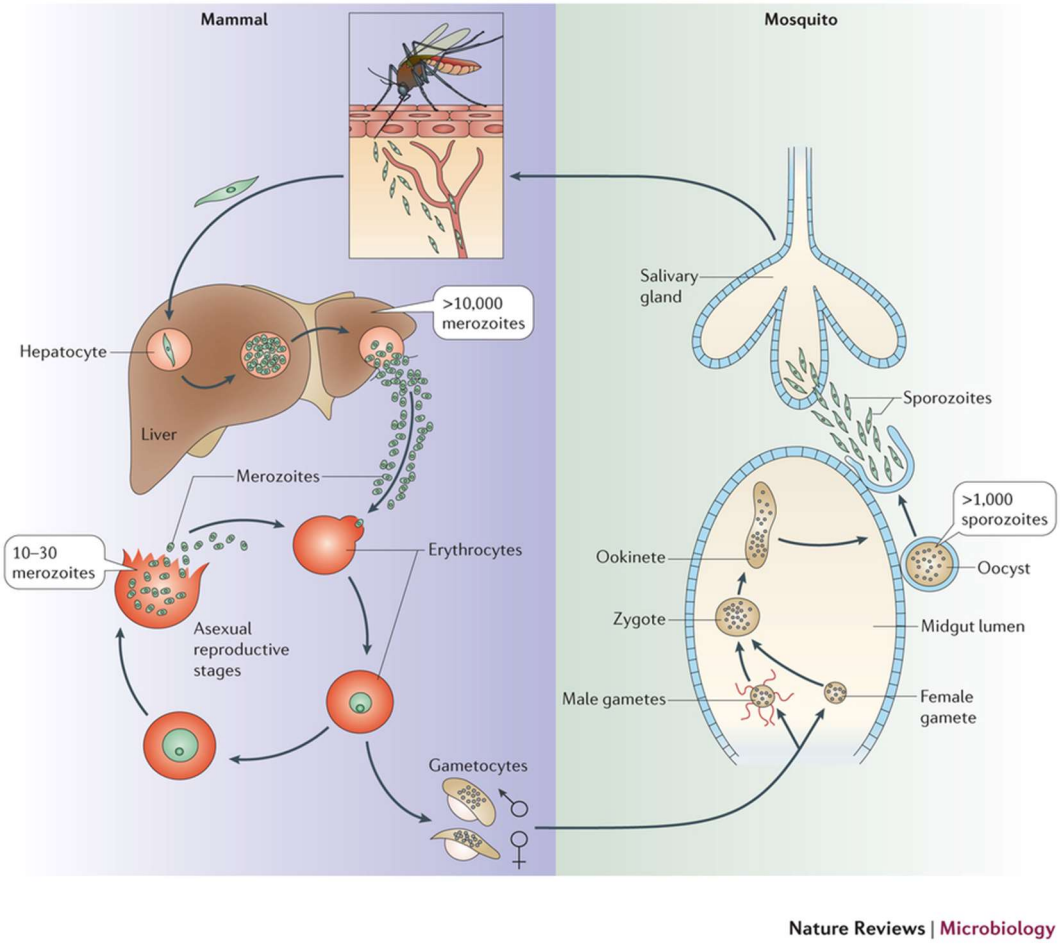

Figure 2 | Plasmodium life cycle. (Ménard *et al*., 2013)

1.1.3. Distribution

Until 1900, malaria was present almost all over the globe. From 1955 to 1965, the World Health Organization (WHO) launched the Global Malaria Eradication Program leading to malaria

eradication in Europe, North America and Australia (Fig. 3). In other endemic countries, due to insufficient technical assistance, funding, and infrastructure, control of the disease was unsuccessful despite continuous efforts. However, with innovation and intervention programs, there are fewer people dying from malaria today than there were twenty years ago. Indeed, 37% incidence and 60% death reduction were notified between 2000 and 2015 (Cibulskis *et al*., 2016).

Malaria was responsible for 219 million cases and 435 000 deaths worldwide in 2017, 61% of which were children under five years old (WHO, 2018). The disease spreads in tropical and subtropical regions and most of the severe cases (99.7%) occur in Africa due to *P. falciparum* infections. This parasite also accounted for the majority of cases in South-East Asia (62.8%), in the Eastern Mediterranean (69%) and in the Western Pacific (71.9%). In South America, *P. vivax* is predominant with 74.1% of cases (WHO, 2019a).

In Brazil, *P. vivax* is responsible for around 85% of malaria cases, affecting the population living in the Amazonian region and causing high morbidity with an important economic impact (Oliveira-Ferreira *et al*., 2010). To make matters worse, the number of reported severe cases due to *P. vivax* has increased in the last years (Costa *et al*., 2011).

Recent progress and efforts have shown encouraging events. Paraguay was certified by the WHO as malaria free in 2018, while Algeria, Argentina and Uzbekistan have made formal requests for certification. In 2017, China and El Salvador reported zero indigenous cases.

It is undeniable that the fight against malaria has made significant progress. However, it is still a deadliest disease worldwide and therefore more is needed for eradication with new tools and new strategies. To achieve this, programs like the Malaria Initiative Eradication (Bill and Melinda Gates foundation) aim to eradicate malaria by 2040 (Fig. 3).

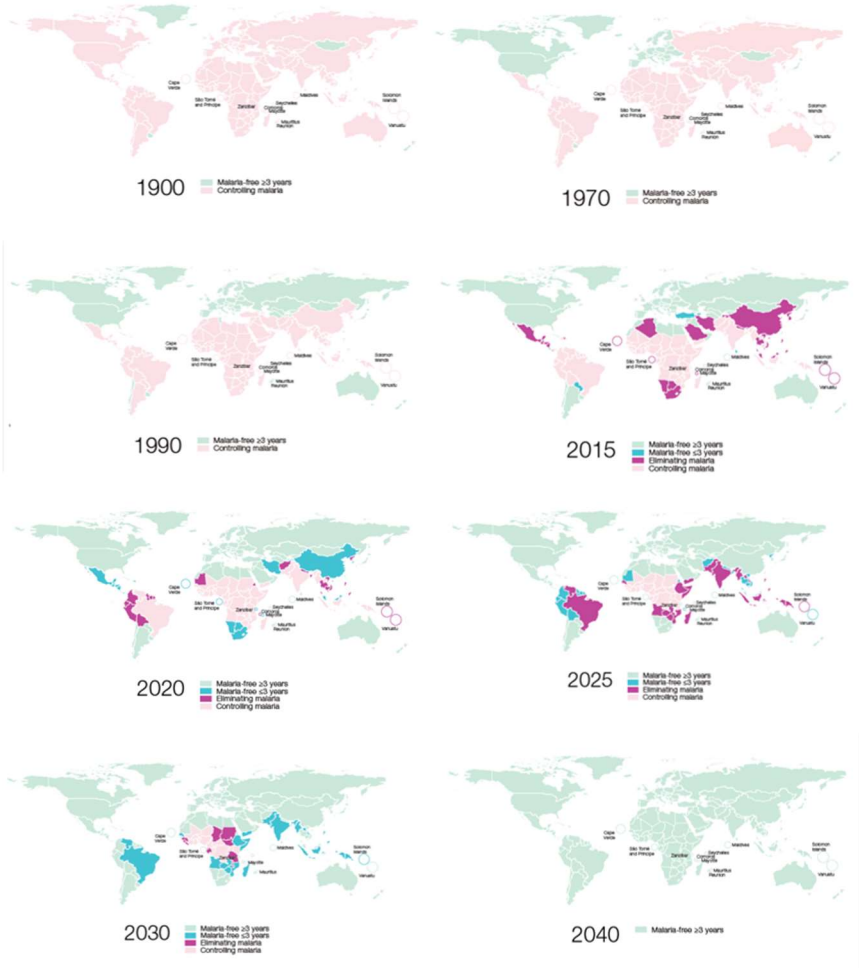

Figure 3 | The shrinking Malaria Map. Malaria source Initiative.

1.1.4. Pathogenesis

Fever, headaches, vomiting, muscle pain among a wide variety of symptoms are reported usually between ten days and four weeks after infection with malaria parasites. The symptoms occur when the erythrocytic schizont egress from the RBC, releasing toxic factors leading to fever. These mild symptoms are not specific but shared with other viral or bacterial infections, and malaria can be misdiagnosed in regions with low endemicity. In endemic regions, usually the diagnostic is more evident, especially in young children presenting thrombocytopenia, hepatosplenomegaly and anemia (Grobusch *et al*., 2005; N. J. White *et al*., 2014).

Administration of correct treatment rapidly eliminates the parasites from the patient's blood, preventing the progression of uncomplicated malaria. However, delays in diagnosis and inappropriate treatment of malaria increase morbidity and mortality (Kain *et al*., 1998; Trampuz *et al*., 2003). *P. falciparum* is the major cause of severe cases of malaria. Complications rapidly progress and can lead to death (WHO, 2019a). Nevertheless, in certain settings and regions,

P. vivax can also induce severe symptoms (Rahimi *et al*., 2014). The progression to severe malaria is mainly due to the parasite ability to induce cytoadherence and rosetting of infected erythrocytes.

1.1.4.1. Cytoadherence and rosetting

Cytoadherence is characterized by the ability of infected RBC (iRBC) to adhere to the vascular endothelium, a phenomenon first observed by Marchiafava and Bignami, in 1892 (Marchiafava *et al*., 1892). Cytoadherence can occur in several organs as the lungs, heart, brain, liver, and kidney, as well as in the subcutaneous adipose tissues and the placenta (Autino *et al*., 2012). *P. falciparum* parasites manipulate the iRBC to cytoadhere to the vascular endothelium by exposing parasite derived proteins at the host cell surface (Newbold *et al*., 1999). In addition, *in vitro* sequestration to some endothelial cell lines and placental cryosections has also been reported with *P. vivax* infected reticulocytes (Carvalho *et al*., 2010).

The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is a multimeric protein encoded by the *var* (variant) gene family (Scherf *et al*., 2008) and plays a major role in cytoadherence and virulence. PfEMP1 is also responsible for the rosetting phenomenon, which is a type of cytoadherence between iRBC and non-infected RBC or platelets (Rowe *et al*., 2009). *P. falciparum* ability to form rosettes correlates with disease severity, while *P. vivax* and *P. ovale* form rosettes but the relation of this with disease severity is still elusive (Angus *et al*., 1996; Udomsanpetch *et al*., 1995).

Severe malaria complications occur more frequently in non-immune individuals and affect the central nervous system (cerebral malaria), the pulmonary system (respiratory failure), the renal system (acute renal failure) and/or hematopoietic system (severe anemia). Likewise, pregnant women, infants, children under five years of age and patients with human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS) are considerably at higher risk of contracting malaria and develop severe disease.

1.1.4.2. Severe malaria

The most important complication in severe malaria is cerebral malaria (CM), a neurological syndrome that may lead patients to coma, presenting elevated intracranial pressure and brain swelling causing seizures, retinopathy and brainstem alterations (Idro *et al*., 2005). The sequestration of iRBC may cause cerebral occlusion of brain capillaries blocking nutrient supply to the brain inducing bleedings and neuronal alterations (Berendt *et al*., 1994). In

addition, inflammatory cytokine production and vascular leakage are characteristic features of CM, eventually resulting in brain hypoxia (Luzolo *et al*., 2019).

Due to cytoadherence ability of the parasite, other organs are the targets for sequestration as the lungs and kidneys. Respiratory failure due to infection by *Plasmodium* spp is another severe form of the disease and the clinical presentations are acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) responsible for an elevated mortality rate (Autino *et al*., 2012). It can induce pulmonary edema where intravascular fluid exacerbates into the lungs due to an increased alveolar capillary permeability (Taylor *et al*., 2006). The pathogenesis mechanisms of ALI/ARDS seem to involve activation of inflammatory pathways.

Malarial acute renal failure (ARF) can occur as an isolated complication or as a component of multiorgan involvement. The precise mechanism of malarial ARF is not known. However, many hypotheses like mechanical obstruction by infected erythrocytes, immune mediated glomerular and tubular pathology, fluid loss and alterations in the renal microcirculation have been proposed (B. S. Das, 2008).

The causative mechanisms of severe anemia are multifactorial, such as hemolysis of infected and uninfected RBC, inappropriate bone marrow response, and other individual factors (Bartoloni *et al*., 2012; Calis *et al*., 2008).

1.1.5. Prevention strategies

Malaria is a preventable and treatable disease. Prevention methods recommended by the WHO are mostly the use of insecticide treated nets (ITN) (or long-lasting insecticidal nets LLINs) to physically drive away mosquitos, usually placed on the top of the beds (WHO, 2019b). The nets are impregnated with pyrethroid insecticide and can be used for three years. Some nets contain piperonyl butoxide having a synergistic effect with pyrethroids. The use if ITN has shown to reduce the rate of uncomplicated episodes of *P. falciparum* but no clear evidence has been shown for *P. vivax*.

In some regions, indoor residual spraying (IRS) can be used to eliminate the mosquitoes and prevent transmission. The use of IRS had a high contribution for malaria elimination in South Europe and the Mediterranean, in Russia, in large parts of Asia and Latin America, as well as in many areas of South Africa (Karunamoorthi, 2011). Highly persistent and having a long residual effect of over six months on most household surfaces, the dichloro-diphenyl-trichloroethane (DDT) remains the best insecticide recommended for IRS (Rehwagen, 2006).

These methods are not, however, hundred percent efficient due to the lack or misuse of the ITNs, and mosquito resistance to the insecticides. Moreover, they may be inefficient for malaria elimination in low incidence regions (Loha *et al*., 2019). Other vector control strategies can be employed or are being proposed, such as larval control, reducing mosquito breeding sites (chemically or biologically), fogging, personal protection, sterile male release, or genetic modification of malaria vectors diminishing the susceptibility of mosquitos to parasites (CDC, 2018).

1.1.6. Treatment of the disease

The first and most effective treatment against malaria was the quinine, an alkaloid isolated from a cinchona tree in 1820 (Achan *et al*., 2011; Tse *et al*., 2019). After first reports of parasite resistance (Bunnag *et al*., 1996), quinine was no longer used as a treatment (restricted use to severe malaria in specific cases). Resistance is the major concern when introducing a new drug against malaria. During the 1950s, the first cases of chloroquine resistance appeared only a decade after its utilization. Its use is nowadays restricted to *P. vivax* infections in regions where no resistance has been reported (Model List of Essential Medicines).

In 1971, Tu Youyou (Nobel Prize in Physiology or Medicine in 2015) isolated artemisinin from the plant *Artemisia annua*, used in traditional Chinese medicine ("Antimalaria studies on Qinghaosu.," 1979) and demonstrated the efficacy against malaria and multi-drug resistant *P. falciparum* strains. As resistance seems slow to appear, artemisinin and its derivatives in combination with other drugs (artemisinin combination therapy, ACT) is the treatment of choice nowadays.

In *P. vivax* infections, chloroquine is still employed in many regions, and the WHO recommends treatment with primaquine, which is efficient against the hypnozoite forms in the liver. However, primaquine is not recommended for pregnant or breast-feeding women neither for glucose-6-phosphate dehydrogenase (G6PD)-deficient patients.

1.2. Erythrocyte invasion by malaria parasites

1.2.1. Merozoite structure and apical organelles

The merozoite of *Plasmodium*, measuring 1-2 μm, is the form invading the erythrocytes (Fig. 4). It has common characteristics with other invading forms of the parasite, like the ookinetes and sporozoites, as well as with *Toxoplasma gondii* tachyzoites (Cowman *et al*., 2006). These

forms share a polarized morphology and secretion of the content of apical secretory organelles: micronemes, rhoptries and dense granules. Rhoptries and micronemes are membrane-bounded organelles containing dense granular content, rhoptries being larger than micronemes (Blackman *et al*., 2001). These two organelles secrete many different proteins involved in: attachment to the host cell membrane; local dismantling of the host cell membrane cytoskeleton; host cell plasma membrane transformation into a PV membrane; and parasite's actin–myosin motor link to the host cell (Blackman *et al*., 2001). After invasion, dense granules have a principal role in modification of the host cell membrane. When invasion is complete, the merozoite resides and develop inside the PV forming a barrier between the host cell cytosol and the parasite surface (Lingelbach *et al*., 1998).

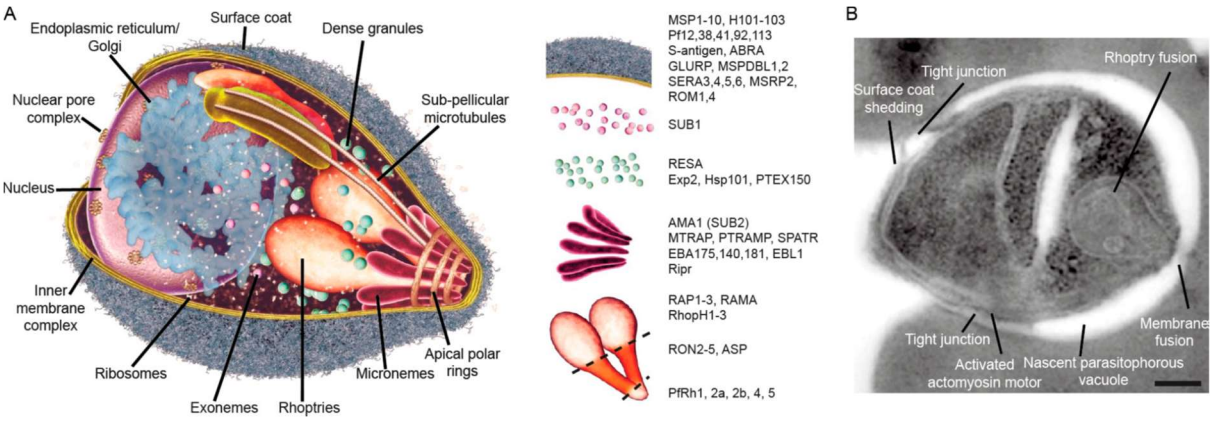

Figure 4 | Three-dimensional diagram of a merozoite and its core secretory organelles. (Cowman *et al*., 2012)

1.2.2. Invasion process

Erythrocyte invasion by *Plasmodium* spp merozoites is a rapid process (~2min) involving high protein trafficking and cell remodeling (Dvorak *et al*., 1975). The process can be separated into various phases: 1- initial merozoite attachment, 2- apical re-orientation of the merozoite, 3- formation of a tight junction and 4- complete invasion and sealing of the PV.

When the first generation of merozoites coming from the liver invade erythrocytes, they maturate into schizonts containing newly formed merozoites. Rupture of these schizonts liberates free merozoites and it is called egress. In the bloodstream, merozoites can reversibly attach to another RBC and immediately the irreversible apical reorientation of the merozoite occurs. A tight junction is formed between the parasite and the RCB membrane, where rhoptry neck proteins (RONs) and the apical membrane antigen 1 (AMA1) are present. The active entry of the parasite is permitted thanks to the actomyosin motor activity and during this invasion

process the parasite forms the PV. The parasite completes the invasion of the RBC when the tight junction is located at the posterior end of the merozoite, and vacuole and RBC membranes are sealed (Fig. 5) (Cowman *et al*., 2012).

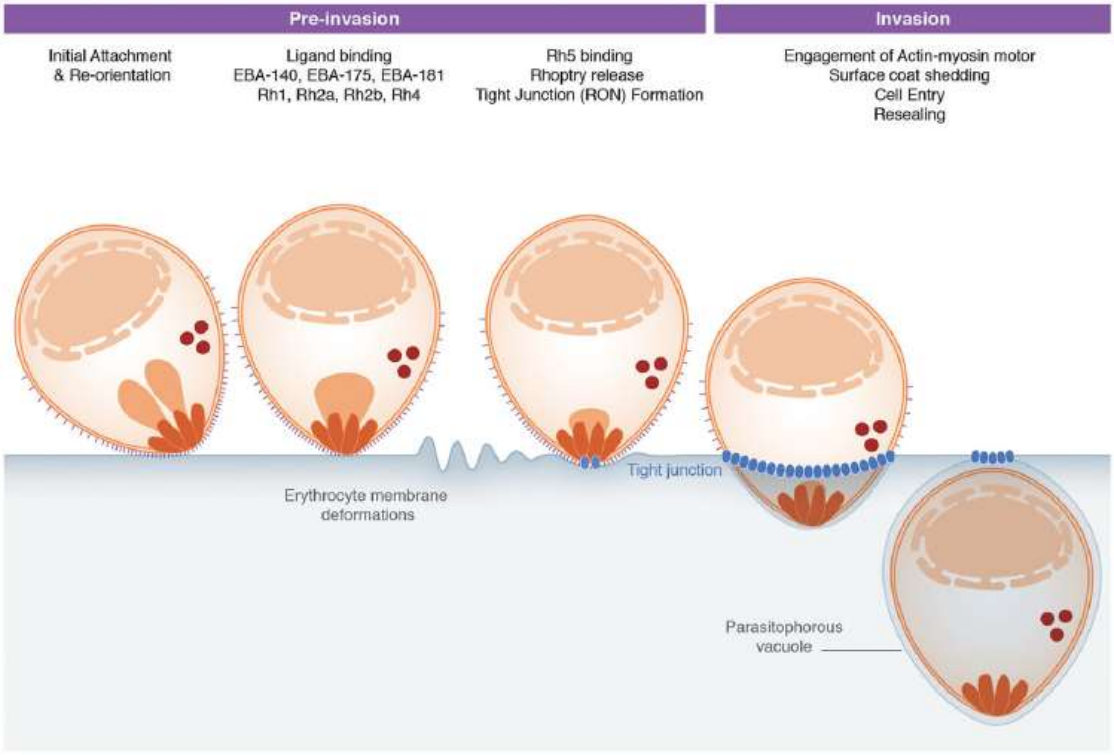

Figure 5 | Overview of erythrocyte invasion by P. falciparum merozoites. (Koch *et al*., 2016)

1.2.2.1. Initial attachment

Merozoite surface proteins (MSPs), which coat the entire free parasite (Kadekoppala *et al*., 2010; Lin *et al*., 2014), are likely involved in merozoite initial attachment to the erythrocyte surface, interacting with band 3 (Goel *et al*., 2003; Li *et al*., 2004) or glycophorin A (Baldwin *et al*., 2015). MSP1 is the largest, most abundant protein of the family (Blackman *et al*., 1990) and the best characterized. MSP1 acts in combination with other MSPs that assemble to form a large protein complex (including MSP6-7 and MSPDBL1-2) (Kadekoppala *et al*., 2010).

MSP1 is a large protein (~200 kDa) synthetized during schizogony that suffers cleavage by the serine protease subtilisin 1 (SUB1) during egress (Koussis *et al*., 2009; Silmon de Monerri *et al*., 2011; Yeoh *et al*., 2007). The protein is then separated into four polypeptides of 83, 30, 38 and 42-kDa forming a non-covalent complex at the merozoite surface (Fig. 6). At the onset of RBC invasion, a second subtilisin, the subtilisin 2 (SUB2) (Harris *et al*., 2005), cleaves $MSP1_{42}$ in two subsequent fragments: a 33-kDa fragment released with the other polypeptides; and a 19-kDa fragment that remains attached to the merozoite membrane by

glycosylphosphatidylinositol (GPI) anchor (Holder, 2009). Kinetics of the MSP1 processing was monitored and shows that the 19-kDa C-terminal region enters the RBC while the 83-kDa not (Boyle, Wilson, *et al*., 2010). The same group showed that blocking MSP1 ligands (heparin-like molecules) impedes the majority of merozoites to attach to erythrocytes (Boyle, Richards, *et al*., 2010). Recently, new data also supports a role for MSP1 during egress (S. Das *et al*., 2015).

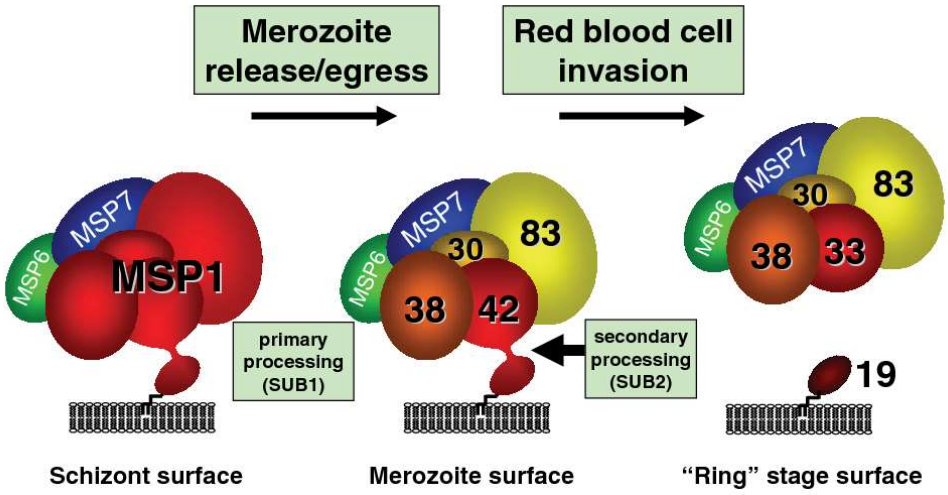

Figure 6 | The assembly and processing of the MSP1 complex. (Holder, 2009).

After attachment, proteins secreted from the apical organelles, micronemes and rhoptries are then the main players through the path of invasion.

1.2.2.2. Apical reorientation and active invasion

The purpose of apical reorientation is to have closer interaction between the apical end of the merozoite and the erythrocyte membrane. Following reorientation, the invasion ligands, such as adhesins, interact with specific RBC surface receptors to allow apical attachment (Fig. 7). These proteins are secreted onto the merozoite apical end from micronemes and anterior necks of the rhoptry organelles. Two main families of proteins are involved in this process: the Duffy binding-like (DBL) or erythrocyte binding-like (EBL) proteins and the reticulocyte binding-like protein homologs (Rh or RBL).

Some DBL receptors have been characterized so far. In *P. vivax*, the Duffy-binding protein (DBP), which was first described in *P. knowlesi*, was found to bind to the Duffy antigen/chemokine receptor (DARC) (Haynes *et al*., 1988; Singh *et al*., 2005). In *P. falciparum*, members of the EBL family, the erythrocyte-binding antigens 175 (EBA175) and 140 (EBA140) bind to O-linked glycans of glycophorins A (Sim *et al*., 1994) and C (Maier *et al*.,

2003) respectively, and the erythrocyte-binding ligand 1 (EBL-1) binds to glycophorin B (Mayer *et al*., 2009) (Fig. 7).

Members of the RBL, RBP1 and RBP2 were identified in *P. vivax* selectively binding to reticulocytes (Galinski *et al*., 1992). They are now known to have orthologs in *P. falciparum*, including PfRh1 (Gunalan *et al*., 2013), PfRh2a (Gunalan *et al*., 2011) and the PfRh5 (Baum *et al*., 2009). Recently, the *P. vivax* RBP2b was shown to bind to transferrin receptor 1 in reticulocytes and seems to define part of the host cell specificity of this parasite (Gruszczyk *et al*., 2018).

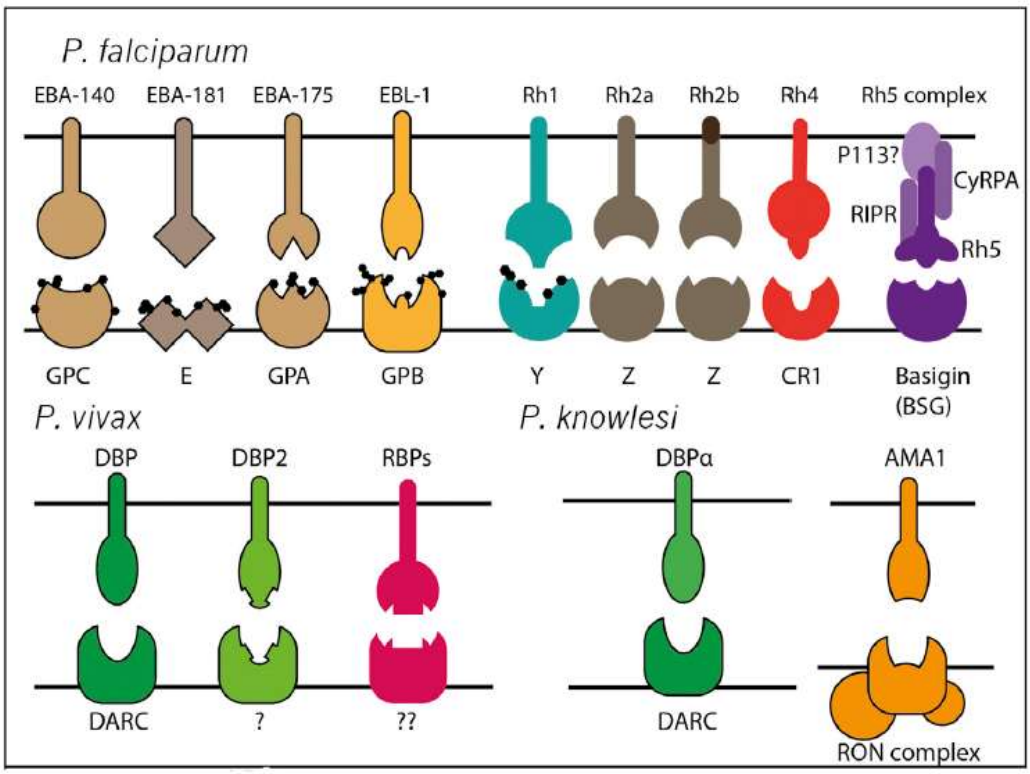

Figure 7 | Erythrocyte receptor and parasite ligand interactions involved in merozoite invasion. *P. falciparum, P. vivax*, and *P. knowlesi.* (Cowman *et al*., 2017).

The active entry of the merozoite into the host cell is thought to be mediated by a complex formed by RON2 and AMA1 (Bargieri *et al*., 2013) (Fig. 8). The rhoptry protein RON2 is inserted into the RBC membrane and acts as a receptor for the microneme protein AMA1 (Richard *et al*., 2010; Srinivasan *et al*., 2011). The current model of invasion pictures the AMA1-RON2 complex as the structure of the tight junction, at which the actin-myosin motor exerts the force that pulls the merozoite body inside the newly formed PV. The motor is part of a submembranar protein complex, called the glideosome. Although well studied among apicomplexan parasites, the molecular mechanisms of invasion of host cells appear more

complex than previously thought. As recently reported, the RON2-AMA1 interaction may not be essential for junction formation and alternative pathways for invasion independent of the actin–myosin motor could also be involved in the process (Bargieri *et al*., 2014).

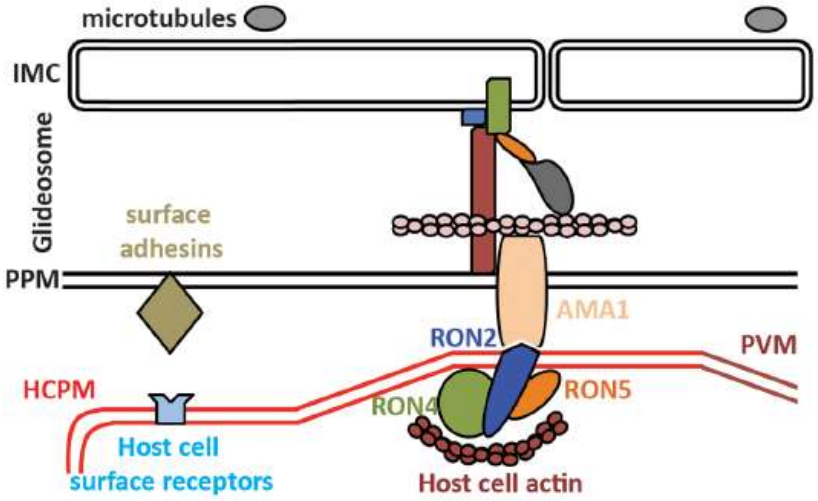

Figure 8 | The model of invasion seen as the junction structured by the AMA1-RON complex. (Bargieri *et al*., 2014).

1.3. Genetically modified rodent malaria parasites

1.3.1. Rodent malaria models

Historical discoveries of malaria biology came from animal studies. As an example, the liver stage of malaria was first identified in birds (James *et al*., 1937; Raffaele, 1936). For many human diseases, animal models have been useful to understand the biology of pathogens, immune responses induced by them, and as a result, improve drug targeting and vaccine approaches. These models include great apes and monkeys, that are being used to a lesser extent due to ethical issues.

In malaria studies, the mouse is the most used model. Between 1948 and 1965 rodent *Plasmodium* species were described in wild rodent animals' natural infections in the African continent (Fig. 9): *P. berghei* (Vincke *et al*., 1948), *P. vinckei* (Rodhain, 1952), *P. chabaudi* (Landau, 1965), and *P. yoelii* (Landau *et al*., 1965), helping experimental malariology (Killick-Kendrick *et al*., 1978). Various strains of each parasite were isolated from African thicket rats *Thamnomys rutilans*, and they differ in pathogenicity, *i.e*. some are more virulent causing lethal infection than others (Landau *et al*., 1978). Parasites such as *P. berghei* and *P. vinckei* and some strains of *P. yoelii* and *P. chabaudi* cause lethal infections in mice, whereas *P. yoelii* 17XNL, *P. chabaudi chabaudi*, *P. chabaudi adami*, and *P. vinckei petterei* infections are resolved after

the initial acute parasitemia or can present small recrudescence for few months (Cox, 1988; Landau *et al*., 1978; Langhorne, 1994).

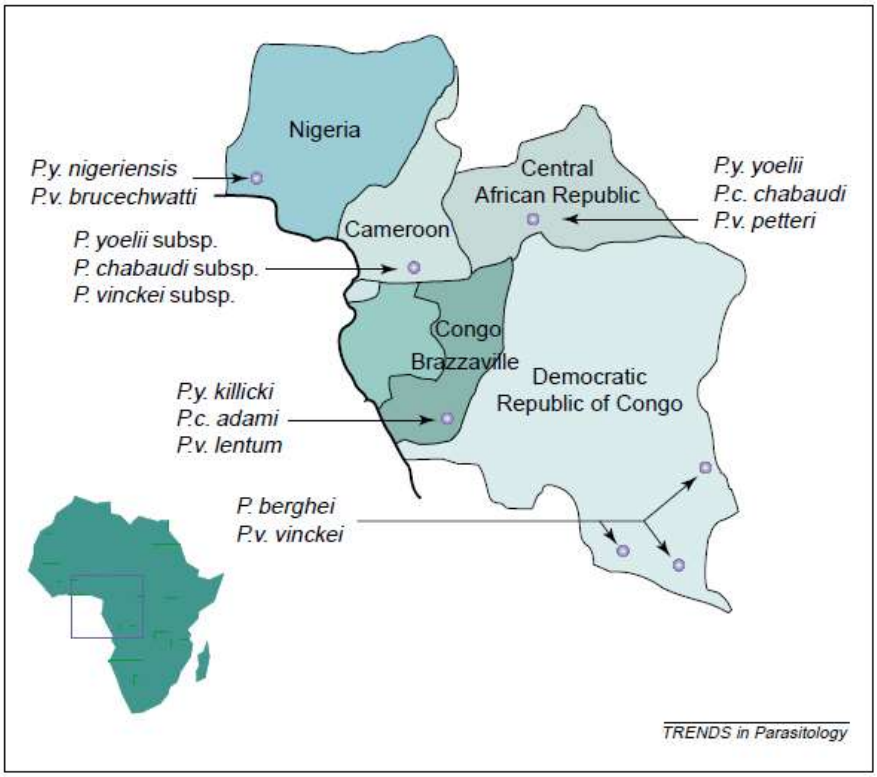

Figure 9 | Countries in which rodent malaria parasites are found. P.c., *P. chabaudi*; P.v., *P. vinckei*; P.y., *P. yoelii*. (Carlton *et al*., 2001).

One of the most interesting advantages of rodent malaria parasites is the experimental access to the entire life cycle allowing exploration of transmission dynamics, vaccines, and drug targets. Similar to all mammalian, the rodent parasites can replicate asexually in the bloodstream and has a sexual replication in the *Anopheles* mosquito. Laboratory mice can be infected after blood inoculation or by an infected mosquito bite. The availability of experimental laboratory mouse that can be inbred, outbred, or even knockout (KO) for specific genes enlarges the possibilities of specific biological studies (J. K. White *et al*., 2013). More recently, humanized mice were created and allow multiple study options (Minkah *et al*., 2018). Classically, inbred C57BL/6 or BALB/c mouse strains are used in malaria research. The parasite pathogenicity can also differ depending on the mouse strain.

Wykes and Good underline some relationship between mouse studies and observations in humans (Wykes *et al*., 2009). For example, the *P. falciparum* 19-kDa C-terminal region of MSP1 ($PfMSP1_{19}$)specific T cells were found to not be well maintained in patients (Egan *et al*., 1997). Studies in mice found that adoptively transferred CD4 T cells stimulated with whole

P. berghei extracts (Hirunpetcharat *et al*., 1998), or specific for PyMSP1$_{19}$ (Wipasa *et al*., 2001), were deleted by apoptosis after infection. This suggests that during a secondary infection the parasite could escape the immune system by inducing the deletion of specific T cells. Therefore, observations using the mouse model can in many instances help explain what is observed in human infections.

1.3.2. Plasmodium genome

The complete *P. falciparum* 3D7 genome was published in 2002 by Gardner *et., al*. (Gardner *et al*., 2002). The nuclear genome was described as 22.9 megabases (Mb) and essentially complete, distributed among 14 chromosomes that vary from approximately 0.643 to 3.29 Mb, with a G+C content of approximately 19%, 5268 genes and approximately 80 gaps. In addition to *P. falciparum,* other genome sequences of *Plasmodium* parasites have been published: the rodent malaria *P. yoelii* 17XNL (Carlton *et al*., 2002), *P. berghei* ANKA and *P. chabaudi* AS (Hall *et al*., 2005), the human malaria parasite *P. vivax* Salvador 1 (Sal 1) strain (Carlton, Adams, *et al*., 2008) and the simian and human malaria parasite *P. knowlesi* H strain (Pain *et al*., 2008). Today, the genome of 22 *Plasmodium* species can be easily assessed in PlasmoDB.org with relatively good annotation.

P. falciparum and rodent malaria genomes are haploid and extremely A + T rich (80.6% on average and close to 90% in introns and intergenic regions in *P. falciparum*) (Gardner *et al*., 2002; Winzeler, 2008) while the *P. knowlesi* and *P. vivax* genomes are more G + C rich (37.5% and 42.3%, respectively) (Carlton, Escalante, *et al*., 2008; Pain *et al*., 2008).

1.3.3. Genetic manipulation

P. berghei is a widely used mouse malaria model and a main tool for reverse genetic studies. The *P. berghei* genome is homologous to the human parasite genomes, which allows various experiments that are difficult to perform with human parasites (Kooij *et al*., 2005).

Transfection is the transfer of exogenous nucleic acid sequences into a eukaryotic cell. Genetic manipulation of *Plasmodium* spp using transfection technology has provided an important tool to aid in the functional study of genes. In 1993, Goonewardene and colleagues were the first to successfully transfect a plasmid deoxyribose nucleic acid (DNA) in *P. gallinaceum*, avian *Plasmodium*, resulting in transient expression of a reporter gene (Goonewardene *et al*., 1993). Few years later, transient *P. falciparum* (Wu *et al*., 1995) and stable *P. berghei* (Van Dijk *et*

al., 1995) transfection were achieved. The ability of the parasite to integrate exogenous DNA into the genome, specifically by homologous recombination, has been favorable for improvement of transfection techniques.

P. berghei transfection has a higher and more efficient transformation rate which makes it a more robust transfection system (Philip *et al*., 2013). Indeed, *P. berghei* transformation occurs in 10-15 days with an efficiency of 10^{-3}-10^{-4} (Janse *et al*., 2006), while *P. falciparum* takes more than a month with a transfection frequency 1000 times less efficient. The recent CRISPR/Cas technology has recently been applied in *P. falciparum* and could reduce time and give higher efficiency of transfections (Ghorbal *et al*., 2014). *P. berghei* transfection target cells are the schizonts containing fully mature merozoites, which are most suitable than ring or trophozoites and that can be obtained in abundance. Also, *P. berghei* schizonts are easy to collect because *in vitro* culture of infected RBC allows synchronization of blood stages until schizonts that are unable to rupture at the end of the full maturation and remain stable for prolonged periods (Janse *et al*., 2006).

Three selection markers are commonly used for *P. berghei* transfection: the pyrimethamine resistant forms of the *Plasmodium* dihydrofolate reductase (*dhfr*) gene and of the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene from *T. gondii*, and the human DHFR gene (hDHFR). The hDHFR confers resistance to pyrimethamine but also to the antimalarial drug WR99210 (Fidock *et al*., 1997; Zhang *et al*., 2002). The *Plasmodium dhfr* as a selection marker gene was isolated from a pyrimethamine-resistant clone of *P. berghei* (Van Dijk *et al*., 1994) that contains a point mutation resulting in an amino acid replacement ($Ser^{110} \rightarrow Asn$) associated with a high level of resistance to pyrimethamine in both human and rodent species of malaria (Cowman *et al*., 1990, 1988; Peterson *et al*., 1988; Tanaka *et al*., 1990).

1.3.4. Transgenic rodent parasites

Manipulation of the genome is a primary tool to study gene function and permits the dissection of the molecular basis of processes of interest. It is also useful to identify essential parasite's proteins that could be used as drug or vaccine targets.

The *Plasmodium* genome is haploid and most of the genes are single-copy, meaning that in a single manipulation event, functional information can be rapidly characterized. The well standardized and very efficient transfection protocol from Janse *et., al.* (Janse *et al*., 2006) has been quite useful for modifying the *P. berghei* genome with multiple purposes. The main goal

of reverse genetics is to characterize the function of a gene of interest. Double cross-over mediated mutagenesis is commonly recommended for gene KO. This strategy leads to a very stable insertion, with no duplicated sequences in the final locus. Gene disruption can affect the asexual replication of the parasite, leading to a lethal phenotype, *i.e.* the parasite is unable to multiply, or to a fitness defect with slow growth manifested by a delay in the erythrocytic development. Besides generating specific KO parasites, it is also possible to add reporter makers cassettes, as fluorescent proteins (green fluorescent protein, GFP; red fluorescent protein, RFP; mCherry), luminescent proteins (luciferase), ovalbumin, or small tags as the hemagglutinin (HA), that are commonly used for specific protein follow up during all steps of the cycle.

The availability of murine model and advances in reverse genetics set new horizons for discoveries. While no laboratory culture is available for *P. vivax* and most of the work on *P. falciparum* is *in vitro*, the genetic tools in *P. berghei* allows *in vivo* rodent model with modified protein expression. The effect of immune sera or specific antibodies on the asexual growth of parasites is commonly evaluated by growth inhibition assays (GIA) (Lunel *et al*., 1989). However, this method does not englobe *in vivo* components of antibody function (complement, cytokines, immune cells). Thus, *in vivo* models are very useful.

Rodent malaria models can be used to express reporter proteins (transgenic parasites) but also human malaria proteins (chimeric parasites) (Salman *et al*., 2015). Many works have then focused on generating rodent malaria parasites expressing *P. falciparum* proteins and fewer *P. vivax* proteins (reviewed in (Othman *et al*., 2017), Table 1).

Table 1 | Transgenic rodent malaria parasites used in malaria vivax vaccine research. (Adapted from (Othman *et al*., 2017).

Chimeric rodent malaria parasites expressing human Plasmodium proteins				
Protein product	***P. vivax* gene**	**Remarks**	**RMgm ID**	**Ref**
PvTRAP	PVP01_1218700	Replacement copy; Pb (ANKA) trap replaced with Pv (Sal-1) trap. No selectable marker.Normal sporozoite production and infectivity	#1103	Bauza et al.,2014
Pv2	PVX_111175	Replacement copy; Pb25 and Pb28 replaced with Pv 25; in Pb (ANKA)	#222	Ramjanee et al., 2007
PvCSP (VK210)	PVX_119355	Replacement copy; Pb (ANKA) csp replaced by PvVK210 csp under control of endogenous Pbcsp promoter and 3′UTR; No drug selectable marker		Salman et al., 2017
PvCSP (VK247)	PVX_119355	Replacement copy; Pb (ANKA) csp replaced by Pv VK247 csp under control of endogenous Pbcsp promoter and 3′UTR; No drug selectable marker Normal sporozoite production and infectivity		Salman et al., 2017
Rodent malaria parasites expressing HMP-RMP fusion proteins				
CSP (VK210)	PVX_119355	The repeat region of Pb (ANKA) csp is replaced with the Pv (210) csp repeat region	#906	Espinosa et al., 2013
CSP (VK210)	PVX_119355	The repeat region ofPb (ANKA) csp is replaced with (part of) Pv (210) csp gene	#1104	Mizutani et al., 2014
CSP (VK247)	PVX_119355	The majority of Pb (ANKA) csp gene is replaced with Pv (247) csp; the fusion gene retains Pb signal sequence (1–20aa) and Pb GPI anchor sequence (372-395aa)	#1443	Mizutani et al., 2016
P25	PVX_111175	The Pb (ANKA)25 and 28 genes replaced with a fusion of Pv25 and Pb 25	#223	Ramjanee 2007

Most applications of the use of transgenic rodent parasites focused on *P. vivax* proteins members of the pre-erythrocytic stages. In these studies, description of the induced immunity

and protection using the fully infectious transgenic parasite are reported. The different chimeric parasites developed seem to represent robust models for the evaluation of protective immune responses against *P. vivax* vaccines formulations, especially based on the circumsporozoite protein (CSP).

Concerning blood stage antigens, and especially MSP1, only few transgenic or chimeric lines of *P. berghei* were created (Mlambo *et al*., 2008). One study constructed a *P. berghei* expressing the $MSP1_{19}$ of *P. falciparum* and showed the implication of antibodies directed against this portion of PfMSP1 in immunity, as antibodies raised in mice infected with the chimeric line were able to block *P. falciparum* in GIA and passive transfer of anti-$PfMSP1_{19}$ to infected mice could control parasitemia to some extent (de Koning-Ward *et al*., 2003). Another group also demonstrated that specific antibody production after immunization with PfAMA1(III)-$MSP1_{19}$ chimeric protein produced in *Pichia pastoris* protects from challenge with the hybrid *P. berghei* expressing $PfMSP1_{19}$ and GFP as a reporter (Cao *et al*., 2009).

2. INTRODUCTION OF PART 1: PVMSP1-19 VACCINE ASSESSMENT

2.1. Malaria vaccine

Vaccination is considered an approach that will complement other strategies for prevention and control of the disease. There are few examples of disease eradication in humankind history. The most successful is clearly the case of Smallpox, which is eradicated since 1980 (Fenner *et al*., 1988). Another example is the one of Poliomyelitis, which hopefully will be eradicated in a few years. In both cases, eradication or efficient control was achieved using effective vaccines. Several concomitant strategies have been developed for malaria vaccine, targeting different stages of the parasite, as represented in the figure 10.

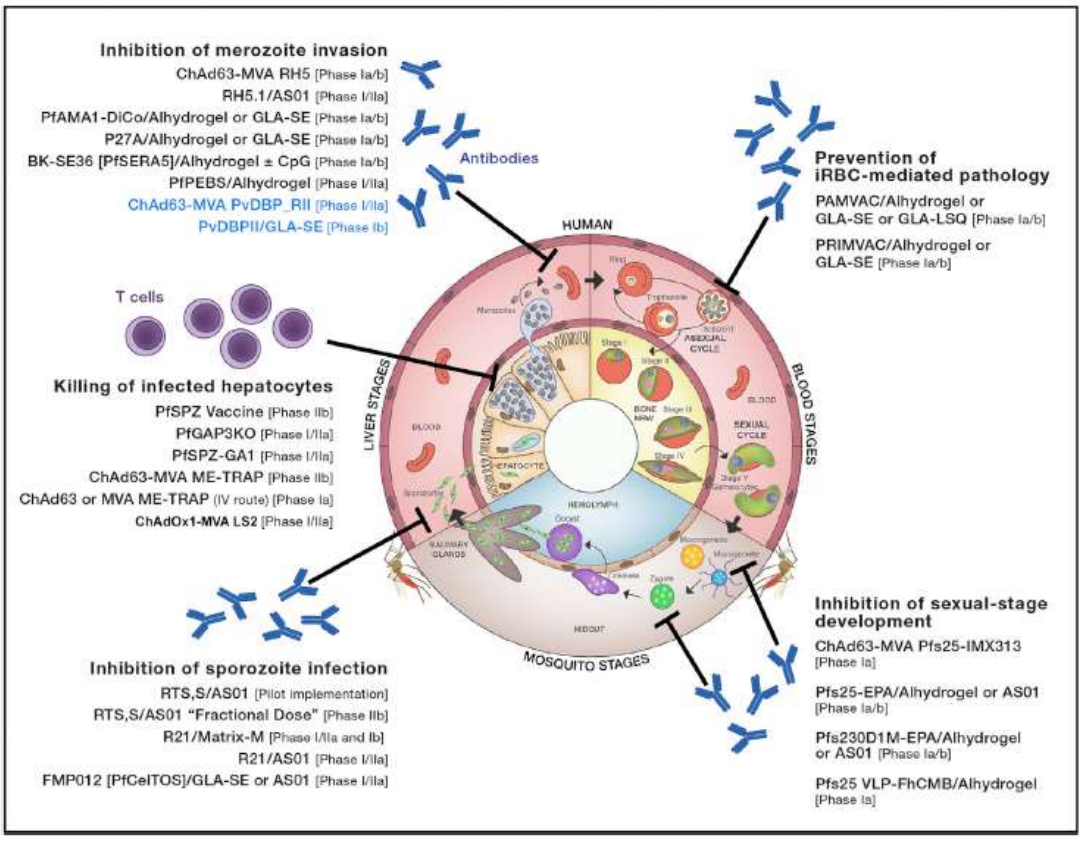

Figure 10 | Malaria Vaccine Candidates in Clinical Development. Vaccines for *P. vivax* are colored blue. (Draper *et al*., 2018).

2.1.1. Liver stage vaccines

From the mid-20th century, evidences of partial immunity induced by killed or inactivated sporozoite in birds (Mulligan *et al*., 1941; Richards, 1966) lead to the use of the same strategy

in other animal species. While killed *P. berghei* sporozoites were not enough to induce protection in mice, vaccination with X-irradiated sporozoites could protect mice against challenge with fully infectious sporozoites (Nussenzweig *et al*., 1967). Few years later, the same was observed in humans (Clyde *et al*., 1973). As a possible target for immunity, the most abundant protein on the surface of sporozoites, the CSP (Cohen *et al*., 2010) was cloned and sequenced (Dame *et al*., 1984). Since then, several vaccine constructs were developed based on the PfCSP.

The most efficient vaccine formulation based on the PfCSP sequence, called RTS,S (Mosquirix™, GlaxoSmithKline), is made of a fragment of PfCSP fused to the hepatitis virus B surface antigen (HBsAg) and mixed with recombinant HBsAg to form virus-like particles, which are then admixed with an adjuvant system (AS01E).

The international community expects the licensing of the first malaria vaccine against *P. falciparum* for the next years. In 2015, the European Medicines Agency approved the use of the vaccine in malaria endemic countries in Africa (Elvidge, 2015; Gosling *et al*., 2016). Subsequently, the WHO recommended pilot implementation studies. The National Regulatory Authorities of Ghana, Kenya and Malawi authorized the RTS,S vaccine for use in the pilot areas, starting in April 2019. The Malaria Vaccine Implementation Program, coordinated by the WHO, has been designed to address several outstanding questions related to the public health use of the vaccine, such as the feasibility of administering the required four doses of the vaccine in children, the vaccine's role in reducing childhood deaths, and its safety in the context of routine use.

The RTS,S/AS01E vaccine has shown around 45% efficacy in protecting children between five and seventeen months of age in the first year after vaccination with average malaria exposure, with efficacy dropping with time and until as little as around 16% in situations with high malaria exposure, even in the first year after vaccination (Olotu *et al*., 2013; M. T. White *et al*., 2015). These results are far from the ones expected for an ideal vaccine. It is clear that a better vaccine, possibly to complement RTS,S, is also urgently needed. Thus, research has focused in the finding of new antigens, especially from the blood stages (merozoites), to compose future new vaccines.

Other vaccine formulations have been developed. For example, the whole organism strategy, as the use of radiation-attenuated sporozoites (RAS) had proven to be very efficient. These sporozoites partially develop into the hepatocyte and die before reaching the bloodstream. The

PfSPZ vaccine (Sanaria®) can be protective for over six months in non-immune volunteers, but it is less efficient in endemic countries where the population has been already exposed. The high number of immunizations (x5), the conservation of live irradiated sporozoites and the administration route (intravenous, *i.v.*) make this vaccine not ideal, but strategies and technologies are under study to overcome these issues (Richie *et al*., 2015). In 2020, the first large clinical trial will be performed with irradiated *P. falciparum* sporozoites in Bioki (an island off the coast of Equatorial Guinea) involving 2,100 individuals.

In addition to irradiated sporozoites, other studies focus on genetically attenuated parasites (GAP) (Kublin *et al*., 2017). These sporozoites can develop until a later liver stage, inducing immune responses against a broader spectrum of antigens.

2.1.2. Blood stage vaccines

As a complement of liver stage vaccine, with the idea of creating an immunity in the blood to control an eventual leak of parasites coming from the liver, blood stage vaccines have been also investigated. Even though there are some blood stage vaccines that demonstrate efficacy in humans, to date no blood stage vaccine has reached phase III clinical trials (Miura, 2016). The main antigens used in these formulations are merozoite proteins involved in attachment and invasion of erythrocytes, alone or multiple antigens in combination with adjuvants (Table 2).

Table 2 | Blood-stage vaccine candidates that showed effects in humans. (Miura, 2016).

Trial	Vaccine formulation	Main outcome	Ref
Combination B (MSP1, MSP2, and RESA)			
Phase IIb	*E. coli* expressed recombinant MSP1 (K1 strain), MSP2 (3D7), and RESA (FCQ-27/PNG) proteins with Montanide ISA720 adjuvant	No clinical protection, but strain-specific reduction in malaria infection: 78 out of 359 (22%) PCR samples showed 3D7 dimorphic form of MSP2 in the control groups, while 30/360 (8%) in the vaccine groups	[96]
AMA1			
Phase IIb	*E. coli* expressed recombinant AMA1 (3D7) protein with GSK $AS02_A$ adjuvant	No clinical protection, but strain-specific reduction in malaria cases. In 22 episodes (out of 271 total episodes observed during the trial) infected with AMA1-3D7 type parasites, 16 cases occurred in the control group, and another 6 cases in the vaccine group.	[97]
Phase IIa	*E. coli* expressed recombinant AMA1 (3D7) protein with GSK $AS02_A$ adjuvant	No significant difference in prepatent period or parasite growth rate after sporozoite challenge. However, significantly lower cumulative parasitemia during Day 7–9 after challenge in the vaccine group ($n = 10$) as compared to the unvaccinated infectivity control ($n = 6$).	[68][b]
MSP3			
Phase Ib	MSP3 long synthetic peptide with aluminum hydroxide adjuvant	Significant reduction in risk of clinical malaria: 1.2 (15 μg dose) and 1.9 (30 μg dose) cases per 100 days per person in the vaccine groups ($n = 15$ each) while 5.3 in the control group ($n = 15$)	[98]
SE36 (SERA-5)			
Phase Ib	*E. coli* expressed recombinant SERA-5 (Honduras-1) protein with aluminum hydroxide adjuvant	Significant reduction in risk of clinical malaria: Hazard ratio = 0.26 after adjustment of age and gender: the vaccine group ($n = 66$) was compared to the control group ($n = 16$) and newly enrolled unvaccinated individuals ($n = 50$)	[99]
MSP1			
Phase IIa	Recombinant chimpanzee adenovirus 63 (ChAd63) and modified vaccinia virus Ankara (MVA) vectors encoding MSP1	Significant difference in prepatent period after sporozoite challenge ($n = 3$ in the vaccine group and $n = 6$ in control) in the initial study, but not in the second study ($n = 9$ in vaccine and $n = 6$ in control)	[85]

[a]Clinical trials which were conducted with a multistage vaccine(s) are not included.
[b]There were other vaccine groups in the trial, but only one group which showed a significant effect is shown.

A blood stage vaccine is convincing principally because epidemiological studies have shown naturally acquired immunity in endemic areas (Bull *et al*., 1998). The lack of acquired blood stage immunity is the main reason why young children (under five years old) are the main victims of the disease. In contrary, older children or adults present levels of protection against severe disease, although remaining susceptible for infection (Marsh, 1992). Thus, repeated infections lead to non-sterile immunity that is directed against the blood stages of the parasite.

The progress of pre-erythrocytic vaccines raises concerns supporting the need for blood stage vaccines. Pre-erythrocytic vaccines would reduce the population exposure to infections. However, if efficacy wanes over time and the control measures fail, population living in endemic areas could develop severe malaria due to the absence of naturally acquired immunity (prevented by the lesser exposure provided by the pre-erythrocytic vaccination).

Developing an effective blood stage vaccine against malaria has been challenging mostly due to correlates of protection often lacking reliability and reproducibility. However, it is important to note that old studies of passive serum transfer in monkeys or humans demonstrate that efficacy of protection is partly mediated by antibodies (Coggeshall *et al*., 1937). In 1990, passive transfer of human purified immunoglobulins G (IgG) mediated parasitemia control (Bouharoun-Tayoun *et al*., 1990). Using specific antigens, it was shown that $MSP1_{19}$ immunization of monkeys protects them against *P. cynomolgy* infection (Perera *et al*., 1998). Therefore, it seems that strong antibody responses may effectively control or reduce parasitemia.

2.2. *P. vivax* vaccine

There seems to be a general agreement that malaria elimination based on vaccines will require the development of a vaccine not only against *P. falciparum*, but also against *P. vivax*. The limitations to study *P. vivax* in laboratories, due to the impossibility of maintaining this parasite growing in continuous laboratory cultures, forced the community to use information gathered from *P. falciparum* to apply in *P. vivax* vaccine development (Carlton, Escalante, *et al*., 2008). Therefore, *P. vivax* vaccine development is mainly focused on orthologs of *P. falciparum* vaccine candidates like CSP, MSP1, and the 25-kDa oocyst/ ookinete surface protein (Pvs25) (Arévalo-Herrera *et al*., 2010).

There are research groups in Brazil and abroad working to develop a *P. vivax* vaccine based on the PvCSP antigen (Teixeira *et al*., 2014). However, it is reasonable to foresee, based on the

results obtained with the clinical trials of RTS,S/AS01E, that a *P. vivax* vaccine based on the PvCSP may not be fully effective by itself. In addition, in a low transmission region context, hypnozoite forms of *P. vivax* are responsible for relapses resulting in blood stage infections and therefore blood stage immunity is needed to prevent symptoms. Thus, the development of a vaccine based on *P. vivax* blood stage antigens is needed and might be a useful and necessary tool for *P. vivax* elimination.

There are three major *P. vivax* antigens as vaccine blood stage candidates: PvMSP1, PvAMA1 and PvDBP. The first two are orthologs of the *P. falciparum* proteins. PvMSP1 and PvAMA1 were shown to have limited polymorphism, and several vaccine formulations with good immunogenicity in mice, rabbits and non-human primates were developed (Bargieri *et al*., 2008; Gentil *et al*., 2010).

The PvDBP is the major *P. vivax*-specific antigen in vaccine development pipelines (Arévalo-Herrera *et al*., 2001). PvDBP was discovered based on the observation that *P. vivax* (and *P. knowlesi*) can only invade RBC that express the Duffy blood group surface receptor (Horuk *et al*., 1993). Thus, the binding of PvDBP to the Duffy receptor is essential for *P. vivax* merozoite invasion, making PvDBP an interesting vaccine candidate. However, the high PvDBP allelic variation complicates vaccine development based on this antigen, because immunity is strain-specific (Ntumngia *et al*., 2012). Nevertheless, research groups are trying to find epitope determinants that can induce specific antibodies capable of recognizing all PvDBP allelic variants that circulate in human infections (Ntumngia *et al*., 2012).

So far, few vaccine formulations based on these three *P. vivax* antigens have undergone clinical trials, and no *P. vivax* human vaccine formulation has ever been successful. However, the lack of a continuous *P. vivax* laboratory culture has so far prevented systematic tests of efficacy of these available vaccine formulations, even in pre-clinical tests, which obviously blocks most of the attempts to progress with these formulations to clinical tests in humans. Therefore, a platform that allows testing these vaccine formulations in pre-clinical experiments is an urgent need.

2.3. $MSP1_{19}$ as a vaccine target

The MSP1 protein plays an essential role in parasite survival, possibly by its involvement in erythrocyte binding and invasion. Indeed, disruption of *P. chabaudi* MSP1 is deleterious for the parasite (O'Donnell *et al*., 2000), a result confirmed in many other *Plasmodium* species.

Another important role of MSP1 was found by conditional gene inactivation. *P. berghei* MSP1 suppression in the sporozoite showed to impair the merozoite formation in the liver (Combe *et al*., 2009). These evidences, in addition to the fact that MSP1 immunization induces protection against lethal *P. yoelii* challenge of mice (Holder *et al*., 1981) and *P. falciparum* challenge of monkeys (Siddiqui *et al*., 1987), led the community to have a great interest in MSP1 as a vaccine target.

The $MSP1_{19}$ is composed of two epidermal growth factor (EGF) like domains containing cysteine residues forming disulfide bonds, determined by the tertiary crystal structure of *P. cynomolgy* $MSP1_{19}$ (Chitarra *et al*., 1999). This specific structure was then characterized in *P. falciparum* (Blackman *et al*., 1991; Morgan *et al*., 1999) and *P. vivax* (Babon *et al*., 2007), and a similar folding for all $MSP1_{19}$ was observed.

The C-terminal region of MSP1 is one of the leading malaria vaccine candidates. Immunity to *P. falciparum* was associated with specific antibodies for the EGF-like domains of MSP1 (Blackman *et al*., 1990; Chang *et al*., 1994; Egan *et al*., 1999, 1996). In different experimental systems, recombinant $MSP1_{19}$ immunization was found to be protective against parasite challenge (Daly *et al*., 1993; Ling *et al*., 1994; Perera *et al*., 1998; Rotman *et al*., 1999). The specificity of antibodies for $MSP1_{19}$ correlated with low levels of blood parasitemia in *P. falciparum* infected patients (Corran *et al*., 2004; John *et al*., 2004; Okech *et al*., 2004). Moreover, monkey immunization with a recombinant protein based on the $MSP1_{42}$ protects against *P. falciparum* challenge (Stowers *et al*., 2002, 2001). The protective immune response induced after this type of immunization is mediated by $MSP1_{19}$ antibodies (Ahlborg *et al*., 2002).

P. vivax $MSP1_{19}$ is particularly immunogenic to a large proportion of individuals in Brazilian endemic regions. This exposed population presents high antibody titers and cellular immune response to $PvMSP1_{19}$ (Soares *et al*., 1997, 1999). Moreover, even a slight exposition to the parasite can induce specific antibodies with a substantial longevity (Morais *et al*., 2005), in some cases as longer as thirty years post infection (Lim *et al*., 2004).

Many vaccine formulations based on the sequence of the PvMSP1 have been developed, and a parasite model allowing to test these formulations would help accelerate vaccine development based on this antigen.

3. INTRODUCTION OF PART 2: BLOOD STAGE ANTIGEN DISCOVERY

3.1. Antigen discovery

3.1.1. Pre and post-genomics era

In the 1980s, advances in molecular biology techniques allowed the discovery of many *Plasmodium* antigens. Cloning of *P. falciparum* complementary DNA (cDNA) or genomic DNA (gDNA) in bacteriophage vectors for expression in *Escherichia coli*, lead to the construction of DNA libraries for antigen screen (Kemp *et al*., 1986). The use of mouse monoclonal antibodies (mAb), infected human or animals' sera was crucial for the characterization of antigens such as the CSP, or the ring erythrocyte surface antigen (RESA) (Cowman *et al*., 1984; Ellis *et al*., 1983). These antigens are usually immunodominant as they induce strong antibody responses, but whether this response mediates protection is debated.

The availability of *Plasmodium* annotated genomes and the use of reverse genetics provided significant data for the identification of protective antigens. Transcriptomics and proteomics have provided information on the expression profiles of malarial proteins during the parasite's life cycle. Using *in silico* analysis, it is possible to characterize antigen expression by the identification of stage specific transcription patterns. This allows to target specific and conserved cross-stage antigens in the parasite's life cycle (Florens *et al*., 2002).

Several proteomic-based experimental platforms have been applied to vaccine candidate discovery and focused on the identification of immunogenic surface-exposed, secreted or membrane proteins of pathogens. Some well-studied vaccine candidates such as MSP1, already described as a GPI anchor, have been identified alongside a long list of secreted, surface-associated or surface-anchored parasite proteins (Gelhaus *et al*., 2005). The potential of inducing protective immune responses with these antigens can then be studied by the search of correlates of protection in cohort studies (Osier *et al*., 2014). The immunoproteomic approach uses specific immunoglobulins that bind to antigens to be identified as potential vaccine targets. In order to identify protein-protein interactions, immunoprecipitation (or pull-down) uses these antibodies. For example, using this method, PfMSP1 was shown to interact with a large protein complex at the merozoite surface (Ranjan *et al*., 2011).

3.1.2. Current blood stage antigen candidates

Most of the blood stage antigens were discovered during the pre-genomic era by classical methods of identification. Three antigens of the merozoite surface are considered the major candidates to compose a blood stage vaccine against *P. falciparum*: MSP1, AMA1 and EBA-175. These antigens, known since decades ago, are involved in steps required for merozoite invasion of RBC and are highly immunogenic in natural infections (Goodman *et al*., 2010). They have been used since then in several vaccine formulations, but despite clear evidence that specific antibodies against these antigens can block merozoite invasion *in vitro*, no human vaccine formulation has ever been successful using these molecules. Research efforts continue trying to understand the requirements needed to make successful vaccines using these antigens. Identification of novel malarial antigens for vaccine development is critical.

3.1.3. Recent antigens

In the past years, research laboratories have also focused in finding new merozoite surface molecules that are essential for merozoite invasion. Recently, using the avidity-based extracellular interaction screen (AVEXIS) system (Bushell *et al*., 2008), in which a panel of recombinant erythrocyte and merozoites surface proteins are screened for specific interactions, the merozoite surface RBP homologue 5 of *P. falciparum* (PfRh5) was identified as a ligand to the erythrocyte receptor basigin (CD147) (Crosnier *et al*., 2011). Antibodies blocking the interaction (anti-CD147 or anti-Rh5) inhibit invasion of erythrocytes by all *P. falciparum* isolates tested so far (Crosnier *et al*., 2011, 2013; Douglas *et al*., 2014). The PfRh5 is therefore being proposed as the newest and most promising target for a vaccine against *P. falciparum* malaria (Wright *et al*., 2014).

Interestingly, PfRh5 strongly interacts in *P. falciparum* extracts with the Rh5-interacting protein (Ripr) (Chen *et al*., 2011), which was localized specifically at the surface of erythrocytes during merozoite invasion, putatively interacting with the Rh5-CD147 complex. As Rh5, Ripr seems to be essential for *P. falciparum* merozoite invasion, because it cannot be genetically deleted (Chen *et al*., 2011). In addition, antibodies raised against Ripr inhibit merozoite invasion by several *P. falciparum* strains (Chen *et al*., 2011; Healer *et al*., 2019). PfRipr is a 123-kDa cysteine-rich protein with ten EGF-like domains, and it localizes to the micronemes in schizonts, where it is processed into two polypeptides. The processed 65-kDa PfRipr pairs remain associated and together with PfRh5 are shed into the supernatant during invasion (Chen

et al., 2011). These proteins are part of a complex PfRh5/PfRipr/CyRPA that is essential for erythrocyte invasion (Fig. 11) (Volz *et al*., 2016).

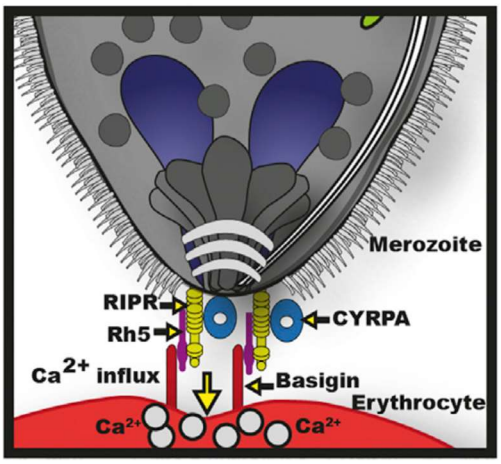

Figure 11 | PfRh5/PfRipr/CyRPA complex at interface between merozoite and erythrocyte. (Volz *et al*., 2016)

3.2. *P. vivax* new blood stage antigens

The search for new *P. vivax* vaccine antigens is limited by the difficulties of maintaining this parasite in continuous laboratory cultures. Novel vaccine candidates are often elected based on those identified in *P. falciparum* studies. In fact, even using adapted *P. falciparum* laboratory strains, which are genetically tractable, the identification of molecules that are essential for *Plasmodium* merozoite invasion and can be targeted by the immune system is complicated. After years of studies, only few *P. falciparum* molecules have appeared as promising targets, like the PfRh5 and PfRipr, as discussed above.

Unfortunately, PfRh5 seems to be specific to *P. falciparum* and *P. reichenowi* (closely related to *P. falciparum*), as orthologs are not found in any other *Plasmodium* species. As opposed to PfRh5, however, Ripr is conserved and orthologs are found in all *Plasmodium* species sequenced so far, including *P. vivax* and *P. berghei*, suggesting that the Ripr/PfRh5/CD147 functional pathway may be also conserved, and that the role played by PfRh5 could be fulfilled by another protein in other *Plasmodium* species.

Thus, the identification of putative PfRh5 functional orthologs in other *Plasmodium* species might guide the identification of a new essential invasion pathway in *P. vivax*, which should become an important and promising vaccine candidate.

4. CONCLUSIONS

- Pb/PvMSP1$_{19}$

* The replacement of *P. berghei* MSP1$_{19}$ by the one of *P. vivax* is possible, in both ANKA and NK65 strains.
* Mutant hybrid parasites obtained infect mice hosts similarly to the respective wild type *P. berghei* ANKA or NK65 strains.
* Mice immunization with PvMSP1$_{19}$ vaccine formulations induces high specific IgG titers.
* The model of BALB/c mice immunization and NK65 hybrid challenge may be a valuable model for PvMSP1$_{19}$ vaccine formulation testing.
* FliC-PvMSP1$_{19}$-PADRE + poly (I:C) was protective in this model.
* No obvious link was possible to make between protection and IgG subclasses or cytokines production.

- Rh5/CyRPA/RIPR invasion pathway

* *P. berghei* Ripr is essential for parasite erythrocytic replication as it is for *P. falciparum.*
* *P. berghei* Ripr localizes at micronemes.
* *P. berghei* Ripr interactome was described.
* Pb235 is a good candidate to be the functional ortholog of the PfRh5 in *P. berghei*.

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