Characterisation of antibody cross-reactivity between *Plasmodium vivax* DBL proteins
and *Plasmodium falciparum* VAR2CSA in sera from Colombian and Brazilian populations

by

Angie Yarleidy Mena Palacios

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in
Environmental Health Sciences

School of Public Health
University of Alberta

Abstract

Background: Malaria in pregnancy (MiP) poses a significant risk to the mother and fetus, causing approximately 10,000 maternal deaths and 200,000 infant deaths each year. A vast majority of these deaths are attributed to the species *Plasmodium falciparum.* MiP can cause severe negative birth outcomes such as maternal anemia, stillbirth, intrauterine growth restriction, and low infant birth weight. During infection with *P. falciparum,* the parasite protein VAR2CSA is expressed on the surface of infected red blood cells which sequester in the placenta. In Africa, maternal antibodies against VAR2CSA are acquired after several infections with the parasite and these antibodies confer protection against placental malaria. In Colombia and Brazil, we observed that non-pregnant populations exposed to *Plasmodium vivax* have high levels of antibodies against VAR2CSA. These sera also have antibodies to PvDBPII, a merozoite protein from *P. vivax*, which shares a DBL domain similar to those in VAR2CSA. We showed that PvDBPII affinity-purified antibodies from human sera recognized VAR2CSA by ELISA, as well as a mouse monoclonal antibody (mAb 3D10) raised against PvDBPII, demonstrating cross-reactivity between these proteins. The purpose of this study was to identify which are the shared cross-reactive epitopes between the DBL proteins from *P. vivax* that contribute to the protective antibodies against *P. falciparum* VAR2CSA.

Methods: Sera from non-pregnant populations from areas of Colombia and Brazil endemic to *P. falciparum* and *P. vivax* were analyzed for reactivity against VAR2CSA, PvDBPII, SD1 and EBP2. Cross-reactivity to VAR2CSA was characterized by ELISA. PvDBPII and SD1-specific antibodies were affinity-purified from pooled human sera from Colombia and the functional activity of these antibodies was evaluated in the inhibition of binding assay (IBA).

Results: Our study population has high levels of antibodies against PvDBPII, SD1, EBP2, and VAR2CSA. I found significant correlations between the antibody levels to PvDBPII, SD1 and VAR2CSA, but no correlation between VAR2CSA and EBP2. PvDBPII and SD1-specific antibodies recognized VAR2CSA by ELISA. SD1 affinity-purified antibodies inhibited the parasite binding to CSA in the IBA.

Conclusions: The findings from this work identified the subdomain 1 from PvDBPII as the potential source for the cross-reactive antibodies with VAR2CSA. This work contributes to our understanding of a new mechanism for natural cross-species immune recognition. This knowledge can be applied to the development of vaccine to protect women against placental malaria.

Preface

Some of the research conducted for this thesis forms part of an international research collaboration, led by my supervisor, Dr. Stephanie Yanow at the University of Alberta. Some of the data from Chapter II was published as Gnidehou S, Mitran CJ, Arango E, Banman S, Mena A, Medawar E, et al. Cross-Species Immune Recognition Between *Plasmodium vivax* Duffy Binding Protein Antibodies and the *Plasmodium falciparum* Surface Antigen VAR2CSA. J Infect Dis. 2019;219(1):110-20.

In Chapter II and Chapter III, the contribution of collaborators appears in the section "Contributions".

Dedication

Dedicated to the memory of my biggest fan, who took care of me in this process as an angel and supported me each time when I needed it the most.

Thanks, Dad

Acknowledgements

Being in Canada doing my master's degree and achieving one of dreams could not be possible without the contribution and support of several people. I am deeply grateful with all of them. First of all, I would like to thank my mom who with her love, wisdom and encouragement has guided me in every aspect of my life. I want to thank my siblings, Yasson, Carlos, Lady and Jhoana and my sister in law, Laura for their unconditional support.

I would like to truly thank my supervisor, Dr. Stephanie Yanow, for her patience, guidance and knowledge. Thank you for opening the doors of your lab to me and allow me to acquire such a great knowledge and experience that would shape my future career path. Thank you for opening the doors to your home and letting me to know the great human being that you are, becoming my Canadian mom and my role model.

I would also want to thank my committee members Dr. Sedami Gnidehou and Joel Dacks for guidance and kindness. I am grateful for the wonderful lab team that I had the opportunity to meet, Nawell Fayad, Katie Mitran, Ken Gavina, Hazel Lugo, Urvi Rai, Maddy Wiebe, Brendan Todd, Shanna Banman, Kim Sharplin and Dr. Ninad Mehta, thank you for the fun times, technical support and company in the lab. Additionally, I want to thank all individuals who collaborated with this work, Drs. Amanda Maestre, Eliana Arango, Luzia Carvalho, Flora Kano, Michael Good, Michael Hawkes, John Adams and Ali Salanti. I would like to thank Formación de Alto Nivel - Nuevo Chocó, Li Ka Shing, NSERC CREATE HPI and COLFUTURO for their support.

Finally, this experience would not be possible without all the special friends that I met during this process, who became my family, but I especially want to thank Marissa, Teresa, Annette and Paolo for always being there for me.

Table of Contents

List of tables

List of figures

Chapter 1: Introduction

Figure 1.1 Global predicted age-standardized parasite prevalence rate for *Plasmodium falciparum* malaria for children two to ten years of age, 2017

Figure 1.2 Global predicted all-age parasite prevalence rate for *Plasmodium vivax* malaria in 2017

Figure 1.3 *Plasmodium falciparum* life cycle.

Figure 1.4 Schematic organization of PfEMP1 protein

Figure 1.5 Schematic organization of VAR2CSA protein

Figure 1.6 Schematic organization of PvDBP protein

Figure 1.7 Schematic organization of EBP2 protein

Figure 1.8 *P. falciparum* sequestration in the placenta

Chapter 2: Expression of PvDBPII protein and, subdomains and serological characterization of human sera from Colombia and Brazil

Figure 2.1 Colombian and Brazilian populations with exposure to *P. vivax* recognized VAR2CSA.

Figure 2.2 Schematic representation of the protein expression and purification system strategy

Figure 2.3 The amino acid sequence of subdomain 1 was identical across Colombian isolates

Figure 2.4 Preparation of inclusion bodies and protein purification using Ni-NTA resin of PvDBPII

Figure 2.5 Refolded and purified recombinant PvDBPII

Figure 2.6 Preparation of inclusion bodies and protein purification using Ni-NTA resin of rSD1+SD2

Figure 2.7 mAb 3D10 against rSD1+DS2

Figure 2.8 Testing different temperatures and incubation periods for optimal protein induction

Figure 2.9 Small scale of high efficiency transformation of rSD1+SD2, and induction with IPTG at 20°C

Figure 2.10 Preparation of inclusion bodies and protein purification using Ni-NTA resin of rSD1+SD2.

Figure 2.11 Testing different concentrations of IPTG, 1% glucose addition and cold shock

List of abbreviations

ACTs – Artemisinin combination therapies

ATS – Acidic terminal segment

AU– Arbitrary unit

CD62P – Glycoprotein P-selectin

CIDR – Cysteine-rich interdomain region

CM – Congenital malaria

CSA– Chondroitin sulphate A

CSPGs – Chondroitin sulphate proteoglycans

DARC – Duffy antigen receptor for chemokines

DBL – Duffy-binding like

EBL – Erythrocyte binding-like

EBP2 – Erythrocyte binding protein 2

ECD – Extracellular domain

ELISA – Enzyme-linked immunosorbent assay

EPCR – Endothelial protein C receptor

GM – Gestational malaria

HIF-1α – Hypoxia-inducible factor 1 alpha

IB – Inclusion bodies

IBA – Inhibition of binding assay

ID – Inter-domain

IPTp – Intermittent preventive treatment in pregnancy

RBCs – Red blood cells; iRBCs – Infected red blood cells; uRBCs – Uninfected red blood cells

KAHRP– knob-associated histidine-rich protein

LBW – Low birth weight

MDA – Mass drug administration

MiP – Malaria in pregnancy

NTS– N terminal segment

OD – Optical density

PCR – Polymerase chain reaction; nPCR – Nested PCR; qPCR – Real-time quantitative PCR

PfEMP1 – *P. falciparum* erythrocyte membrane protein 1

PfMSP1 – *P. falciparum* merozoite surface protein 1

PHIST– *Plasmodium* helical interspersed subtelomeric

PGF – Placental growth factor

PM – Placental malaria

PTB – Pre-term birth

PvDBP – *P. vivax* Duffy binding protein

RDTs – Rapid diagnostic tests

rSD1+SD2 – Recombinant SD1+SD2 subdomains of PvDBPII

SD1 – Subdomain 1; SD2 – Subdomain 2; SD3 – Subdomain 3

SMIs – Submicroscopic infections

TMD – Transmembrane domain

VEGF – Vascular endothelial growth factor; VEGFR – VEGF receptor

WHO – World Health Organization

Chapter 1: Introduction

1.1 General epidemiology of malaria

Malaria is an infectious disease caused by a protozoan parasite belonging to the genus *Plasmodium (P.)* (WHO. 2018). There are five *Plasmodium* species that cause the disease in humans: *P. falciparum, P. vivax, P. malariae, P. ovale* and its subspecies (*P. ovale curtisi* and *P. ovale wallikeri*) (Sutherland et al. 2010, J 1927), and *P. knowlesi* (White 2008). *P. falciparum* is the most prevalent and pathogenic species (WHO. 2018, White et al. 2014) .

Malaria represents a huge public health burden, as half of the world's population is at risk of infection. The disease mostly affects infants, children under 5 years of age, and pregnant women in high-transmission malaria areas. According to the World Malaria Report (2018) (WHO. 2018), there were 90 countries endemic for malaria in 2017, with the majority of cases and deaths in sub-Saharan Africa, followed by Asia, the Americas, Europe, and Oceania. There were 219 million cases of malaria (95% confidence interval (CI): 203-262 million) and more than 435,000 deaths in 2017. These statistics are similar to the number of deaths (445,000) and cases (217 million) of malaria reported in 2016 (WHO 2017). Additionally, for 2017, the WHO estimated that 99.7% of malaria cases in the Africa region, 71.9% in the Western Pacific, 69% in the Eastern Mediterranean and 62.8% in South-East Asia were attributed to *P. falciparum*. On the other hand, 74.1% of malaria cases in the region of the Americas were caused by *P. vivax* (WHO. 2018).
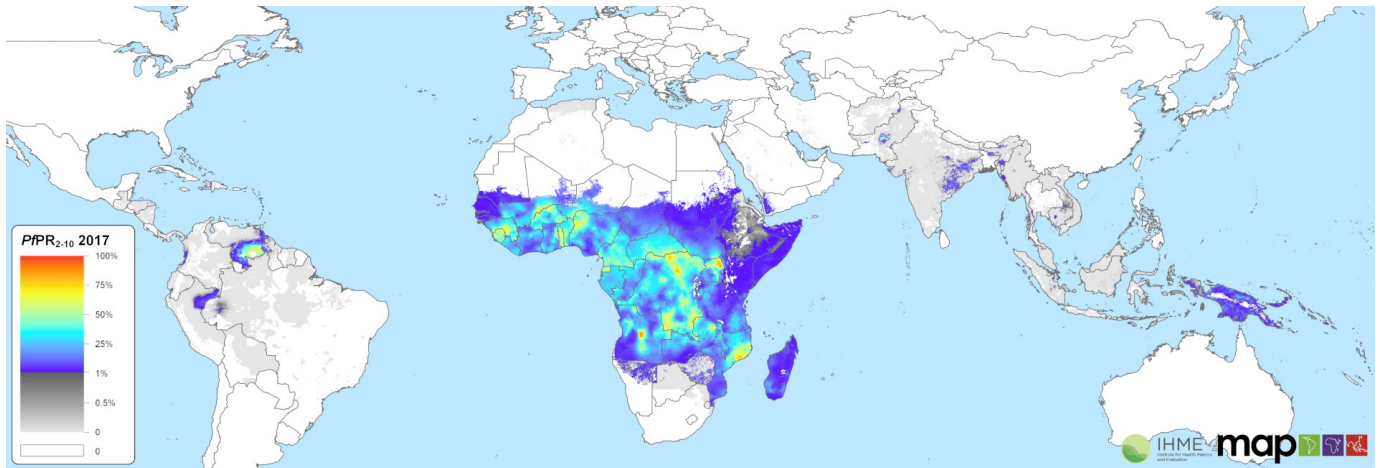
Figure 1.1 Global predicted age-standardized parasite prevalence rate for *Plasmodium falciparum* malaria for children two to ten years of age (*Pf*PR2-10) in 2017. This contemporary map was produced by Malaria Atlas Project.
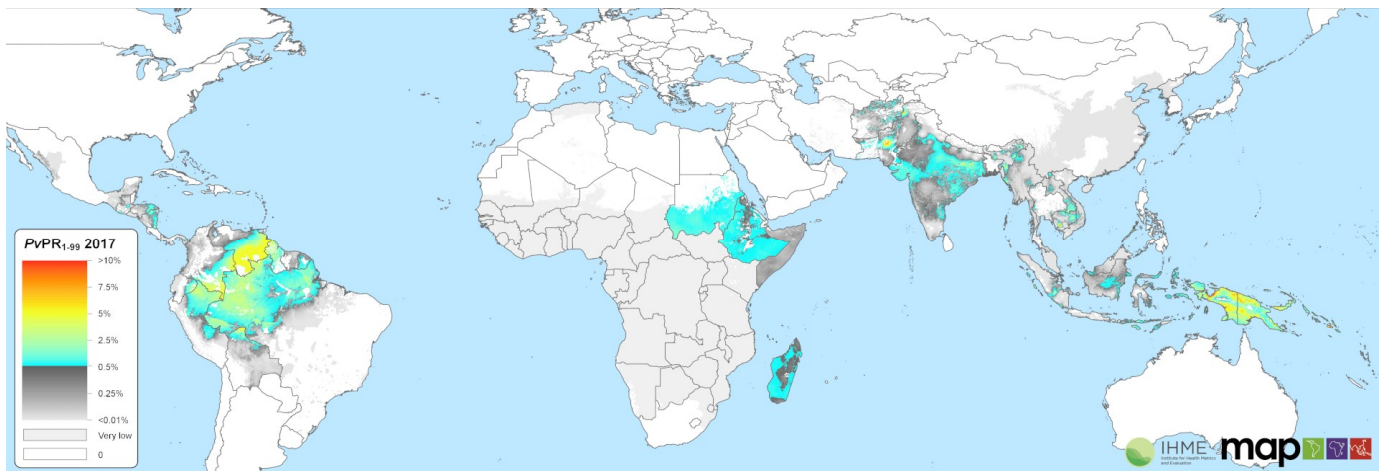
Figure 1.2 Global predicted all-age parasite prevalence rate for *Plasmodium vivax* malaria (*Pv*PR1-99) in 2017. This contemporary map was produced by Malaria Atlas Project.

1.2 The parasite life cycle
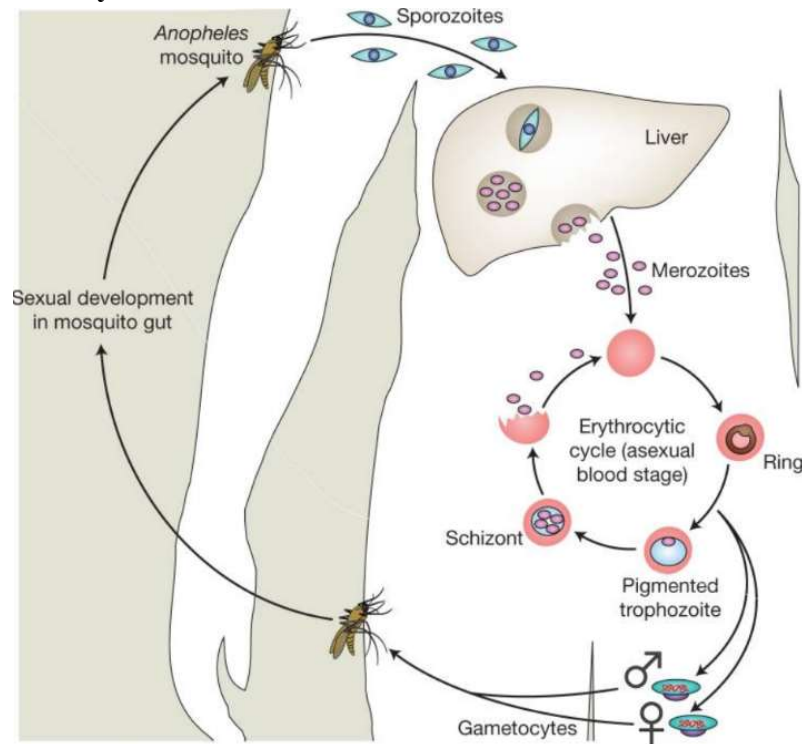
Figure 1.3 *Plasmodium falciparum* life cycle. Adapted figure from Rowe JA *et al.,* 2009.

The life cycle of the protozoan parasite (Fig. 1.3) (Rowe et al. 2009, Cowman et al. 2016) begins with a bite from an infected female mosquito of the genus *Anopheles*. Sporozoites from this mosquito are injected into the circulatory system of the host through subcutaneous tissue. While some sporozoites are eliminated by the immune system, a small number of parasites evade the immune response and reach the liver where they establish an infection, with no clinical symptoms in the host. *P. vivax* and *P. ovale* can also develop into a dormant stage known as hypnozoites. These hypnozoites can cause a relapse infection weeks or years after the first infection (White 2011). After 8 to 10 days, the sporozoites mature to thousands of merozoites and travel from the liver to the blood, rapidly infecting erythrocytes. This gives rise to the asexual erythrocytic cycle. During this cycle, merozoites differentiate into the ring-stage, which in turn develop into pigmented trophozoites and finally into schizonts. About 16 to 32 new merozoites will burst from

fully matured schizonts and go on to infect new erythrocytes and restart the cycle. The symptoms of malaria in humans arise during this intraerythrocytic cycle. At this stage, some parasites develop into gametocytes, the sexual forms, that can be taken up by mosquitoes during a blood meal (Rowe et al. 2009, Miller et al. 2002, Beri, Balan, and Tatu 2018). The gametocytes differentiate into female (macrogametocyte) and male (microgametocyte) forms. In the mosquito midgut, the gametocytes develop into female and male gametes, fusing to form a zygote which later develops into a motile ookinete (Arai et al. 2001, Cowman et al. 2016). Subsequently, the motile ookinete travels to the midgut epithelial cells and matures into oocyst forms. Thousands of small elongated sporozoites are released from the oocyst stage. The elongated sporozoites migrate to the salivary glands of the mosquito. The infected female *Anopheles* mosquito injects the sporozoites into the next human host during its blood meal, starting the cycle over again (Cowman et al. 2016).

1.3 Malaria disease

Plasmodium infections in humans can cause uncomplicated or severe disease. The principal clinical symptoms for uncomplicated malaria are fever (body temperature above 38°C), sweats, general malaise, headache, nausea, and vomiting. These clinical symptoms are caused by the asexual erythrocytic stage parasites. Alternatively, manifestations of severe malaria can lead to cerebral malaria, hemoglobinuria, severe anemia, and acute respiratory distress syndrome among others (Prevention 2018).

1.3.1 Diagnosis

Diagnosis of malaria is only possible during the erythrocytic stage of the infection. The WHO recommends Giemsa-stained thick and thin blood smears or Rapid Diagnostic Tests (RDTs) (Agudelo-García, Arango-Flórez, and Carmona-Fonseca 2017, WHO 2019). Giemsa-stained thick and thin blood smears examined by light microscopy are the "gold standard method" for parasite detection in endemic areas. However, it is not the most sensitive and accurate method for diagnosis, especially since it strongly relies on the technician's expertise (Ohrt et al. 2008, Kimura et al. 1995). RDTs detects parasite antigens in the blood; but these are not ideal as they are unable to discriminate between non-falciparum species and are not sensitive enough for detection of low-level parasitemia, particularly in asymptomatic infections (plasmodial infection without symptoms and signs) (Murphy et al. 2013). However, in resource-limited areas with high *P. falciparum* burden, RDTs play a decisive role in the treatment course for children under 5 years old. New technologies have been developed that include the use of molecular techniques to detect parasite nucleic acid, such as the polymerase chain reaction (PCR), nested PCR (nPCR), and real-time quantitative PCR (qPCR); these methods detect low parasitemia levels which cannot be identified by microscopy, known as submicroscopic infections (SMIs) (Kimura et al. 1995, Coleman et al. 2006).

SMIs are usually asymptomatic, but they may pose serious health risks. These infections are particularly important from a public health perspective because they often represent more than 50% of infections in some areas with low prevalence of parasitemia by microscopy (Tshibola Mbuyi, Bouyou-Akotet, and Mawili-Mboumba 2014). These infections are a potential reservoir for parasite transmission to mosquitoes. Patients with SMI are approximately 0.3 times as infectious to mosquitoes as patients with malaria detectable by microscopy (Slater et al. 2019);

this perpetuates the parasite life cycle and increases the incidence of malaria infection in the population. SMI detection and epidemiology have become increasingly important, due to the role these infections play in maintaining transmission of malaria, acting as a reservoir of infection in people who may not even have clinical manifestations of the disease (Cheng, Cunningham, and Gatton 2015, McMorrow, Aidoo, and Kachur 2011). Ongoing transmission hinders malaria elimination efforts (Cheng, Cunningham, and Gatton 2015). While the WHO does not recommend treatment for SMIs, Slater et al., 2019, states that SMI is a marker for higher exposure and risk of future infection. Studies have shown that patients with untreated SMI later develop infections with high parasitemia (Nguyen et al. 2018, Galatas et al. 2018), making evident the need for diagnosis and treatment of submicroscopic infections.

1.3.2 Treatment

Treatment of malaria is complex and varies depending on the affected population (children, adults, pregnant women, or travelers), region, and disease severity. According to the WHO 'Test, treat and track' guidelines (WHO 2013), antimalarial treatment should begin only when a patient has a positive blood smear or RDT (WHO. 2015). Prior to the turn of this century, chloroquine, amodiaquine, and sulfadoxine-pyrimethamine were the recommended antimalarial drugs to treat infections caused by *P. falciparum*. However, all of these medications showed therapeutic failure and parasites acquired resistance to the medications (Garcia-Bustos and Gamo 2013, Hwang et al. 2006).

Currently, artemisinin combination therapies (ACTs) are the first-line treatment for uncomplicated *P. falciparum* malaria in countries where there is no artemisinin resistance reported. It is crucial to always use artemisinin in combination with partner drugs, to prevent drug resistance (Fairhurst and Dondorp 2016, WHO. 2015). The single use of an antimalarial drug applies a strong

selection pressure over the parasite population, selecting and increasing in number, mostly the resistant microorganisms, which are a small proportion of the parasite burden (WHO 2000). However, when different drugs are administered together, all the parasites are attacked and the selection pressure is reduced, ensuring the effectiveness of the drugs over time (WHO 2000, 2018). In other words, the parasites are under attack through different concerted mechanisms, rendering them more sensitive to antimalarials (WHO 2018).

Artemisinins have potent activity against rings, trophozoites, schizonts (asexual stages) and immature gametocytes (sexual stages) of *P. falciparum* (WHO 2000, Garcia-Bustos and Gamo 2013). These blood stages digest hemoglobin while developing inside the erythrocyte. The iron released from this digestion process cleaves the endoperoxide moiety of artemisinins, leading to the formation of reactive oxygen species that target nucleophilic groups in parasite proteins and lipids (Garcia-Bustos and Gamo 2013, WHO 2000). Additionally, artemisinin binds several parasite proteins crucial for parasite survival proving to be effective in killing the parasite (Fairhurst and Dondorp 2016). Hitherto, the proposed targets of artemisinin action are *Pf*ATP6 enzyme, the *Plasmodium falciparum* ortholog of mammalian sarcoendoplasmic reticulum Ca^{21}-ATPases (SERCAs) (Eckstein-Ludwig et al. 2003), and most recently, the *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K) (Mbengue et al. 2015).

1.3.3 Drug resistance

Drug resistance has been documented for all classes of antimalarial drugs, including those derived from artemisinin (WHO 2010). *P. falciparum* has documented resistance to all antimalarial drugs currently in use (amodiaquine, chloroquine, mefloquine, quinine, and sulfadoxine-pyrimethamine) and, more recently, to artemisinin derivatives. The geographical distribution and spread rates vary considerably (WHO 2010). *P. vivax* has rapidly developed resistance to

sulfadoxine-pyrimethamine in many areas, while its resistance to chloroquine is largely confined to Indonesia, Papua New Guinea, Timor-Leste and other parts of Oceania (MYCDCGP 2019). There are also reports of resistance in Brazil and Peru (MYCDCGP 2019). *P. vivax* remains susceptible to chloroquine in most of Southeast Asia, the Indian subcontinent, the Korean Peninsula, the Middle East, North East Africa and most of South and Central America (WHO 2010). In addition, *P. malariae* resistance to chloroquine has been documented (Maguire et al. 2002).

Genetic mutations are a common factor leading to antimalarial drug resistance and occur spontaneously in nature. Sinha *et al.* (Sinha, Medhi, and Sehgal 2014) state that the start of resistance occurs in two phases. During the first phase, a *de novo* mutation is required and allows for parasite survival in the presence of the drug. In the second phase, parasites with the new genetic trait are selected and consequently multiplied, resulting in a new parasite population resistant to antimalarial drugs (Sinha, Medhi, and Sehgal 2014). Other factors that influence antimalarial drug resistance are over-prescription, inadequate prescription, and poor quality drugs, especially in Southeast Asia (Fairhurst and Dondorp 2016).

In Pailin, Western Cambodia, the first emergence of artemisinin resistance was reported in 2009 as a 100-fold reduction in parasite clearance rate (Dondorp et al. 2009). Artemisinin resistance is defined as "a parasite clearance half-life ≥5 hours following treatment with artesunate monotherapy or an ACT" (Fairhurst and Dondorp 2016). Artemisinin resistance has spread across Southeast Asia, mainly caused by mutations (polymorphisms) that change the primary amino acid sequence of the propeller region of the kelch motif-containing gene, known as K13, acting through upregulation of the pathways that respond to unfolded proteins (Fairhurst and Dondorp 2016, Tun et al. 2015, Rosenthal 2018).

1.4 Malaria in pregnancy

P. falciparum infections in adults living in endemic areas usually do not develop into clinical malaria (history of fever accompanied by peripheral parasitemia) since exposure to *P. falciparum* in infancy and childhood eventually leads to acquired immunity (Rowe and Kyes 2004). In contrast, pregnant women, especially when they are pregnant for the first time, are among the most vulnerable populations at risk of malaria infection (Doritchamou et al. 2016). Malaria in pregnancy (MiP) can be defined as follows (Agudelo-García, Arango-Flórez, and Carmona-Fonseca 2017, Rogerson et al. 2007, Carmona-Fonseca and Maestre 2009):

- Gestational malaria (GM): confirmed by the presence of *Plasmodium* spp. parasites in maternal peripheral blood by thick blood smear or another positive diagnostic test;

- Placental malaria (PM): confirmed by the presence of *Plasmodium* spp. parasites or hemozoin in placental blood or placental tissue. PM depends on the occurrence of GM, but both are not always detected simultaneously; and

- Congenital malaria (CM): this pathology affects the fetus and is confirmed by the presence of *Plasmodium* spp. in cord blood at delivery or in fetal peripheral blood within 30 days after delivery. CM is caused by PM.

MiP due to *P. falciparum* is well documented; on the other hand, there are fewer studies on *P. vivax* GM, PM, and CG. Knowledge is also limited with regards to MiP caused by the less prevalent species (*P. ovale, P. malariae,* and *P. knowlesi*) (Rogerson et al. 2007).

MiP can cause severe adverse outcomes including maternal anemia, stillbirth, intrauterine growth restriction, histopathological changes in the placenta, miscarriage, low birth weight (LBW), preterm birth (PTB), and maternal and infant death (Desai et al. 2007, Steketee et al. 2001,

Arango, Maestre, and Carmona-Fonseca 2010). These complications arising from MiP result in approximately 10,000 maternal deaths and 200,000 infant deaths each year (Schantz-Dunn and Nour 2009). Approximately 125 million pregnancies are at risk of malaria infection each year worldwide (for pregnant women living in both high and low malaria transmission areas) (Dellicour et al. 2010). Based on a study from 2007, 55 and 30 million of pregnancies occurred in areas with stable and unstable *P. falciparum* transmission, respectively; 40 million occurred in areas with only *P. vivax* transmission, while 53 million were observed in areas where both species coexisted (Dellicour et al. 2010). It is important to note that the vast majority of cases in Africa are attributed to *P. falciparum*, while outside this continent, most cases are attributed to *P. vivax* (Dellicour et al. 2010, Rijken et al. 2012, Lopez-Perez et al. 2016).

SMI usually occurs early in pregnancy and several studies conducted in pregnant women with SMI from sub-Saharan Africa have shown an association between SMIs and poor birth outcomes such as LBW and PTB (Cottrell et al. 2015, Adegnika et al. 2006, Cohee et al. 2014). These findings emphasize the need for new interventions to treat and prevent future infections in pregnant women with SMI.

1.4.1 Control efforts for MiP

Currently, several strategies are in place to protect pregnant women living in endemic areas from malaria (WHO. 2017). 1) Early diagnosis of malaria can be achieved through frequent antenatal screening by microscopy. 2) Intermittent Preventive Treatment in pregnancy (IPTp) involves prophylactic medication with sulfadoxine-pyrimethamine during pregnancy (2 to 4 full treatments). 3) Vector control through the use of mosquito bed nets treated with effective insecticides and indoor residual spraying at home. 4) Individual precautions to prevent bites from the *Anopheles* vector, like wearing insecticide and avoiding having a small pool of water inside

and around the house. 5) Design of the houses with walls and mosquito nets. 6) Increased awareness and public education of these preventative measures for pregnant women and their families. Collectively, these control measures can support disease control and contribute to the prevention of MiP (Agboghoroma 2014, Marchesini, Costa, and Marinho 2014). However, preventive measures and adequate protocols are not always tailored to the individual community and the risk of malaria infection in vulnerable populations, including pregnant women, remains high. Poor living conditions of the inhabitants in these areas and government policies that do not favor the lower socio-economic strata also complicate the elimination of MiP (Agboghoroma 2014, Marchesini, Costa, and Marinho 2014).

1.4.2 Treatment during MiP

1.4.2.1 Uncomplicated *P. falciparum* malaria

The WHO recommends treating pregnant women in the first trimester with seven days of quinine combined with clindamycin. ACT is only recommended if it is the only treatment available or if treatment of quinine with clindamycin fails (WHO. 2015).

During the second and third trimesters, MiP should be treated with ACT for seven days. The combination should include a short acting artemisinin component (artemether, artesunate, or dihydroartemisinin) which decreases parasitemia considerably within the first three days, plus a longer acting drug (lumefantrine, piperaquine, amodiaquine, or mefloquine) which kills the remaining parasites in the blood and prevents recrudescent malaria (Tarning 2016).

1.4.2.2 Uncomplicated *P. vivax, P. malariae, P. ovale, P. knowlesi* malaria

During the first trimester, pregnant women should not be treated with ACT. Treatment with quinine is given for chloroquine resistant *P. vivax* malaria (WHO. 2015). Primaquine is used to

kill hypnozoites and prevent relapse but is contraindicated in pregnant women as it is a teratogenic drug (WHO. 2015).

1.4.2.3 Severe malaria

Treatment for severe malaria consists of intravenous artesunate for a least 24 hours and until patients can tolerate oral medication. ACT is administrated for three days (WHO. 2015). Despite this, the case fatality rate for severe *P. falciparum* malaria is 20% (Trampuz et al. 2003).

1.4.3 MiP in Latin America

In Latin America, 2.9 million pregnancies are at risk of malaria each year (Dellicour et al. 2010). Insufficient surveillance programs in countries like Colombia and Brazil result in incomplete and inaccurate information about the prevalence and/or incidence of malaria in these regions, including MiP (WHO 2016). However, MiP research conducted in Colombia showed that *P. vivax* is the most prevalent parasite (60%) (Pineros-Jimenez et al. 2011, Carmona-Fonseca and Maestre 2009), with the exception of two regions, Nariño and Chocó, where the predominant species is *P. falciparum* (Lopez-Perez et al. 2016). Brazilian studies found that *P. vivax* is the most prevalent species and caused 85% of cases in pregnant women (Parekh et al. 2007, Marchesini, Costa, and Marinho 2014, Dombrowski et al. 2018). Malaria detection in these countries is quite difficult due to an increased rate of SMIs where the parasitemia is very low. For this reason, it is necessary to use molecular techniques that detect the genetic material of the parasites but these methods are not available in health facilities (Moraga C et al. 2015).

1.5 Pathogenesis of *P. falciparum* MiP

During *P. falciparum* infections, parasites have the ability to sequester as mature infected red blood cells (iRBCs). Sequestration occurs when the parasite expresses adhesins on the surface of the iRBCs allowing the cells to bind to different human receptors, leading to sequestration in the microvasculature of several organs and tissues such as lung, muscle, brain, placenta, adipose tissue and heart (Rowe et al. 2009). This phenomenon allows the parasite to evade clearance by the spleen and avoid the host immune response. The iRBCs can cytoadhere through several host receptors: CD36 and the endothelial protein C receptor (EPCR) on endothelial cells (Udeinya et al. 1981, Smith and Deitsch 2004); the glycoprotein P-selectin (CD62P) on activated platelets (Udeinya et al. 1981); CR1 on erythrocytes, which causes rosetting with uninfected red blood cells (uRBC) (Udomsangpetch et al. 1989); ICAM-1 on brain endothelium; and chondroitin sulphate proteoglycans (CSPGs) in the placenta (Rowe and Kyes 2004).

In MiP, a specialized form of adhesion takes place where the iRBCs bind to the receptor CSA, a type of CSPG. This leads to sequestration of iRBCs in the placenta (placental malaria) and infiltration of immune cells (macrophages and monocytes) in the intervillous placental space. Often the parasite density in the placenta is higher than in the peripheral blood (Rogerson et al. 2003). The intervillous placental space is an important source of nutrients to ensure fetal development. This space is located between the microvilli and is formed by a low-flow pool of maternal blood (Botella Llusiá 1992). The decidua consists of cells resulting from the connective tissue or trophoblasts of the maternal endometrium that covers the fetus. It is abundant in spiral arteries that are invaded by the endovascular trophoblasts of the placenta. These arteries feed the fetus with nutrients and gases. Also, the decidua secretes hormones, immune cells, growth factors and cytokines with tolerogenic phenotypes (Botella Llusiá 1992).

By histopathology, MiP caused by *P. falciparum* produces placental lesions such as increased fibrin deposits, which may lead to focal syncytial necrosis, hemozoin deposits, and ultimately to delays in fetal growth and/or fetal loss (Rota et al. 2006). Sequestration also leads to placental angiogenic modifications linked to the vascular changes of the microvilli, decreasing the available space for the exchange of fetal-placental blood flow (Ismail et al. 2000). In addition, long-term placental angiogenic alterations may contribute to LBW and fetal death in the uterus. All of these outcomes are important causes of perinatal morbidity and mortality (Ismail et al. 2000). Furthermore, the marked irregular thickening of trophoblastic basement membranes and the infiltration of maternal macrophages and monocytes (an increase of syncytial knobs) hinders oxygen flow to the placenta (Boeuf et al. 2008). In the same way, the thickness of the trophoblastic basement membranes and the number of monocytes in the placenta have been correlated with the number of plasmodial infections in pregnancy (Ataide et al. 2015). The accumulation of inflammatory cells and iRBCs in the intervillous space cause placental and fetal hypoxia because of the oxygen consumption of the cellular infiltrates and/or blood perfusion. The hypoxia-inducible factor 1 alpha (HIF-1α) is increased mainly by hypoxic conditions in the placenta, but non-hypoxic conditions induced by cytokines, growth factors and the renin-angiotensin system also may increase HIF-1α (Patel et al. 2010). The expression of VEGF is induced due to a decrease in oxygen pressure (hypoxia) (Ferrara 2004); VEGF is a pro-angiogenic factor that favors the growth of new blood vessels and also favors greater oxygen transport (Ferrara 2004). Once VEGF production in blood is stabilised, production of VEGFR (its receptor) is also increased. The placental growth factor (PGF) and VEGFR promote arteriogenesis stimulating the smooth muscle cells, which are crucial for the generation of new mature and functional vessels (Luttun et al. 2002). In placentas with plasmodial infection, there is an increase in the RNA expression of HIF-1α, VEGF, VEGFR-

1, and sVEGFR-1, and a decrease in PGF (Boeuf et al. 2008). Those factors indicate that the placenta is not getting sufficient oxygen, which affects the flow of nutrients (Luttun et al. 2002, Patel et al. 2010). The sequestration of iRBCs in the placenta induces monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage inflammatory proteins (MIP-1α and MIP-1β) production (Suguitan et al. 2003), accumulation of proinflammatory cytokines (TNF and IFNɣ), and complement activation (Fried et al. 1998).

1.5.1 *Var* genes

P. falciparum has the ability to sequester iRBCs in the placenta due to a surface variant antigen, VAR2CSA, which is an important protein for the pathogenesis of placental malaria. This protein is expressed on the iRBC surface, which binds to the glycosaminoglycan CSA (Fried and Duffy 1996, Beeson et al. 2000), expressed in the syncytiotrophoblast of the placenta (the epithelial layer that covers the intervillous space allowing maternal/fetal exchange) (Salanti et al. 2004, Duffy et al. 2006, Ataide, Mayor, and Rogerson 2014).

VAR2CSA is a member of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of cytoadherent proteins. These proteins are expressed by around 60 *var* genes (Dahlback et al. 2007, Gardner et al. 2002). Only one PfEMP1 variant is expressed on the surface of the iRBC at a time; the parasite will switch on a singular *var* gene and switch off the rest of the genes, leading to clonal antigenic variation (Rowe and Kyes 2004) and evasion of host immunity.

The *var* genes can be classified in five classes (*upsA, upsB, upsC, upsBC, upsD,* and *upsE*) or into groups, as A, B and C and two intermediate groups B/A and B/C (Kirchner, Power, and Waters 2016, Kyriacou et al. 2006). All the *upsB var* and *upsBC var* genes (but one) or group A and B genes localize to subtelomeric regions, while the *upsC var* genes or group C are located in the centromeric regions (Rask et al. 2010, Smith 2014). The classes *upsA*, *upsD* and *upsE var* genes

are also in the subtelomeric region but are transcribed in the opposite orientation to the *upsB var* genes. Of note, the *upsD* and the *upsE* classes each contain just one *var* gene, *var1csa*, and *var2csa* respectively, which bind to CSA.

Switching on and off the surface antigen PfEMP1 allows *P. falciparum* to maintain infections in the host (Ralph, Scheidig-Benatar, and Scherf 2005). The expression of *var* genes is monoallelic, meaning that the parasites transcribe one dominant *var* gene while silencing other alleles (Scherf et al. 1998, Chen et al. 1998). *Var* promoters artificially removed from their chromosomal context are activated by default (Deitsch, del Pinal, and Wellems 1999), and for silencing, genetic rearrangements are not required (Scherf et al. 1998), consistent with an epigenetic control mechanism. It has been found that *var* genes expression can be modified by movements in the *var* loci subnuclear position, which can be associated with the displacement of the gene to a transcriptionally permissive perinuclear area. Additionally, silencing is aided by the proximity of the subtelomeres to the nuclear periphery (Ralph, Scheidig-Benatar, and Scherf 2005).

1.5.2 PfEMP1 protein and expression patterns

The PfEMP1 protein is composed of three main units: the intracellular acidic terminal segment (ATS), the transmembrane domain (TMD), and the extracellular domain (ECD). The *var* genes consist of two exons; the first one codes for the extracellular and highly variable domain (ECD). The second exon encodes the amino acids that are conserved in the intracellular acidic terminal segment (ATS) and the transmembrane domain (TMD) (Fig. 1.4) (Smith 2014).

The ECD contains a set of sub-domains, known as Duffy-binding like (DBL) domains (named because of the similarity with the Duffy binding protein from *P. vivax*), and the Cysteine-rich interdomain region (CIDR), a degenerate form of the DBL domains (Smith 2014). The DBL

domains are cysteine-rich regions, and each of them has 12 to 16 cysteine residues within approximately 300 amino acids. Additionally, DBL domains are categorized into six variant types from the N-terminus: DBLα, DBLβ, DBLγ, DBLδ, DBLε, and DBLζ or DBLX (heterologous domain). The CIDR regions are classified into CIDRα, CIDRβ, and CIDRγ (Smith et al. 2000, Smith 2014).

Once the PfEMP1 protein is translated, it is exported to the RBC membrane via the Maurer's cleft (Golgi-like membranous vesicle) in the cytoplasm which is mediated by the N terminal segment (NTS) (Smith 2014). Within the vesicle, there is a family of proteins known as *Plasmodium* helical interspersed subtelomeric (PHIST) that bind to the intracellular acidic terminal segment (ATS) of the PfEMP1 during transport to the membrane of the iRBC. The PfEMP1 proteins are concentrated at the surface of the RBC membrane, resulting in "knob-like" structures, or protuberances on the membrane (Smith et al. 2000, Smith 2014). To complete the transportation of the PfEMP1 from the vesicle to the membrane, another protein must be synthesized, knob-associated histidine-rich protein (KAHRP). This protein binds the vacuole to increase the RBC structural thickness and adhesion of PfEMP1 in the knobs (Watermeyer et al. 2016). Close to 10,000 knobs can be found on the surface of one iRBC (Quadt et al. 2012).

Figure 1.4 Schematic organization of PfEMP1 protein, showing its N terminal segment (NTS), the Cysteine-rich inter-domains regions (CIDR), a C2 domain, the Duffy binding like domains (DBL), a transmembrane domain (TM), and an intra-cellular acidic terminal segment (ATS).

1.5.3 VAR2CSA

VAR2CSA is central to the pathogenesis of placental malaria. This gene is fairly conserved among different parasite isolates (Salanti et al. 2003) and importantly, transcription of *var2csa* is

upregulated following parasite selection on CSA (Salanti et al. 2003). This was demonstrated by Salanti *et al* and Duffy *et al* (Fried and Duffy 2002) when they evaluated the parasite clone NF54 and the CSA-selected line NF54CSA using primer pairs to amplify all the *var* genes in the genome by qPCR (Salanti et al. 2003). This gene was not identified until 2003, because it was not found in the first genetic screens for the *var* genes transcribed by CSA-binding parasites, as it does not contain DBLα (used to detect *var* gene expression by PCR) (Taylor, Kyes, and Newbold 2000). The VAR2CSA protein is characterized by DBLX domains, which do not match any of the previously recognized DBL domain types, and three DBLε domains. Additionally, the expression pattern for a CSA-binding ligand is consistent with the transcription of *var2csa* (Rowe and Kyes 2004).

VAR2CSA is a 350 kDa protein composed of six DBL domains, and several inter-domain (ID) regions that have an important role in the immunogenicity and adhesion of VAR2CSA (Fried and Duffy 2015), a transmembrane domain (TM) and an acidic terminal segment (ATS) (Fig. 1.5). The CSA-binding sites in VAR2CSA were mapped to DBL domains and antibodies to these recombinant domains inhibited parasite adhesion to CSA (Khunrae et al. 2010). Based on these findings, two VAR2CSA candidate vaccines were developed: PriMalVac that includes DBL1X-3X region and PlacMalVac that contains ID1-DBL2X-ID2a domains from VAR2CSA. These vaccines use recombinant proteins, which share overlapping regions in the ID1-DBL2x region but have haplotypes from different parasites strains (3D7 for PriMalVac and FCR3 for PlacMalVac (CORDIS_EU 2019, Chêne et al. 2016). These vaccines are currently in Phase I vaccine trials.

Figure 1.5 Schematic organization of VAR2CSA protein, showing its N terminal segment (NTS), four inter-domains regions (ID), six Duffy binding like domains (DBL), a transmembrane domain (TM), and an intra-cellular acidic terminal segment (ATS).

1.6 Pathogenesis of *P. vivax* MiP

The effects of *P. vivax* PM are relatively understudied. There are few studies of the histological effects of *P. vivax* on placental tissue; for this reason, the specific pathways of placental damage are not clear. Carvalho *et al* (2010) demonstrated that *P. vivax* iRBCs can cytoadhere to endothelial cells and placental cryosections (Carvalho et al. 2010, Carvalho et al. 2011). However, in a recent investigation in Colombia, the most frequent histological findings in *P. vivax* infections were increased fibrin deposits and increased monocytes in the intervillous space (Carmona-Fonseca, Arango, and Maestre 2013). Another study conducted in an area of low malaria transmission in Brazil showed that women with *P. falciparum* and *P. vivax* infections had a significant increase in the thickness of the placental barrier and the number of monocytes in the intervillous space compared to placentas of women who were not infected (Souza et al. 2013). In contrast, a study conducted in a low transmission area in northwestern Thailand that evaluated placentas infected with both *P. falciparum* and *P. vivax* determined that infections with *P. vivax* did not cause necrosis and did not increase fibrin deposits compared to infections with *P. falciparum* (McGready et al. 2004). Collectively, these studies highlight that the effects of *P. vivax* on the placental tissue remain unclear and further research is needed on the pathophysiology of *P. vivax*.

1.6.1 *Vir* genes

The multigene *vir* gene superfamily in *P. vivax* may be involved in antigenic variation in this species. The Sal-I genome counts approximately 346 *vir* genes, localized in subtelomeric regions (Fernandez-Becerra et al. 2009, del Portillo et al. 2001). Each gene includes one to three exons; the first exon does not have a signal peptide sequence, the second exon codes for a predicted transmembrane domain and conserved cysteine residues, and the third exon encodes a putative cytosolic domain (Singh, Gupta, and Pande 2014). The function of the VIR proteins encoded by the *vir* genes is still unclear and more studies are needed to elucidate their role. However, it is believed that they are important for cytoadherence and in the chronicity of parasite infection (Singh, Gupta, and Pande 2014).

1.6.2 DBL domain proteins from *P. vivax*

P. vivax is the most prevalent malaria parasite infecting humans outside Africa. However, the biology of the parasite is not fully understood due to limitations in studying the parasite *in vitro* (Hester et al. 2013b). When merozoites invade new RBCs, critical molecular interactions take place between the receptor in the RBC and the ligand from the merozoites. The *P. vivax* merozoite invades reticulocytes, and mostly infects people positive for the Duffy blood group antigen (Fy)/Duffy antigen receptor for chemokines (DARC) (Ntumngia et al. 2016, Cheng et al. 2013); however, there are reports of *P. vivax* malaria in Duffy-negative people (Menard et al. 2010, Mendes et al. 2011). Additionally, the merozoites appear to be dependent on two ligands to complete the invasion process: the Duffy binding like protein (PvDBP) and the Erythrocyte binding protein 2 (EBP2) (Ntumngia et al. 2016, Cheng et al. 2013) through the adhesive DBL domain.

DBP and EBP2 are part of the Erythrocyte Binding-Like (EBL) protein superfamily, consisting of ligands found on the surface of infected RBCs. The EBL proteins are composed of six regions in the extracellular domain that include a cysteine-rich DBL domain in region II, a type I transmembrane domain, and a short cytoplasmic domain (Howell, Samudrala, and Smith 2006, Batchelor, Zahm, and Tolia 2011).

Erythrocyte invasion can be described as a four-step process: attachment, reorientation, junction formation and entry. Surface merozoite proteins are required for the attachment step, while the other three steps involve proteins from the micronemes, rhoptries and dense granules, which are intracellular organelles from the apical complex of the merozoite (Howell, Samudrala, and Smith 2006).

1.6.2.1 PvDBP

PvDBP is a 140-kDa microneme protein that facilitates merozoite invasion of RBCs by binding DARC and forming a tight junction to initiate invasion (Choe et al. 2005). PvDBP dimerizes with a DARC molecule through the DBL domain forming a heterodimer which matures into a heterotetramer, consisting of a 2:2 complex of PvDBP and DARC proteins (Batchelor, Zahm, and Tolia 2011).

The region II (the DBL domain) consists of 330 amino acids (aa) with 12 conserved cysteines forming disulfide bridges, and several aromatic residues (Adams et al. 1992). The DBL domain is divided into three subdomains (SD1, SD2 and SD3) (Fig. 1.6) (Chen et al. 2016). Subdomain 1 is the smallest (40 aa), in which cysteines 1 and 4, and 2 and 3 form disulphide bonds. Subdomain 2 includes cysteines 5 and 6 and it is a four-helix bundle. Subdomain 3 is the largest,

includes cysteines 7 to 12 and forms a second helical bundle. SD2 appears to be the most polymorphic subdomain (Batchelor, Zahm, and Tolia 2011, Xainli, Adams, and King 2000).

Patients exposed to *P. vivax* commonly have naturally acquired antibodies to PvDBP that block the invasion of erythrocytes by *P. vivax* (Tran et al. 2005, Grimberg et al. 2007); these antibodies are associated with protection against *P. vivax* infection. The recombinant protein is a leading vaccine candidate to protect against *P. vivax* infection (de Sousa et al. 2014). Currently, there are two malaria vaccines in phase Ia clinical trials which target the invasion pathway of *P. vivax* by developing antibodies against PvDBP and blocking the invasion of RBCs (Payne et al. 2017). Both vaccines are a replication-deficient chimpanzee adenovirus serotype 63 (ChAd63) and an attenuated orthopoxvirus modified vaccinia virus (MVA) (Payne et al. 2017). The vaccines were well tolerated in 24 volunteers in different doses, showing that they can be safely administered in humans. The participants induced B and T cells responses and some patients who received one MVA boost had an antigen-specific serum antibody response (Payne et al. 2017).
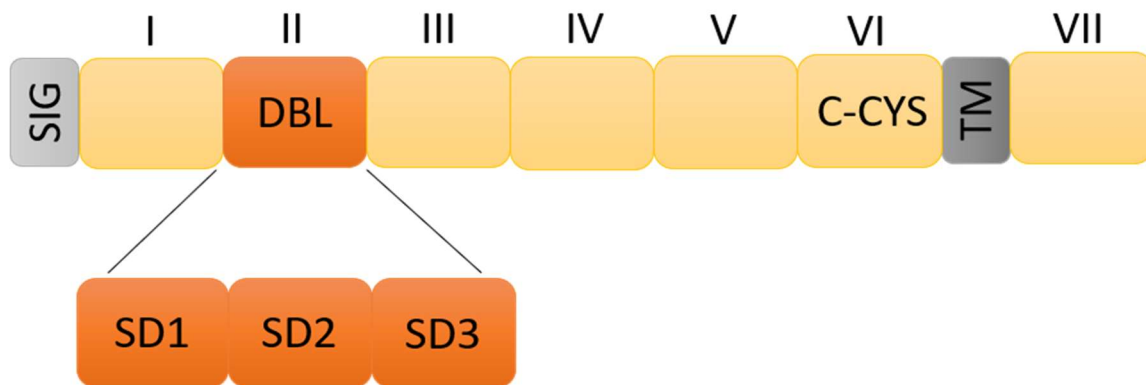
Figure 1.6 Schematic organization of PvDBP protein, showing its signal sequence (SIG), six regions (regions III to V are low complexity regions) a singular DBL domain (Region II), a C- terminus conserved cysteine-rich domain (Region VI) and a transmembrane domain (TM). The Region II is divided into three subdomains. SD1 has two intrasubdomain disulfides. SD2 has one intrasubdomain disulfide and SD3 three intrasubdomain disulfides.

1.6.2.2 EBP2

EBP2 (Fig. 1.7) was not discovered until 2013, since it is not present in the *P. vivax* reference strain (Sal 1) and was only discovered following whole genome sequencing of patient isolates (Chan et al. 2012, Hester et al. 2013b). For this reason, little is known about the function of EBP2, except that it plays a role in invasion (Hester et al. 2013b).

It is well-known that an interaction between DARC and the DBL domain must occur to establish RBC invasion. However, new evidence shows that *P. vivax* infection can occur in Duffy blood group-negative patients (Menard et al. 2010, Cavasini et al. 2007), suggesting an alternative invasion mechanism in these patients, possibly mediated by EBP2. Ntumngia *et. al* (Ntumngia et al. 2016) evaluated the function of EBP2 in reticulocyte invasion of merozoites from *P. vivax*. EBP2 preferentially bound young (CD71high) Duffy-positive (Fy+) reticulocytes. However, EBP2 did not bind to Duffy-negative reticulocytes, suggesting it is not involved in an alternative invasion pathway in Duffy-negative individuals. Notably, EBP2 and PvDBP are antigenically distinct and thus cannot be functionally inhibited by anti-PvDBP antibodies (Ntumngia et al. 2016).

Figure 1.7 Schematic organization of EBP2 protein, showing its signal sequence (SIG), a singular DBL domain (cysteine-rich domain), a C- terminus conserved cysteine-rich domain (C-CYS) and a transmembrane domain (TM).

1.7 Maternal immunity
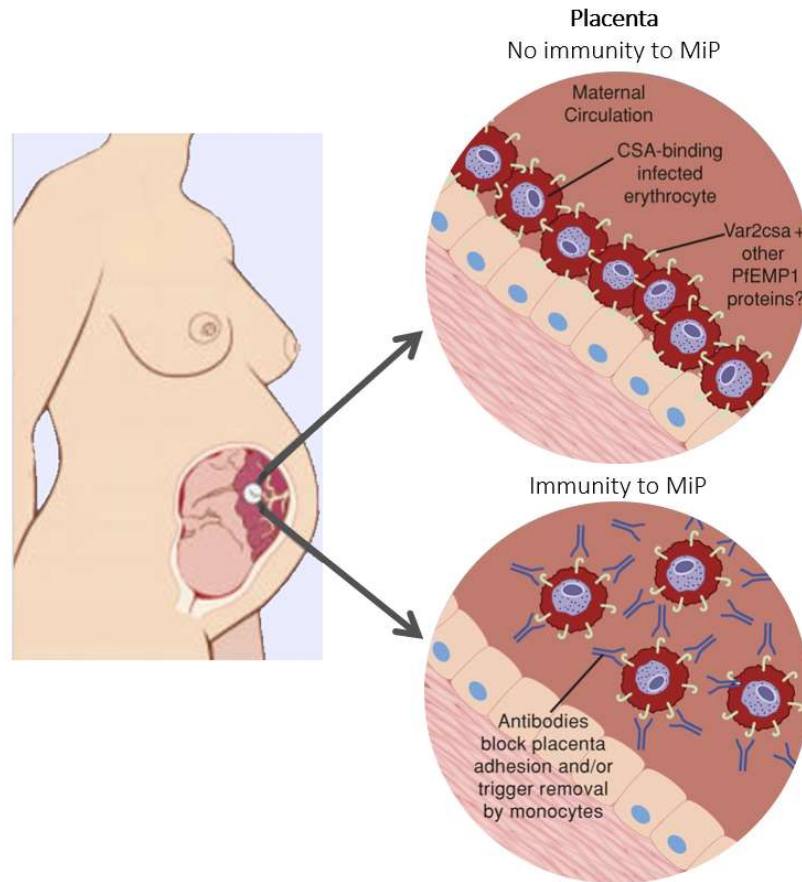
Figure 1.8 *P. falciparum* sequestration in the placenta (43). Adapted figure from Smith JD, Deitsch KW *et al.,* 2004

In MiP, immunomodulatory processes within the body act to both limit parasitic infection and simultaneously promote fetal tolerance (Lindsay et al. 2000, Desai et al. 2007). Exclusively during MiP caused by *P. falciparum*, the parasite protein VAR2CSA is expressed on the surface of iRBCs, allowing the parasite to bind to CSA in the placenta (Salanti et al. 2004). In Africa, women in their first pregnancy (primigravid) have very low levels of antibodies to VAR2CSA and are most vulnerable to placental malaria and poor clinical outcomes (Smith and Deitsch 2004, Duffy and Fried 2003, Rogerson et al. 2003, Desai et al. 2007). With successive exposures to VAR2CSA, women develop antibodies targeting this protein, and these are associated with

protection from PM, as the antibodies to VAR2CSA can block the adhesion of VAR2CSA to the placenta (Fig 1.8) (Duffy and Fried 2003). The findings from several studies in Africa consistently show that the development of protective anti-VAR2CSA antibodies are related to pregnancy history with protective VAR2CSA antibodies significantly higher in multigravid compared to primigravid women (Oleinikov et al. 2007). As a result, primigravid women have a higher risk of developing PM and birth complications compared to the multigravid women. However, some studies conducted in Africa showed that antibodies against VAR2CSA can be detected in non-pregnant populations like men, children, and teenagers at a low frequency (Beeson et al. 2007, Oleinikov et al. 2012, Babakhanyan et al. 2015, Fodjo et al. 2016). The origin of these antibodies is not understood.

1.7.1 VAR2CSA antibodies and Colombia and Brazil

Contrary to the findings from studies in Africa, our research group observed no negative birth outcomes associated with malaria infection, and healthy mothers in Colombia infected with *P. falciparum* or *P. vivax* during pregnancy (Gavina et al. 2018). Unexpectedly, primigravidae women, men and children had high frequencies and levels of functional antibodies against VAR2CSA. Furthermore, protection from maternal anemia was associated with VAR2CSA antibodies that blocked parasite adhesion *in vitro* (Gnidehou et al. 2019). These functional antibodies were observed even in primigravid women, consistent with our previous findings that non-pregnant and primigravid populations from Colombia had high levels of antibodies against VAR2CSA that can block adhesion to CSA (Gnidehou et al. 2014).

Our research group proposes that the co-circulation of *P. falciparum* and *P. vivax* species in Colombia gives rise to antibodies against VAR2CSA in non-pregnant populations and these could originate from *P. vivax*; opposite to sub-Saharan Africa where the *P. vivax* malaria

prevalence is very low. Human sera from men and children from Colombia and Brazil only exposed to *P. vivax* have antibodies to VAR2CSA, and this protein shares DBL domains similar to the one in PvDBP. This shared DBL domain may be responsible for the observed cross-reactivity. We showed that PvDBPII affinity-purified antibodies from human sera recognized VAR2CSA by ELISA, as did a mouse monoclonal antibody (mAb 3D10) raised against PvDBPII, demonstrating cross-reactivity between these proteins (Gnidehou et al. 2019). It is important to note that PvDBP is not a direct orthologue of VAR2CSA, but the DBL domain, particularly within SD1, shares structural similarity with the six DBL domains of VAR2CSA. We propose that a conserved structural epitope in SD1 mediates cross-reactivity between these proteins.

1.8 Hypothesis and purpose

In this thesis, I will test the hypothesis that cross-reactive epitopes within DBL proteins from *P. vivax* contribute to protective antibodies against *P. falciparum* VAR2CSA. The purpose is to identify which are the shared epitopes between proteins that are involved in the cross-reactivity.

1.8.1 Objectives

The project has the following specific objectives:

1. To produce recombinant PvDBP and subdomains for immunological experiments.

2. To characterize the reactivity of sera from Colombians and Brazilians against PvDBP, SD1, EBP2, and VAR2CSA by ELISA.

3. To affinity-purify PvDBP and SD1 antibodies from Colombian and Brazilian sera, in order to test by ELISA if the affinity-purified antibodies recognize VAR2CSA.

4. To measure antibody activity against known functional correlates of protection from placental malaria by IBA.

1.8.2 Importance of the study

This research project aims to identify key epitopes within PvDBP and EBP2 that mediate antibody recognition of VAR2CSA. This project also seeks to assess the protective function of the cross-species antibodies against placental malaria. The knowledge generated from this research will contribute to our understanding of the immune mechanisms underlying cross-species immune recognition of *P. falciparum* in pregnancy. Potential cross-species immunity is highlighted by the protection found in our clinical studies. This research ultimately attempts to translate these findings into a vaccine candidate that mimics the natural protection observed in our Latin American populations. The outcome of our study will be the identification of an epitope in PvDBP (SD1) or EBP2 with the capacity to provide immunity against *P. falciparum* in pregnant populations for the development of a vaccine against placental malaria.

Chapter 2: Expression of PvDBPII protein and subdomains, and serological characterization of human sera from Colombia and Brazil

Contributions

H. Lugo contributed to the ELISA data presented in Figures 2.11, 2.13 and 2.14. S. Banman was a technician in the lab who tested some of the sera in Figure 2.11. C. Mitran tested some of the Brazilian sera against VAR2CSA presented in Figure 2.12. C. Hamel tested the primigravid women for VAR2CSA in figure 2.14. A. Maestre, E. Arango, L. Carvalho, F. Kano provided the human sera for the study. J. Adams kindly provided the pET21a+PvDBPII plasmid and EBP2 recombinant protein. A. Salanti provided the full-length VAR2CSA protein. S. Yanow, A. Mena designed the research. S. Yanow and S. Gnidehou assisted with the data analysis.

Portions of this chapter have been published:

Gnidehou S, Mitran CJ, Arango E, Banman S, Mena A, Medawar E, et al. Cross-Species Immune Recognition Between *Plasmodium vivax* Duffy Binding Protein Antibodies and the *Plasmodium falciparum* Surface Antigen VAR2CSA. J Infect Dis. 2019;219(1):110-20.

2.1 Introduction

More than half of all malaria cases in the region of the Americas, Oceania, and Asia are attributed to *P. vivax* (WHO. 2018). Despite the global importance of this species, it is difficult to study the biology of this parasite since it cannot grow in continuous culture. However, the use of mammalian cell systems expressing parasite ligands has revealed the complex process by which *P. vivax* merozoites invade human erythrocytes through specific molecular interactions with host receptors (Satchwell 2016). To date a single invasion mechanism for *P. vivax* had been described, which involves interaction between the DARC receptor and the DBL from Duffy binding like protein (PvDBP), creating a tight junction that allows the merozoite to enter the RBC. PvDBP was discovered as a microneme protein that plays an important role in this process by binding to the DARC receptor located in the membrane of the RBC (Batchelor, Zahm, and Tolia 2011). This interaction is through the DBL domain (region II) in PvDBP (referred to subsequently as PvDBPII), which is a conserved domain rich in cysteine (Adams et al. 1992). Binding of PvDBPII to DARC is critical for infection, based on observations in Africa where the Duffy-negative phenotype is frequent in populations of different ethnicities and where *P. vivax* malaria transmission is rare (Welch, McGregor, and Williams 1977). Additionally, previous studies have shown that African subjects who are heterozygous carriers of a Duffy-negative allele have half the amount of DARC on erythrocytes compared to wild-type homozygotes and their susceptibility to *P. vivax* blood stage infection is reduced (Zimmerman et al. 1999, Kasehagen et al. 2007).

PvDBP is a member of the EBL protein superfamily, which contains one other member from *P. vivax,* EBP2. PvDBP and EBP2 have similar DBL domains with 50% sequence identity; interestingly the similarity of the DBL domains from *P. vivax* and *P. knowlesi* is around 70% (Ntumngia et al. 2016). Due to its recent discovery (Hester et al. 2013a), the literature on the EBP2

is scarce, but its role in the erythrocyte invasion by *P. vivax* merozoites has been reported, and it also binds only to Duffy-positive RBCs (Hester et al. 2013a, Ntumngia et al. 2016). DBL domains are also found in PfEMP1 proteins and *P. knowlesi* DBL proteins, sharing high identity between them (Adams et al. 1992). Those DBL domains can bind different human receptors like ICAM-1 and CSA, among others, causing sequestration of iRBCs in the brain and placenta, respectively (Rowe and Kyes 2004). It is therefore important to understand the different structural interactions of the DBL domains and the host receptors to design vaccines or therapies that can interfere or block these interactions.

In our research group, we discovered that exposure to *P. vivax* induces antibodies that cross-reacted with VAR2CSA and may confer protection from MiP (Gnidehou et al. 2019). We showed that serum from Colombian and Brazilian populations with exposure to *P. vivax* recognized VAR2CSA (Figure 2.1A); additionally, these antibodies were functional because they blocked iRBC adhesion to CSA *in vitro* (Figure 2.1B) (Gnidehou et al. 2019). Further, we affinity-purified PvDBPII antibodies from sera pooled from non-pregnant Colombian populations with previous exposure to *P. vivax,* and these PvDBP-specific antibodies recognized VAR2CSA (Gnidehou et al. 2019) providing evidence of cross-reactivity between these two proteins by ELISA.
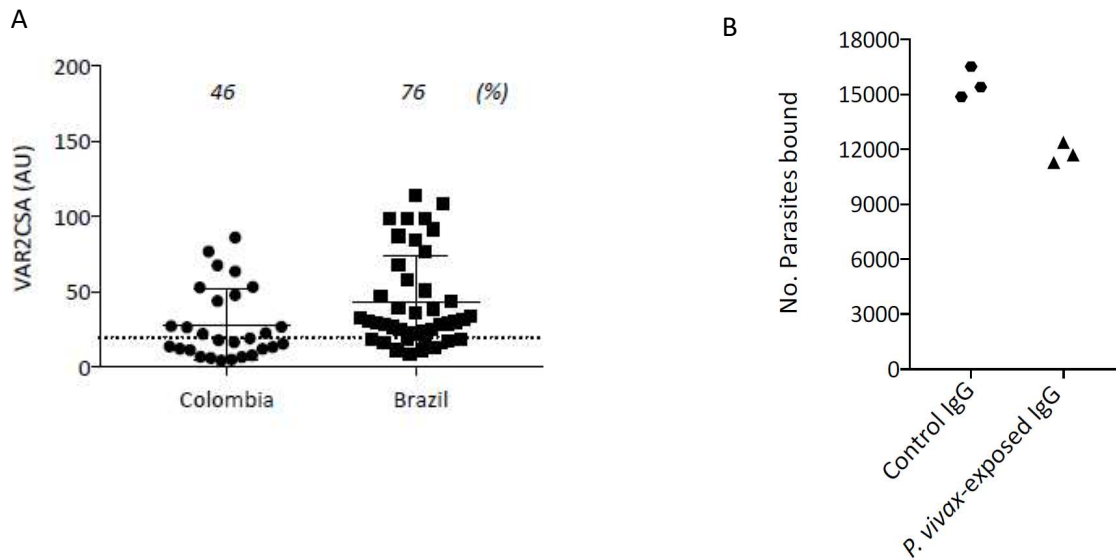
Figure 2.1 Colombian and Brazilian populations with exposure to P. vivax recognized VAR2CSA. (A) Sera from Colombian and Brazilian individuals exposed only to *P. vivax* recognized VAR2CSA by ELISA. (B) IgG purified from a pool of Colombian sera blocked parasite adhesion to CSA *in vitro* relative to a non-specific IgG control. Figures from Gnidehou, Mitran *et al.,* 2019.

In addition to human PvDBP antibodies, we also tested mouse monoclonal antibodies raised against the DBL domain of PvDBP. We discovered that one mAb, 3D10, recognized VAR2CSA by ELISA and inhibited the binding of iRBC to the CSA receptor *in vitro* (Gnidehou et al. 2019). Our next step was to map the epitopes in PvDBPII involved in the cross-reactivity. Based on previous work (Chen et al. 2016, Gnidehou et al. 2019) , the epitope in PvDBP that is recognized by 3D10 was mapped to subdomain 1 (SD1).

My objective for this Chapter was to express SD1 in order to perform a serological characterization, and later affinity-purify antibodies to this epitope from human sera. I used both peptide and recombinant protein approaches to express SD1. For the recombinant proteins, I expressed PvDBP region II (rPvDBPII), and the smaller fragment spanning SD1 and subdomain

2 (rSD1+SD2). I used the *E. coli* prokaryotic system for expression of recombinant proteins. This expression system is widely used because it is simple to manipulate and highly amenable to the rapid expression of large amounts of protein. Fraser et al in 1997 (Fraser et al. 1997), first expressed the regions II and IV of PvDBP as GST fusion proteins in *E. coli*. Although the protein was immunogenic, it did not have binding activity to DARC. Then, in 2000 Dutta et al (Dutta et al. 2000) found that the region II of PvDBP was mainly in the inclusion bodies (IB), the insoluble fraction of *E. coli* culture, but this protein did not bind to the RBCs. It wasn't until 2001, that Singh et al (Singh et al. 2001) developed a protocol that produced well-folded and functional PvDBPII using a prokaryotic system. In this protocol the authors used urea to denature the IB, followed by a refolding process under redox conditions for 36 hours at 10°C, finishing with dialysis for 48 hours. The recombinant PvDBPII was immunogenic in rabbits and the protein bound to DARC on RBCs. I adapted this protocol to express and purify PvDBPII-derived proteins.

In this Chapter, I describe the results of various strategies to express the vivax-derived antigens and the serological characterization of human sera from non-pregnant populations from Colombia and Brazil against PvDBPII, SD1, EBP2, and VAR2CSA by ELISA.

2.2 Materials and methods

2.2.1 rPvDBPII and rSD1+SD2 protein expression

2.2.1.1 Plasmids

Region II of the DBL domain from PvDBP was cloned into a pET21a+ expression vector (Novagen Cat# 69740-3). This plasmid adds an N-terminal T7 tag and a C-terminal peptide containing six tandem histidine residues while conferring ampicillin resistance for bacterial expression. The plasmid expressing the rSD1+SD2 subdomains of PvDBPII was synthesized by GenScript using a pET30a expression vector (Novagen Cat# 69909-3). The protein has a histidine tag at the N-terminus to facilitate purification on a nickel column, using kanamycin as a selective marker.

2.2.1.2 Expression and purification of subdomains 1 and 2 from PvDBPII
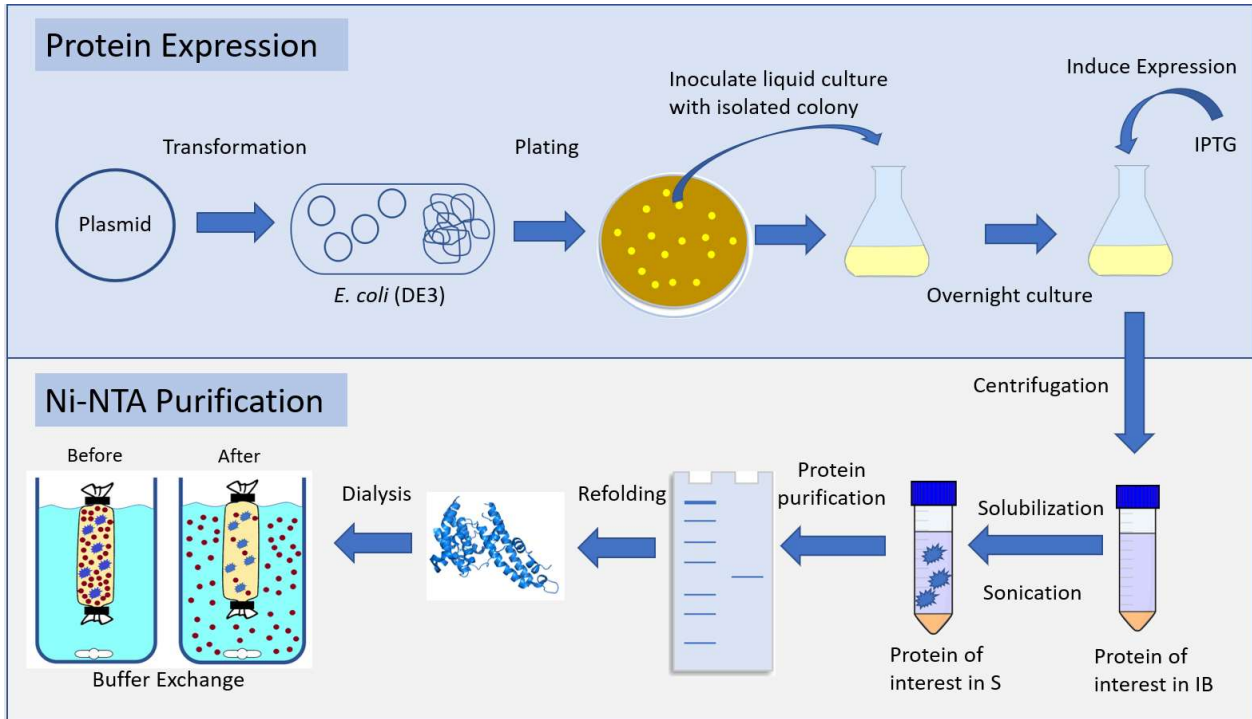
Figure 2.2 Schematic representation of the protein expression and purification system strategy. The desired sequence is cloned into a bacterial expression vector and transformed into *E. coli* BL21 bacteria strains. After the visualization of colonies on an agar plate, a colony is inoculated into growth media and incubated overnight. Expression is induced after IPTG addition. The PvDBP protein will be found in the IB of the culture. The IB are lysed by sonication and then solubilized, releasing the proteins into the supernatant (S). The protein is purified using a HisPur Ni-NTA resin, refolded and dialyzed. PvDBP imagen (DBP:4NUV)

I transformed pET21a+PvDBPII and pET30a+SD1+SD2 plasmids into BL21 (DE3) competent *E. coli* cells. I transformed ten ng of pET21a+PvDBPII plasmid into 50 µL of *E. coli* BL21 (DE3) cells. I allowed the transformation to occur for 30 minutes at 4°C, on ice. Subsequently, I heat-shocked the bacteria for 10 seconds at 42°C and immediately placed it on ice. I added SOC Medium (enriched bacterial medium; 250 µL) to the bacteria. I incubated the suspension was at 37°C for one hour while shaking horizontally. Next, I spread 50 µL and 200 µL of the transformed bacteria on two different agar plates containing 100 µg/mL ampicillin for PvDBPII and 50 µg/mL kanamycin for rSD1+SD2. I incubated both plates at 37°C overnight along with one control plate (without transformed bacteria).

I checked growth by visualization of colonies on the plate. I inoculated individual colonies from the overnight cultures into separate flasks of fresh liquid LB containing the required antibiotic (100 µg/mL ampicillin for the PvDBPII experiments and 50 µg/mL kanamycin for rSD1+SD2 experiments) and cells were grown overnight at 37°C while shaking horizontally at 220 rpm. I prepared a negative control flask with LB containing only ampicillin 100µg/mL or kanamycin 50 µg/mL.

When the absorbance (A_{600}) reached 0.6, I induced protein expression with different concentrations of isopropyl-1-thio-β-galactopyranoside (IPTG) in 10 L of bacteria culture. The cultures were allowed to grow for 4 hours at 37°C. Subsequently, I centrifuged the culture for 15 minutes at 6000 x g. I discarded the supernatant and washed the pellet by adding 200 mL of cold PBS to remove LB buffer. I homogenized the bacterial pellet and centrifuged it again for 10 minutes at 6000 x g, discarding the supernatant and conserving the pellet at -80°C.

2.2.1.3 Preparation of inclusion bodies (IB)

I lysed the cell pellet in ice-cold lysis/IB Buffer 1 (50 mM Tris, pH 8, 0.5 M NaCl, 0.2 mM EDTA, 3% sucrose, 1% Triton X-100). I added lysozyme (200 µg/mL), PMSF (1 mM) and DNase (20 µg/ml) to the lysis/IB Buffer 1 and homogenized the solution. The process was followed by 15 minutes of incubation on ice and sonication. After sonication, the solution was centrifuged at 30,000 x g for 30 minutes at 4°C. Then, I resuspended the cell pellet twice in ice-cold IB Buffer 2, (50 mM Tris, 0.5 M NaCl, 0.2 mM EDTA, 3 M urea and 1 mM PMSF) in order to release the insoluble protein products in the IB. I sonicated the suspension again and incubated it for 15 minutes at room temperature while rocking, then centrifuged it at 30,000 x g for 30 minutes at 4°C. I stored the supernatants for further experiments and collected the cell pellet containing the inclusion bodies, repeating the above twice.

I denatured the highly concentrated protein in the IB (cell pellet) in urea to obtain enough protein. I mixed each gram of insoluble protein with 20 mL of fresh solubilizing buffer, (10 mM Tris, pH 8, 0.2 M NaCl, 10 mM NaH_2PO_4, 8 M urea and 5 mM of BME). I broke up the pellet by sonication. I incubated the suspension overnight at room temperature while rocking. The following day, I centrifuged the suspension at 30000 x g at 4 ºC for 30 minutes and collected the supernatant (soluble phase). This phase contained the soluble PvDBPII recombinant protein. I kept the suspension at -20°C until purification.

2.2.1.4 Protein purification

I added HisPur Ni-NTA resin (Thermo Scientific) at a 1:10 ratio to equilibration buffer (solubilizing buffer). I mixed the suspension for 30 minutes at room temperature to achieve total suspension of the resin. Subsequently, I added ten resin-bed volumes of inclusion bodies in

solubilizing buffer to the resin and mixed it on an end-over-end rotator for 30 minutes. I centrifuged the suspension for two minutes at 700 × g and saved the supernatant for downstream analysis. Next, I washed the suspension with 10 mL of wash buffer 1, (20 mM of phosphate buffer, pH 7.8, 0.5 M NaCl, 10 mM imidazole and 8 M urea) and 10 mL of wash buffer 2, (20 mM of phosphate buffer, pH 6.3, 0.5 M NaCl, 10 mM imidazole and 8 M urea). I saved the supernatant for downstream analysis.

To elute the bound His-tagged proteins, I added elution buffer (20 mM of phosphate buffer, pH 7.8, 0.5 M NaCl, 300 mM imidazole, 8 M urea, and 5 mM BME) to the resin at room temperature for five minutes and I performed a final centrifugation at 700 × g for two minutes. I analyzed all PvDBPII and rSD1+SD2 fractions by SDS-PAGE to visualize the purity of the fractions. Then I pooled and concentrated the selected fractions, buffer exchanged them into a 1x PBS buffer, pH 7 with an Amicon concentrator (10 kDa cut-off) and stored them at -20°C.

2.2.1.5 Refolding and dialysis process

I refolded the purified protein by adding refolding buffer 1, (50 mM Tris, pH 7.2, 1 M urea 0.5 M NaCl, 1 mM EDTA, 0.5 M arginine, 2 mM cysteine, 0.25 mM cystamine dihydrocholide, 1 mM reduced glutathione and 0.25 mM oxidized glutathione) and refolding buffer 2, (β-cyclodextrin), keeping a 7:3 ratio respectively. I used reduced and oxidized glutathione solutions to enable disulfide bond formation; I flushed the protein with nitrogen gas. The protein was refolded. In order to have a proper refolding process, I diluted the protein to a final concentration of 50 µg/mL and refolded it for 48 hours at 4°C.

I dialyzed proteins in 50 mM phosphate, pH 6.5 and 1 M urea for 36 hours at 4°C and I did three exchanges of the buffer. I dialyzed approximately 1 L of dialyzed protein into 8 mL at 0.39

mg/mL, then buffer exchanged it into a 1x PBS buffer, pH 7 with an Amicon concentrator (10 kDa cut-off) and stored it at -80°C. I analyzed the final refolded PvDBPII by SDS-PAGE with DTT (50 mM) and without DTT; the well-refolded PvDBPII without DTT will show faster mobility than the denatured protein.

2.2.1.6 SD1 peptides

To design a peptide based on SD1, I first investigated whether SD1 contains polymorphisms that could affect the reactivity of sera to this sequence. gDNA from 22 isolates from Colombian patients with acute *P. vivax* infection was purified previously in our lab using a QIamp DNA Blood Mini Kit (QIAGEN). I then used primers 5'-CCGTTATGAAGAACTGCAACTACA-3' and 5'-GAATGTGGCGGTGAATATCGAA-3' to amplify a 1098 bp fragment of PvDBPII and sequenced in both directions by Sanger sequencing. I found that the SD1 sequence is highly conserved across these samples (Fig. 2.3). Based on these data, the following peptide was synthesized (by Synpeptide Co., China): SNTVMKNCNYKRKRRERDWDCNTKKDVCIADRRYQLCMK. I used this peptide for all serology assays in this Chapter.
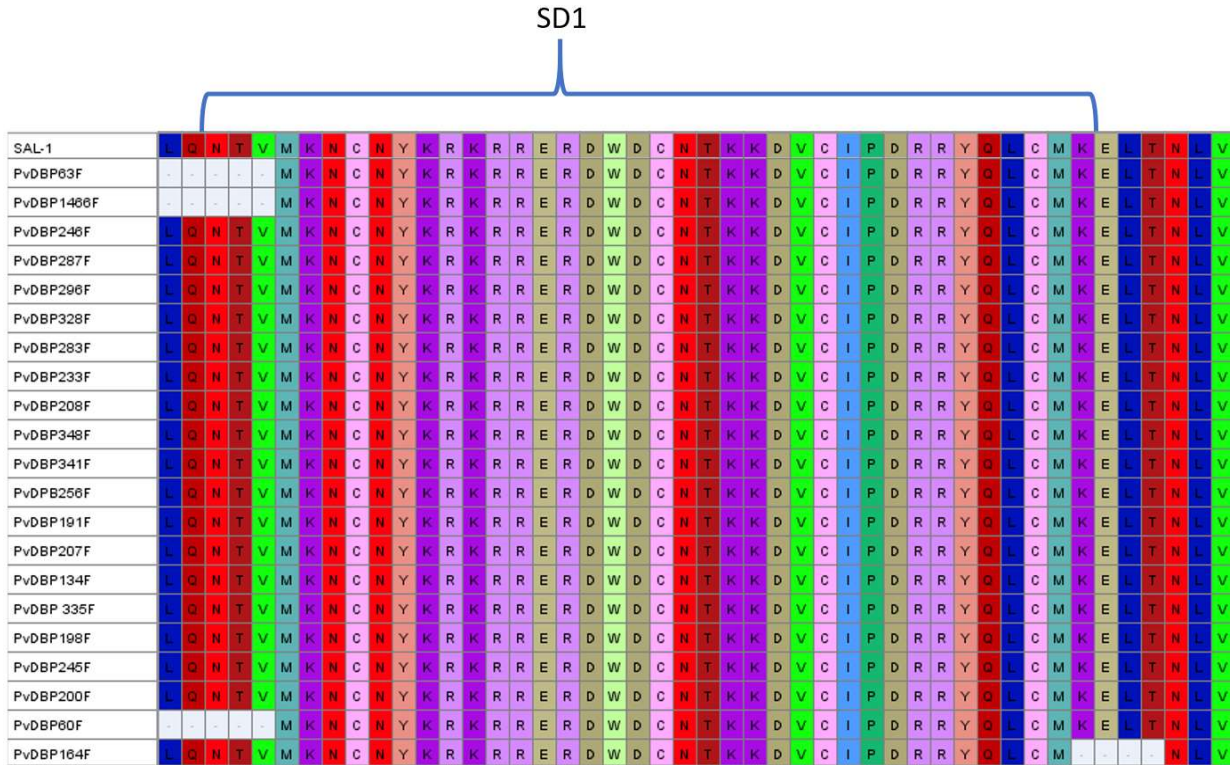
Figure 2.3 The amino acid sequence of subdomain 1 was identical across Colombian isolates. SD1 of PvDBPII is 100% conserved in 22 parasites isolated from Colombia compared to the *P. vivax* reference strain Sal 1.

2.2.2 Serological characterization of Colombian and Brazilian populations

2.2.2.1 Study population

Samples from Colombia and Brazil included in this study were described previously (Gnidehou et al. 2019). The study population included 124 symptomatic and 49 asymptomatic men and children, 42 symptomatic and 57 asymptomatic non-pregnant women, and 49 primigravid women from Colombia; and 123 males from Brazil (Table 2.1). We were particularly interested in measuring the level of antibodies in these populations to demonstrate that the acquisition of VAR2CSA was not pregnancy-specific. Additionally, we included five samples from a *P. vivax* outbreak in Souza, Brazil, between April and May 2003, described previously (Ceravolo et al.

2009). We were particularly interested in testing these samples because these patients were only ever exposed to *P. vivax*.

Table 2.1 Study population

Country, town	Malaria status	Population characteristic	Participants, No.	Sex: M, F
Colombia				
Puerto Libertador	Exposed to *P. falciparum* and *P. vivax*	Men and children (Symp. & Asymp.)	173[a]	170, 3[b]
		Non-pregnant women (Symp. & Asymp.)	99	-
		Primigravid women	49	-
Medellin	No exposure	Men and women	50	25, 25
Brazil				
Souza	Exposed to *P. vivax*	*P. vivax* outbreak	5	N/A
Rio Pardo	Exposed to *P. vivax*	Men and children	117	-
Belo Horizonte	No exposure	Men and women	12	8, 4
Canada				
Edmonton	No exposure	Men and women	22	5, 17

Abbreviations: Symp, symptomatic. Asymp, Asymptomatic. N/A, not available
[a]Includes 17 children and 154 male adults.
[b]Females were $12 \leq$ years old.

2.2.2.2 ELISA

The ELISA protocol was performed as follows:

I coated the recombinant proteins and SD1 peptide at 0.5 μg/mL, diluted in 1x PBS and incubated overnight at 4ºC. The next day, I added 275 μL of 4% BSA/1x PBS to each well to wash out unbound antigen. I added another 275 μL of 4% BSA/1x PBS to each well and incubated at

37ºC to block. I washed the plates once manually with washing buffer, 1x PBST (0.1% Tween 20). I diluted the primary antibody (human sera) in 2% BSA/1x PBS (Table 2.2) and added 100 µL to each well. I incubated the plate at room temperature for one hour and removed the sera from the plate by flicking, followed by five manual washes with 275 µL of 1x PBST (0.1% Tween 20). I diluted the secondary antibody (goat anti-human IgG-HRP, Abcam Cat #98624) in 2% BSA/1x PBS according to Table 2.3 and added 100 µL to the plate. After one hour of incubation at room temperature, I washed the plate manually five times with washing buffer. I developed the reaction by adding 100 µL of 3,3',5,5' tetramethylbenzidine TMB (Sigma-Aldrich) and incubated for 30 minutes at room temperature. I stopped the reaction was stopped once the positive control was a strong blue colour by adding 100 µL of 0.5N H_2SO_4 to the TMB. Finally, I read the OD at 450 nm and 570 nm in an Espire 2300 multilabel reader (PerkinElmer). Every plate I included negative and positive serum controls, along with a control of antigen plus secondary antibody alone. The negative sera controls were used to subtract background. I ran every sample in duplicate. I converted ODs to arbitrary units (AU) relative to a pool of positive sera from previous studies that were run on each plate, according to the formula: $AU = [(OD_{test\ sera} - background)/ (OD_{positive\ pool} - background)] * 100$ for SD1, rPvDBPII and EBP2. For VAR2CSA, the ODs were converted to arbitrary units (AU) relative to a single positive serum from previous studies that was run on each plate, according to the formula $AU = [(OD_{test\ sera} - background)/ (OD_{positive\ pool} - background)] * 126.1954$. For the VAR2CSA AU calculation, I multiplied our results by 126.1954 (OD of the positive sera sample) in order to link the new data to previous VAR2CSA data, developed under different assay conditions in our lab.

The background was the OD of the Canadian pool from every plate. The cut-offs for PvDBPII, SD1 and EBP2 were determined based on the mean OD of individual Canadian sera

(21-22 sera), plus two standard deviations and converted to AU against the positive control on the same plate. The cut-off for VAR2CSA was determined by setting the mean OD of individual Canadian sera as zero (20 sera), converted to AU against the positive control on the same plate.

The full-length VAR2CSA (FCR3) was produced in insect cells and shared by Dr. Salanti (Khunrae et al. 2010). The recombinant protein EBP2 was produced in *E. coli* and shared by Dr. Adams (Ntumngia et al. 2016). The recombinant PvDBPII protein was expressed and purified in this Chapter (Figure 2.5).

Table 2.2 Dilutions of primary and secondary antibodies

Antigen	Dilution of primary antibody	Dilution of secondary antibody
PvDBPII	1/100	1/40000
EBP2	1/100	1/40000
SD1	1/200	1/30000
VAR2CSA	1/1000	1/25000

2.2.2.3 Statistical analyses

I plotted the data using Prism software (version 7; GraphPad). We investigated the association of VAR2CSA with PvDBPII, SD1, and EBP2 using Spearman's rank correlation coefficient to measure the association between AUs of individual sera against the different proteins.

2.3 Results

2.3.1 rPvDBPII and rSD1+SD2 protein expression

2.3.1.1 Expression and purification of rPvDBPII

I expressed the region II of PvDBP protein from *P. vivax* in *E. coli* competent cells. This protein tends to accumulate in the IB in *E. coli* as a misfolded and insoluble protein (Fig 2.4A). I purified the protein using a Ni-NTA resin (Fig. 2.4B), then refolded, dialyzed and concentrated it.

Figure 2.4 Preparation of inclusion bodies and protein purification using Ni-NTA resin of rPvDBPII. A, SDS-PAGE gel stained with Coomassie brilliant blue of preparation of the inclusion bodies. Proteins in the four washes (W1 to W4) show a elution of non-specifically bound proteins. PC is the positive control. Lane IB shows the inclusion bodies containing a strong band of 37 kDa. B, SDS-PAGE gel of the purification with Ni-NTA resin. FT corresponds to the flow through after adding the inclusion bodies. PC corresponds to the positive control and F1 to F5 correspond to the different purified fractions eluted from the column.

Refolded and purified rPvDBPII was separated by SDS-PAGE gel and stained with Coomassie brilliant blue. rPvDBPII migrated with the expected mobility of approximately ~39 kDa and it showed a slower migration after reduction with DTT (Fig. 2.5). Also, the protein was recognized by 3D10 in several ELLISAs experiments.

A

MW -DTT +DTT

250
150
100
75
50
37
25
20
15
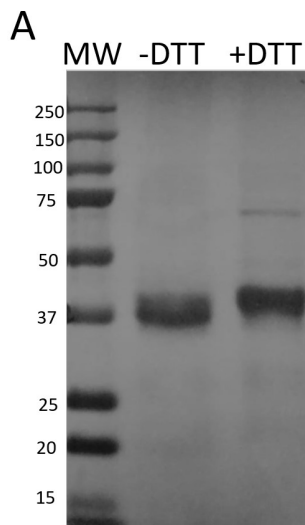
Figure 2.5 Refolded and purified recombinant PvDBPII. A, SDS-PAGE gel stained with Coomassie brilliant blue showing the mobility of recombinant PvDBPII before and after reduction with dithiothreitol (DTT). The rPvDBPII protein has slower mobility in under reducing conditions, demonstrating the presence of disulfide bonds. Final concentration of the refolded and purified recombinant PvDBPII protein was 0.39 mg/mL.

2.3.1.2 Expression and purification of subdomains 1 and 2 from PvDBPII

This protein was first expressed commercially (by Genscript) but the company failed to refold the protein correctly. A small amount of unfolded protein was sent to us and I used this as a positive control. To purify rSD1+SD2 myself, I prepared the IB and performed the purification using the same protocol described previously for rPvDBPII. During the preparation of the inclusion bodies for rSD1+SD2 (Fig 2.6A), our desired protein was apparently expressed (protein close to 20 kDa); however, non-specific proteins were also expressed at the same amount as our desired protein. The same pattern of non-specific bands appeared after nickel purification and the 20 kDa protein was lost between the third and fifth fractions (Fig 2.6B).
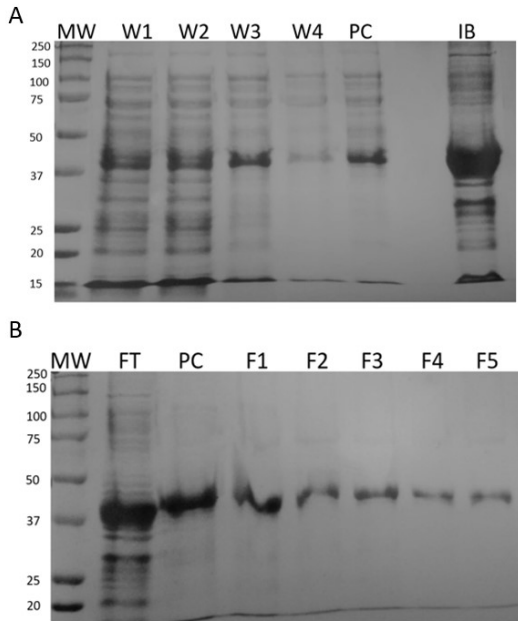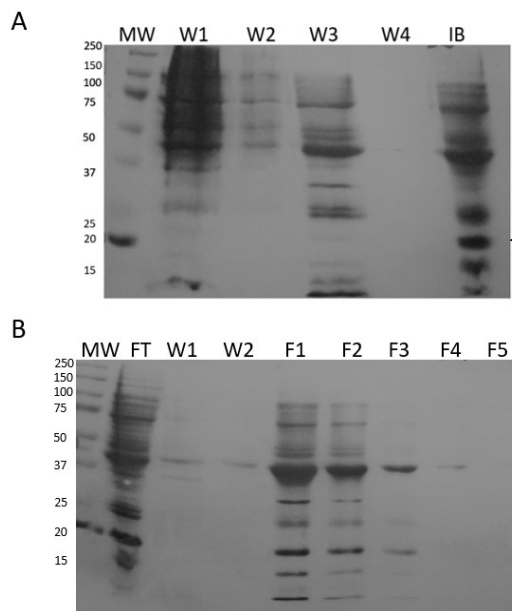
Figure 2.6 Preparation of inclusion bodies and protein purification using Ni-NTA resin of rSD1+SD2. A, SDS-PAGE gel stained with Coomassie brilliant blue of preparation of the inclusion bodies showing the four washes (W1 to W4) and the IB. In the IB, there are two strong bands close to 37 kDa and 20 kDa (arrows). The band close to 20 kDa could correspond to SD1+SD2. B, SDS-PAGE gel of the purification with Ni-NTA resin. FT corresponds to the flow through after adding the inclusion bodies. W1 and W2 correspond to the washes and F1 to F5 correspond to the different purified fractions. A strong band can be observed close to 37 kDa and a second band is observed close to 20 kDa. During repeated experiments, the two bands disappeared after the fourth elution fraction.

Due to the difficulties to express and purify rSD1+SD2, I tested different conditions such as temperature, IPTG concentration, time of incubation, the addition of glucose, and bacterial cold shock in order to optimize expression of the protein. I tested samples by SDS-PAGE (Fig. 2.7A) and western blot (Fig. 2.7B). I tested the overnight culture before and after induction to check if there was an increase in the intensity of the band at the expected size of 20 kDa. As shown in figure 2.7A, there was an increase in the intensity of the desired band, but there was also an increase in the non-specific bands. The first elution fraction from the nickel purification in lane 4 revealed an intense, non-specific band between 37 and 39 kDa, and very weak band for rSD1+SD2. There were no bands in lane 5, which corresponds to the fifth fraction from the nickel purification. In the western blot (Fig 2.7B), I tested the mouse monoclonal antibody (mAb 3D10) raised against rPvDBPII against the protein fractions. The antibody strongly recognized the positive control (lane 1), the overnight culture after induction (lane 3) and the first fraction from the nickel purification, but the monoclonal antibody also recognized a non-specific extra band in lane 3 and it recognized the protein even before induction (lane 2). In order to clarify the observed protein recognition in

the overnight culture by 3D10 (lane 2), an experiment was performed testing overnight culture of transformed bacteria and overnight culture of non-transformed. Figure 2.7C is showing that 3D10 did not recognized the overnight culture of the non-transformed bacteria (lane 2), but it recognized the transformed bacteria (lane 3). This recognition could be explained by leaky expression from the promoter used in the pET30a expression vector (Miroux and Walker 1996).

Figure 2.7 mAb 3D10 against rSD1+DS2 SDS-PAGE gel stained with Coomassie brilliant blue (A) and western blot testing the mAb 3D10 (B) showing the positive control in lane 1, the overnight culture (-IPTG) in lane 2, the overnight culture (+IPTG) in lane 3, the elution fraction 1 in lane 4 and in lane 5 the fraction 5 from Ni-NTA purification (C) western blot testing the mAb 3D10 showing the positive control in lane 1, the overnight culture of transform bacteria (lane 2) and the overnight culture of non-transform bacteria (lane 3)

optimize protein expression (Fig. 2.8). Figure 2.8A shows an SDS-PAGE gel of the induction with IPTG at 20°C with the following incubation periods: 2 hours, 4 hours, 6 hours and overnight. Figure 2.8B shows an SDS-PAGE gel of the induction with IPTG at 37°C and the following incubation periods: 1 hour, 2 hours, 3 hours, and 4 hours. The most abundant and specific protein was expressed when the induction was performed at 20°C for 4 hours (Fig. 2.8A lane 6).
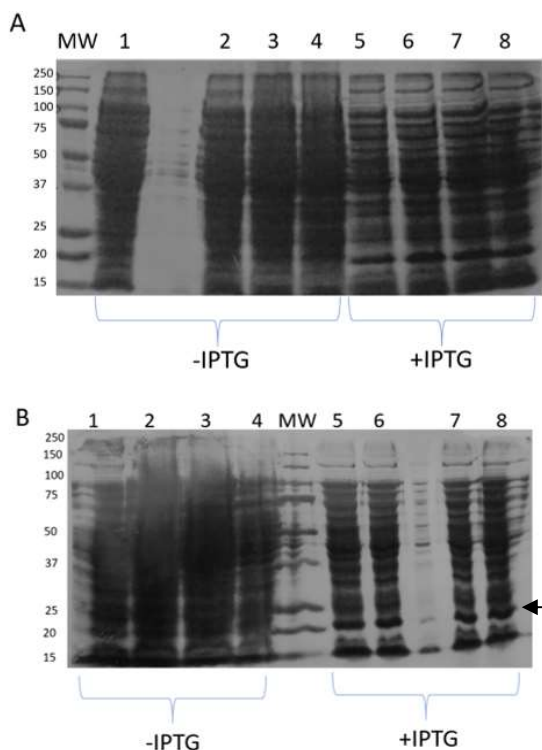
Figure 2.8 Testing different temperatures and incubation periods for optimal protein induction. A, Induction at 20°C. Lanes 1 and 5 correspond to growth after 2 hours, before (1) and after (5) induction with IPTG. Lanes 2 and 6 correspond to growth after 4 hours, before (2) and after (6) induction with IPTG. Lanes 3 and 7 correspond to growth after 6 hours, before (3) and after (7) induction with IPTG. B, Induction at 37°C. Lanes 1 and 6 correspond to growth after 1 hour, before (1) and after (6) induction with IPTG. Lanes 3 and 7 correspond to growth after 2 hours, before (3) and after (7) induction with IPTG. Lanes 4 and 8 correspond to growth after 3 hours, before (4) and after (8) induction with IPTG, and lines 5 and 9 correspond to growth after 4 hours, before (5) and after (9) induction with IPTG.

After selecting the incubation period and temperature for induction, I performed all the following experiments under these conditions. I performed a small-scale experiment to track the protein at every step of the protocol to ensure that the protein was expressed. Once the transformation (Fig. 2.9A) and induction (Fig. 2.9B) protocols were repeated, I ran a SDS-PAGE gel to detect the protein, again showing bands close to 20 kDa compatible with the size of rSD1+SD2. Next, a preparation of IB and nickel purification was repeated (Fig. 2.9 and 2.10); however, the desired protein was completely lost, and only non-specific proteins were obtained.
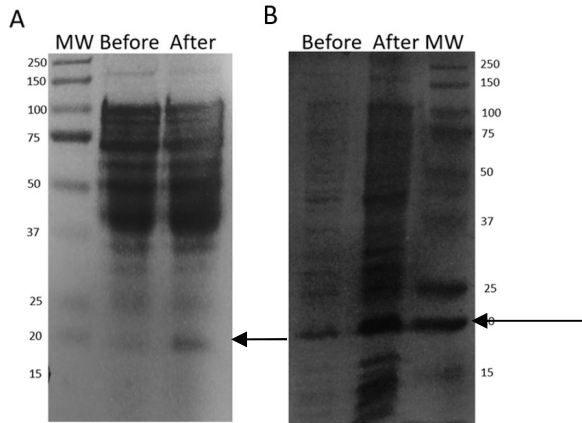
Figure 2.9 Small scale of high efficiency transformation of rSD1+SD2, and induction with IPTG at 20°C. A, a small-scale experiment of the *E. coli* culture before and after transformation with the plasmid pET30a+SD1+SD2. B, the same culture from (A) before and after induction with IPTG, showing bands close to 20kDa.
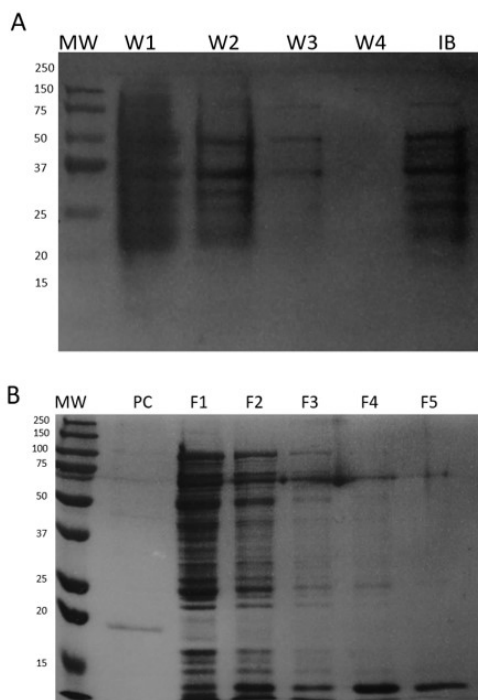
Figure 2.10 Preparation of inclusion bodies and protein purification using Ni-NTA resin of rSD1+SD2. A, SDS-PAGE gel stained with Coomassie brilliant blue of a small-scale experiment preparation of the IB at 20°C, showing the four washes (W1 to W4), where no clear bands appear. B, SDS-PAGE gel of the purification with Ni-NTA resin. PC corresponds to the positive control and F1 to F5 correspond to the different eluted fractions, where no clear bands are observed close to 20kDa.

I did a final attempt to express and purify the protein by testing different conditions (Fig. 2.11). Two concentrations of IPTG, 1mM and 0.1mM were tested, to be sure that the expression system was not being saturated by the concentration of IPTG. Also, I tested the addition of glucose to the cultures, as glucose reduces constitutive activity of the lacO promoter, and helps reduce non-specific expression. The addition of glucose can help with toxic proteins for the cells that are hindering the protein expression. Additionally, I tested a bacterial cold shock, to address the

possibility that the rSD1+SD2 was a soluble protein, given that cold shock at 4°C increases the probability of obtaining soluble proteins.

Table 2.3 Testing different concentrations of IPTG, glucose addition and cold shock

| Lane | Conditions | | | | | | | |
| | 1 mM IPTG | | | | 0.1 mM IPTG | | | |
	Cold shock	1% Glucose	Before induction	After induction	Cold shock	1% Glucose	Before induction	After induction
2	+	+	+	-	+	-	+	-
3	+	+	-	+	+	-	-	+
4	+	-	+	-	+	+	+	-
5	+	-	-	+	+	+	-	+
6	-	-	+	-	-	-	+	-
7	-	-	-	+	-	-	-	+
8	-	+	+	-	-	+	+	-
9	-	+	-	+	-	+	-	+

Figure 2.11 Testing different concentrations of IPTG, 1% glucose addition and cold shock. A, induction with 1mM IPTG. B, induction with 0.1mM IPTG. Lane 1 corresponds to the positive control in both graphs. The experiments were performed at 20°C.
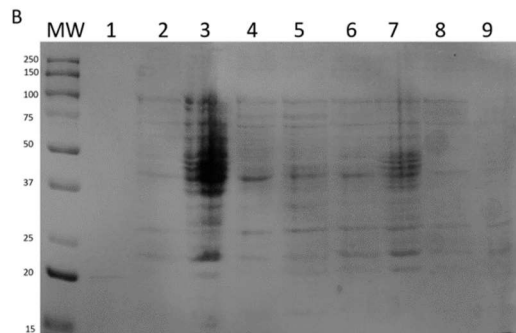
Recombinant PvDBPII protein was expressed and purified using an *E. coli* expression system following the protocol from Singh et al and adapted in John Adams' lab (Singh et al. 2001).

The mobility of the purified rPvDBPII by SDS-PAGE gel was consistent with an apparent molecular weight of ~39 kDa, indicating that purified rPvDBPII had the expected protein size. Moreover, the protein was recognized by ELISA using the mAb 3D10. rPvDBPII was purified in order to be used in the characterisation of human sera from Colombia and Brazil, by ELISA. As efforts to purify rSD1+D2 using *E. coli* were unsuccessful, a synthetic SD1 peptide was used.

2.3.2 Serological characterization of Colombian and Brazilian populations

2.3.2.1 Seroreactivity to malaria antigens

Once I generated the different proteins and peptides that are potential antibody targets, we next used these reagents to characterize sera from Colombian and Brazilian populations for reactivity to these antigens. Specifically, we tested whether non-pregnant populations from Colombia and Brazil living in malaria-endemic areas have antibodies against VAR2CSA, rPvDBPII, SD1, and EBP2. In symptomatic men and children from Colombia (Table 2.3), the seropositivity for VAR2CSA was 48%, 61% for rPvDBPII, 42% SD1 and 77% EBP2; and the seropositivity for the asymptomatic population was 48% for VAR2CSA, 43% for rPvDBPII, 37% for SD1 and 67% for EBP2.

For the symptomatic non-pregnant women from Colombia, the seropositivity was 78.5% for VAR2CSA, 62.5% for rPvDBP, 43% for SD1 and 77.5% for EBP2; and for the asymptomatic population was 34% for VAR2CSA, 50% for rPvDBPII, 47% for SD1 and 49% for EBP2. Additionally, we tested 49 primigravid women from Colombia, finding that 67% were positive for VAR2CSA, 50% for rPvDBP, 45% for SD1and 54%% positive EBP2.

For the non-pregnant Brazilian population, we tested 117 samples in total of which 69 (59%) were positive for VAR2CSA; 40 (43.2%) were positive for SD1, 93 (79.5%) were positive for rPvDBPII, and 86 (73.5%) were positive for EBP2.

Table 2.3 Non-pregnant Colombian samples tested for serum reactivity to VAR2CSA, PvDBPII, SD1, and EBP2

		VAR2CSA No. pos/total (%)	PvDBPII No. pos/total (%)	SD1 No. pos/total (%)	EBP2 No. pos/total (%)
Symptomatic	Men and children	59/123 (48%)	73/120 (61%)	52/124 (42%)	94/122 (77%)
	Women	33/42 (78.5%)	25/40 (62.5%)	18/42 (43%)	31/40 (77.5%)
Asymptomatic	Men and children	21/44 (48%)	19/44 (43%)	18/49(37%)	31/46 (67%)
	Women	16/47 (34%)	28/56 (50%)	23/49 (47%)	28/57 (49%)

After measuring the levels of antibodies in our different populations, we used Spearman's correlation to examine the strength of association between the antibodies to different antigens in sera from Colombia (Fig. 2.12, 2.13 and 2.14) and Brazil (Fig. 2.15). We found positive correlations across all our populations comparing antibodies against VAR2CSA and rPvDBPII, against VAR2CSA and SD1, and against rPvDBPII and SD1. However, there was no correlation between antibodies to VAR2CSA and EBP2 for the men and children from Colombia (Fig. 2.12 C). The *r* value was 0, which corresponds to no correlation.

Samples collected during the *P. vivax* outbreak samples from Souza, Brazil, were tested for antibodies to all of the antigens (Fig. 2.16). None of the sera were positive for SD1 antibodies, one out of five was positive for VAR2CSA antibodies and four of them were positive for rPvDBPII antibodies. No further statistical analyses were performed for the outbreak samples because of the small sample size.
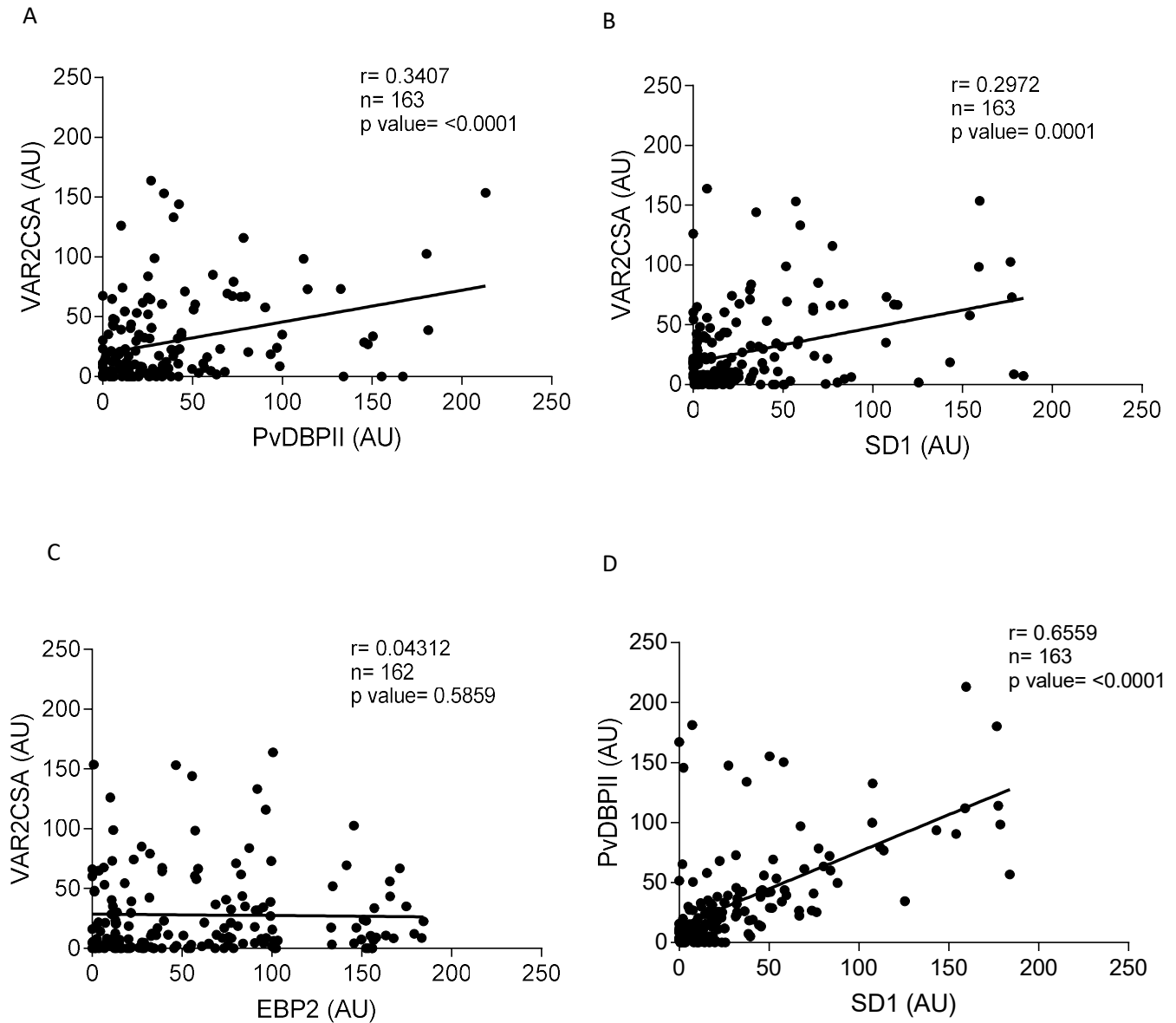
Figure 2.12 PvDBPII and SD1 antibodies correlate with VAR2CSA from Colombian men and children. Sera from symptomatic and asymptomatic men and children from Colombia were tested for reactivity to full-length VAR2CSA (based on detection of the FCR3 allele), PvDBPII, SD1 and EBP2 by ELISA. Values are expressed as AUs, relative to the positive control on each plate. After measuring the antibody levels of the different proteins in the populations, Spearman's rank correlation was applied to look for relationships between; *A,* the reactivity to VAR2CSA and rPvDBPII; *B,* the reactivity between VAR2CSA and SD1; *C,* the reactivity between VAR2CSA and EBP2 and *D,* the reactivity between rPvDBPII and SD1. Non-linear regression was used to generate a fitted line.
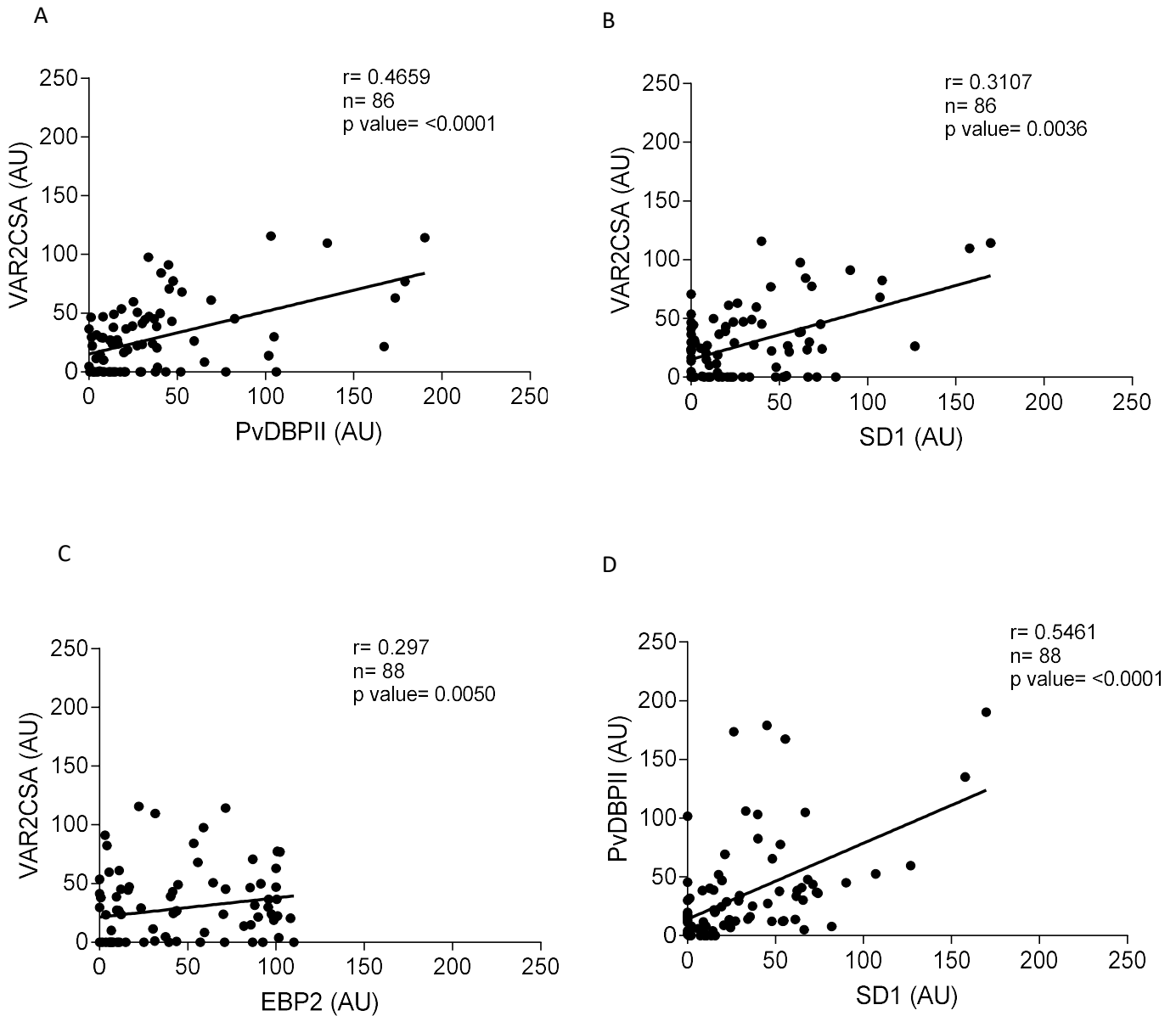
Figure 2.13 PvDBPII and SD1 antibodies correlate with VAR2CSA from non-pregnant Colombian women. Sera from symptomatic and asymptomatic non-pregnant women *(A-C)* from Colombia were tested for reactivity to full-length VAR2CSA (based on detection of the FCR3 allele), PvDBPII, SD1 and EBP2 by ELISA. Values are expressed as AUs relative to the positive control on each plate. After measuring the antibody levels of the different proteins in the populations, Spearman's rank correlation was applied to look for relationships between; *A,* the reactivity to VAR2CSA and rPvDBPII; *B,* the reactivity between VAR2CSA and SD1; *C,* the reactivity between VAR2CSA and EBP2, and *D,* the reactivity between rPvDBPII and SD1. Non-linear regression was used to generate a fitted line.
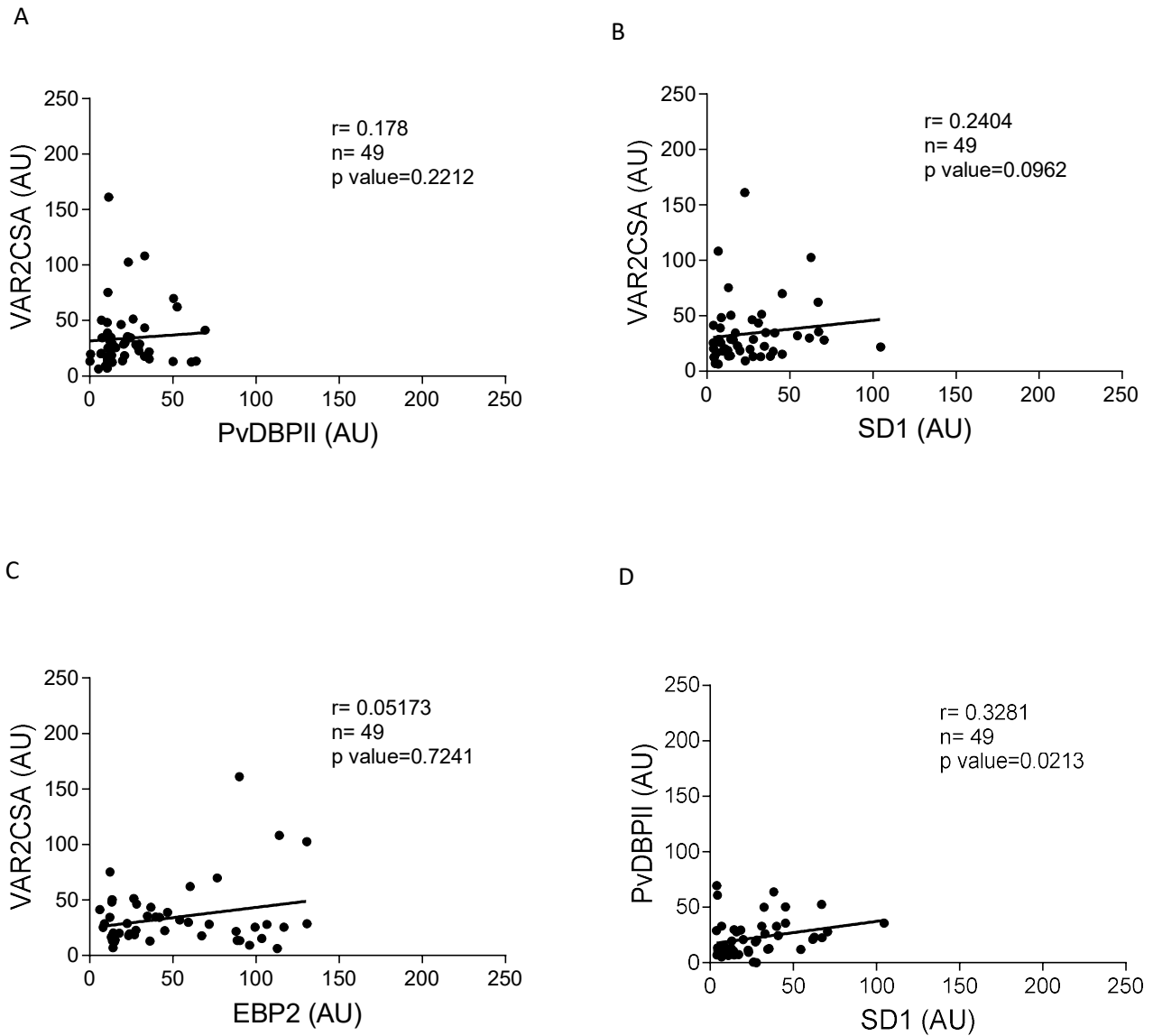
Figure 2.14 Correlations between PvDBPII, SD1 and EBP2 antibodies and VAR2CSA antibodies from primigravid women from Colombian. Sera from primigravid women from Colombia were tested for reactivity to full-length VAR2CSA, PvDBPII, SD1 and EBP2 by ELISA. Values are expressed as AU, relative to the positive control on each plate. After measuring the antibody levels of the different proteins in the population, Spearman's rank correlation was applied to look for relationships between *A*, the reactivity to VAR2CSA and rPvDBPII; *B*, the reactivity between VAR2CSA and SD1; *C*, the reactivity between VAR2CSA and EBP2, and *D,* the reactivity between rPvDBPII and SD1. Non-linear regression was used to generate a fitted line.
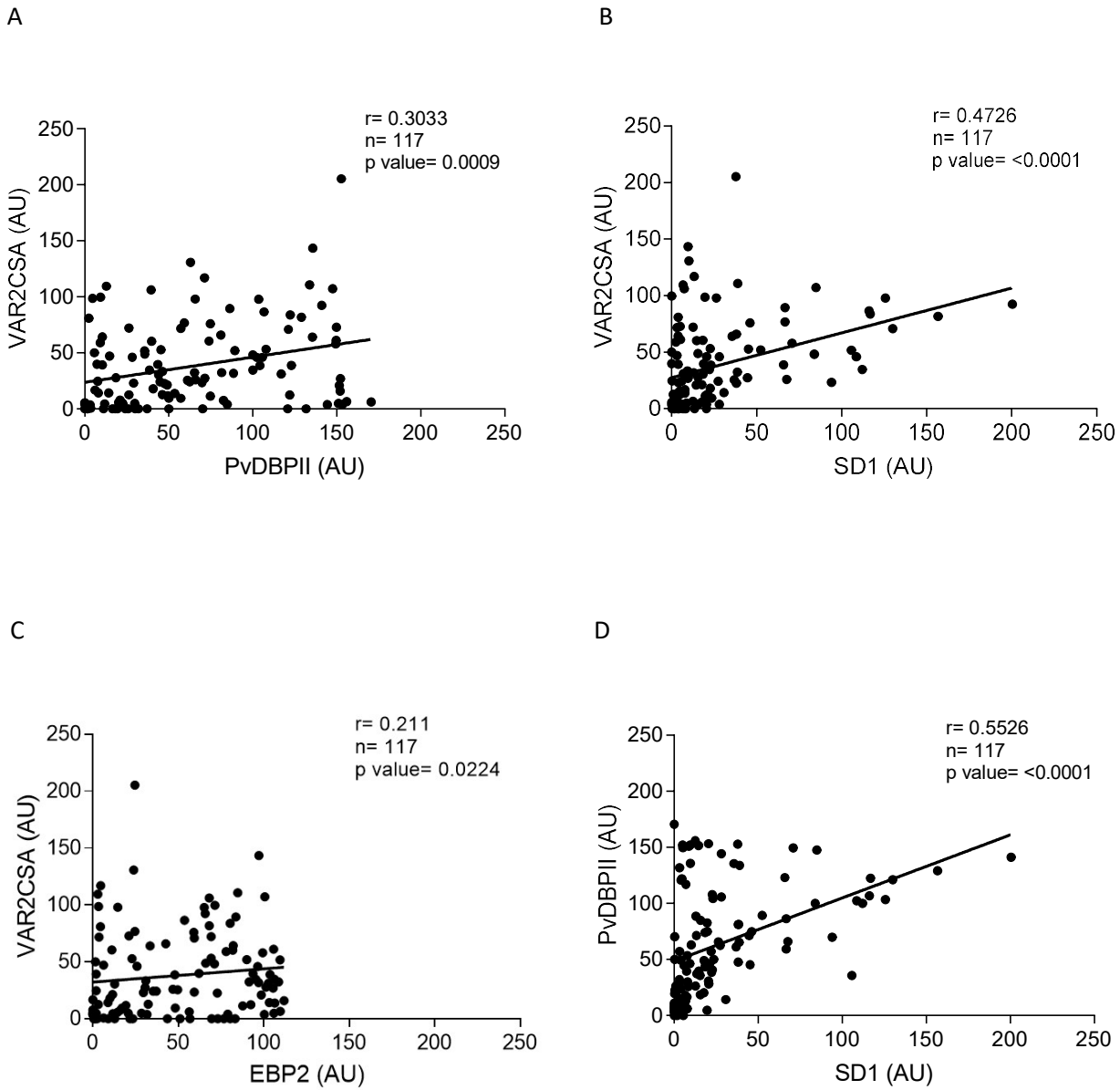
Figure 2.15 PvDBPII and SD1 antibodies correlate with VAR2CSA in male Brazilian populations.
Sera from Brazilian males were tested for reactivity to full-length VAR2CSA (based on detection of the FCR3 allele), PvDBPII and SD1 by ELISA. Values are expressed as AUs, relative to the positive control on each plate. After measuring the antibody levels of the different proteins in the population, Spearman's rank correlation was applied to look for relationships between, *A*, the reactivity to VAR2CSA and rPvDBPII, *B*, the reactivity between VAR2CSA and SD1, *C*, the reactivity to VAR2CSA and EBP2 and *D*, the reactivity to rPvDBPII and SD1. Non-linear regression was used to generate a fitted line.

A

B

C
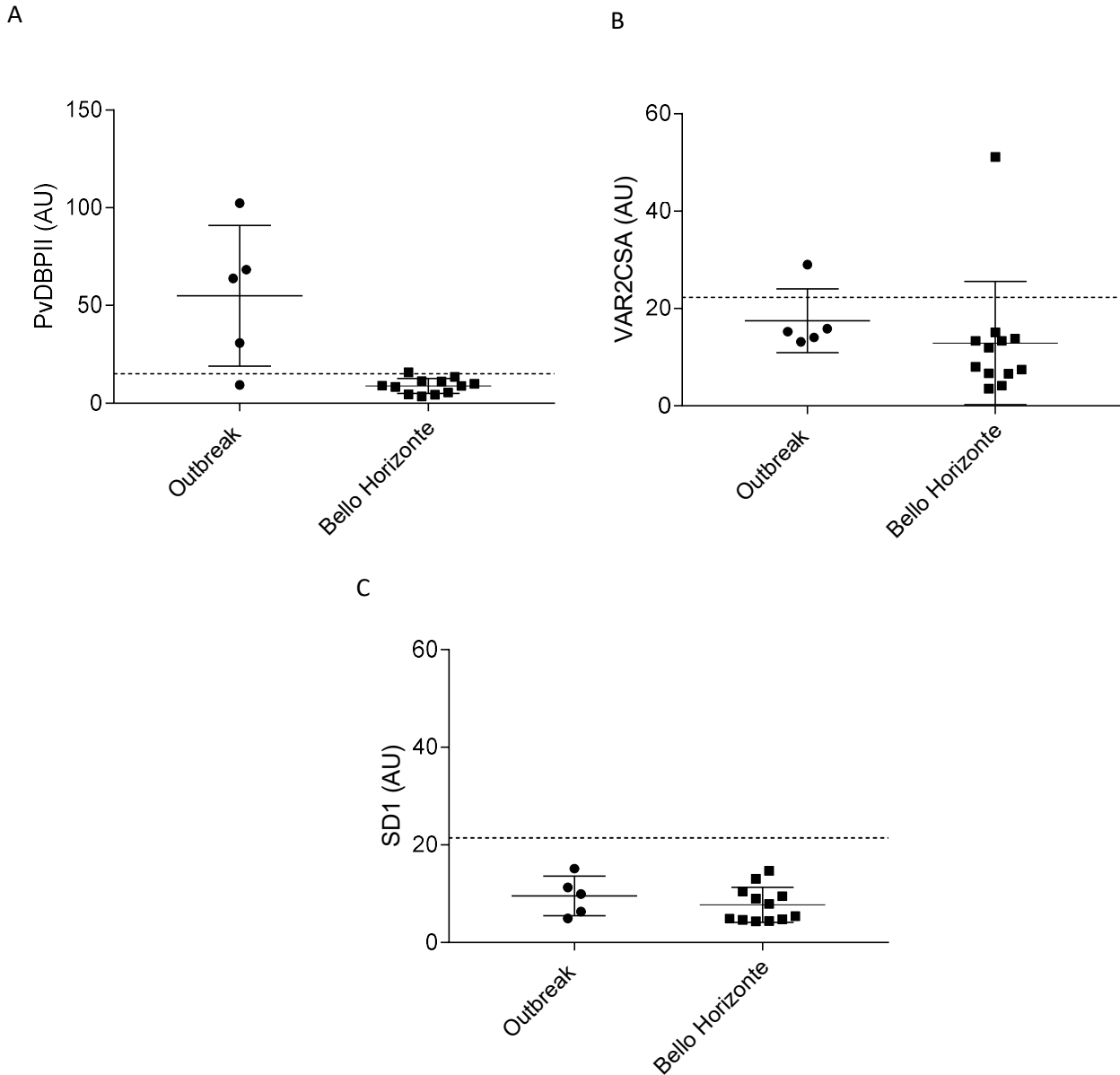
**Figure 2.16 Sera from the Souza outbreak were tested for reactivity to PvDBPII (A), VAR2CSA (B), and SD1 (C).** Sera from Belo Horizonte, where there is no malaria, were used as negative controls. Dashed-line is indicating the cut-off for each antigen.

2.4 Conclusions

This research shows that men and children from areas endemic for *P. falciparum* and *P. vivax* in Colombia and Brazil have high levels of antibodies against PvDBPII, SD1, EBP2, and full-length VAR2CSA and that EBP2 from *P. vivax* is unlikely to be involved in the cross-reactivity with VAR2CSA.

In all the experiments performed for rSD1+SD2 expression, an intense band appeared after transformation and induction with IPTG with a mass close to 20 kDa, but unfortunately, I could not ensure that expression of the protein was specific since the intensity of background bands was equally high. Additionally, the *E. coli* cells showed a band close to 20 kDa in the SDS- Page before transformation, suggesting that *E. coli* expresses a protein with a similar size as rSD1+SD2. However, when the non-transformed bacteria was tested against 3D10 by western blot, there was no recognition of any protein. This can be explained by the fact that many promoters, especially lactose promoters have leaky expression because they are not very tightly regulated, showing some degree of expression before adding the inducer. This leakiness produces an incomplete protein expression, because the leakiness can cause loss of the plasmid or plasmid instability (Miroux and Walker 1996).

The main findings from this Chapter are the serological reactivity of different populations to the various malaria antigens. I tested symptomatic and asymptomatic men and children from areas endemic for *P. falciparum* and *P. vivax* in Colombia and Brazil. I found that these populations have high levels of antibodies against PvDBPII, SD1, EBP2, and full-length VAR2CSA. Most interestingly, we observed a positive correlation between the antigens VAR2CSA, PvDBPII, and SD1, but we did not observe a correlation between antibodies to VAR2CSA and EBP2 in our Colombian study population. In our Brazilian study population, I

observed a very weak correlation between antibodies to VAR2CSA and EBP2. These findings suggest that the EBP2 protein from *P. vivax* is not involved in the cross-reactivity with VAR2CSA. Also, the high frequency of SD1 antibodies in these populations suggests that antibodies to SD1 are naturally acquired with exposure to *P. vivax*. Our ELISA data point to SD1 as the key epitope associated with cross-reactivity to VAR2CSA.

On another hand, I observed a positive correlation between all the antigens including EBP2 in the symptomatic and asymptomatic non-pregnant women from Colombia (Fig.2.13). This population has a slightly stronger correlation between the antigens compared to men and children, but it is important to mention that the obstetric history of these patients is unknown. As a result, we cannot be sure whether the antibodies against VAR2CSA were acquired following exposure to this protein in pregnancy or due to cross-reactive antibodies from *P. vivax*. In addition, EBP2 is more immunogenic than PvDBPII (Ntumngia et al. 2016). At this point, it is hard to interpret the ELISA data for the primigravid women due to the small sample size, but nevertheless, there is a similar pattern of correlations as the men and children.

Finally, it is difficult to interpret the results from the samples collected during the *P. vivax* outbreak. We predicted that these patients would have antibodies to SD1 and PvDBPII and therefore have antibodies to VAR2CSA. Instead, we found that they were positive for PvDBPII but not SD1 and only one was positive for VAR2CSA. One limitation is that the number of samples is very small (n=5). Additionally, the *P. vivax* outbreak occurred over a short time period (50 – 60 days) (Ceravolo et al. 2009). These patients may be seronegative for SD1 as they were treated rapidly with anti-malarial drugs (chloroquine plus primaquine) to control the infection, and a second round of treatment was given to control relapses and/or recrudescence (Ceravolo et al. 2009); as a result, the patients may not have had enough time to generate antibodies to SD1 and

VAR2CSA. However, these data also could suggest that antibodies against SD1 are generated from a different source other than PvDBPII. This hypothesis could be explored in future studies.

Chapter 3: Affinity-purification and functional characterization of human PvDBPII and SD1 antibodies

Contributions

A. Maestre and E. Arango provided the human sera for the study. A. Salanti kindly provided the full-length VAR2CSA protein.

3.1 Introduction

Malaria in pregnancy is a public health problem due to the negative outcomes that are associated with this condition, such as low birth weight, fetal growth restriction, stillbirth, and maternal anemia (Desai et al. 2007, Steketee et al. 2001). These outcomes primarily result from *P. falciparum* infection because this parasite can sequester iRBCs in the placenta and adhere to CSA (Smith and Deitsch 2004).

In Africa, where malaria transmission is stable, women can control placental infection once they have developed protective antibodies after one or two pregnancies. This immunity can protect the fetus as antibodies block the interaction of iRBCs expressing the surface protein VAR2CSA with CSA (Desai et al. 2007, Smith and Deitsch 2004). In previous studies, our group observed a high level of VAR2CSA antibodies in men and children from Latin America. Moreover, we showed that the human sera from this population also have functional antibodies that can block adhesion of the parasite to CSA *in vitro* (Gnidehou et al. 2014, Gnidehou et al. 2019).

The origin of the VAR2CSA antibodies in non-pregnant populations is still unknown. However, we showed that VAR2CSA antibodies can come from exposure to *P. vivax*, through the microneme protein PvDBP which shares a DBL domain similar to those in VAR2CSA (Gnidehou et al. 2019). We propose that this shared DBL domain is responsible for the observed cross-reactivity (Gnidehou et al. 2019). Our next step was to map the cross-reactive epitope in PvDBPII that was recognized by the monoclonal, 3D10, raised against PvDBPII. The epitope was mapped to the SD1 of this protein.

In Chapter II, we sequenced SD1 from PvDBPII and found 100% identity across Colombian isolates. Additionally, we described the serological characterization of non-pregnant populations against VAR2CSA, PvDBPII, and SD1 in Chapter II, and observed positive

correlations between the antibody reactivity against PvDBPII and VAR2CSA, and against SD1 and VAR2CSA. These findings point to SD1 as an important epitope mediating cross-species immune recognition of VAR2CSA.

The purpose of this Chapter is to affinity-purify PvDBPII and SD1 antibodies and test the functional activity of the affinity-purified antibodies. There are currently no suitable animal models of MiP in which to evaluate antibody function against placental malaria (Doritchamou et al. 2017). Instead, *in vitro* assays are used as correlates of function, to measure inhibition of iRBC binding to CSA by antibodies acquired naturally or following immunization (Doritchamou et al. 2017). One of the current methods is *the 96 well assay* which consists of a static assay where the iRBCs treated with hypoxanthine bind to CSA-coated wells. Importantly, in all functional assays which measure the binding inhibition to CSA, the test or control sera must be pre-incubated with the late stage trophozoites before the parasites are added to the coated CSA (Nielsen and Salanti 2015). This assay enables several plates to be run simultaneously. The washing is performed by a pipetting robot or an electronic pipette allowing the elimination of unbound cells by washes during this experiment. This reduces assay to assay variation and it is not influenced by person variation (Nielsen and Salanti 2015, Pehrson et al. 2017). After the washing the parasites are counted by liquid scintillations and the degree of the parasite binding is measure by the level of tritiated hypoxanthine in the parasite DNA (Pehrson et al. 2017, Nielsen and Salanti 2015). However, the 96 well assay is the most expensive method to implement due to the machines that are required for washing and reading (Nielsen and Salanti 2015). *The micro capillary assay or flow assay* consists of capillary tubes coated with CSA that binds to the iRBCs. A wall pressure, similar to a capillary blood vessel is applied to the capillary tubes using peristaltic pump. This method mimics the binding under physiological conditions in a small blood vessel. The binding inhibition is measure

by a software which counts the number of bound iRBCs and images recorded by a microscope connected to a video camera (Nielsen and Salanti 2015). Unfortunately, small blood clots or excessive adhesion in a channel reduce significantly the flow rate in that channel and affect the adjacent capillaries (Nielsen and Salanti 2015, Pehrson et al. 2017). A recent method is *the placental perfusion assay*, which tests the binding of iRBCs to placental tissue *in vivo*. This method allows to evaluate the natural parasite binding to intact human placental tissue under physiological flow conditions (Pehrson et al. 2017, Pehrson et al. 2016). Additionally, the intervillous space is intact and unfixed, that permits the adhesion of iRBCs to the syncytiotrophoblast and the intervillous space. The proportion of iRBCs in the maternal circulation is determined by flow cytometry (Pehrson et al. 2017, Pehrson et al. 2016). Some of the limitations reported for this method are the assay depends on the acquisition of placentas immediately after delivery and the binding inhibition appears to be a concentration dependent manner, because the binding inhibition increased as the serum concentration increased, this could be explain by the fact that in the placenta the density of CSA around the iRBCs is higher compared to the *in vitro* assays (Pehrson et al. 2016). The Petri dish assay or inhibition of binding assay (IBA) tests the erythrocyte binding on a plastic surface allowing maximal interaction between the cell and the receptor. The washing is performed manually; washing buffer is added to cover the bottom of the plate and gently rotated (manually or using a rocker). The counting can perform manually or using different software options. One limitation with this assay is that it is not possible to remove the entire volume of washing buffer, increasing the contamination of unbound cell in the binding spots. The Petri dish assay requires minimal equipment, can be easily applied across different laboratories and it does not require large sample amounts (Nielsen and Salanti 2015, Pehrson et al. 2017, Saveria, Duffy, and Fried 2015).

A major limitation for *in vitro* functional assays is the difficulty of translating the infected erythrocyte binding inhibition to the *in vivo* situation. A study that compared different binding assays found that even though they were using the same highly standardized parasite strain, the methods had a considerable inter and intra assay variation. From this experiment, the Petri dish and the flow assays showed a higher variation than the 96 well and the placental perfusion assays (Pehrson et al. 2017). The selection of the assay varies on the research needs.

In this Chapter, I used the Petri dish assay to measure the functional activity of affinity-purified SD1 and PvDBPII antibodies purified from Colombian sera.

3.2 Material and methods

3.2.1 Samples

In order to perform the affinity-purification, I pooled samples characterized in Chapter II. I selected 63 samples with the highest reactivity to PvDBPII and VAR2CSA to perform the PvDBPII affinity-purification (Table 3.2). I selected 49 samples with the highest reactivity to SD1 and VAR2CSA to perform the SD1 affinity-purification (Table 3.3). The same volume of sera was taken from each sample for a total pool of five mL; 80 µL and 115 µL from each individual serum were taken to make the PvDBPII pool and the SD1 pool, respectively. It should be noted that some samples were included in both the PvDBP and SD1 pools if they had high reactivity to PvDBPII, SD1 and VAR2CSA. It was necessary to work with pools of sera due to limited volume of the individual samples from our study.

Table 3.2 Selected samples for PvDBPII affinity-purification

Sample code	Sex	PvDBP AU	VAR2CSA AU
Y0404AEMM	M	14.348	35.678
Y0408HCSX	M	36.322	25.162
Y0409JJRA	M	81.413	91.797
Y0411MGGM	M	33.002	48.869
Y0412WJVP	M	41.951	23.732
Y0415MFTC	M	18.927	33.540
Y0418DAMM	M	33.712	25.846
Y0420CMCO	M	49.773	25.103
Y0433PAML	M	170.176	30.718
Y0436BRHN	M	129.180	58.049
Y0439LFGR	M	201.656	121.683
Y0440NJVH	M	58.144	47.740
Y0450EEST	M	36.742	86.828
Y0452JCSP	M	72.861	21.816
Y0458JEEN	M	30.816	27.328
Y0470NJUD	M	77.072	80.796
Y0471AAVM	M	140.976	27.511
Y0472JRHA	M	43.355	63.584
Y0474JCAJ	M	16.843	41.715

Table 3.3 Selected samples for SD1ss affinity-purification

Sample code	Sex	SD1 AU	VAR2CSA AU
Y0406LAOU	M	42.126	45.554
Y0408HCSX	M	25.162	41.345
Y0409JJRA	M	91.797	79.352
Y0411MGGM	M	48.869	69.415
Y0412WJVP	M	23.732	23.722
Y0418DAMM	M	25.846	37.186
Y0420CMCO	M	25.103	53.126
Y0434LMAB	M	21.300	33.001
Y0436BRHN	M	58.049	107.123
Y0439LFGR	M	121.683	155.118
Y0450EEST	M	86.828	36.617
Y0452JCSP	M	21.816	27.594
Y0472JRHA	M	63.584	23.050
Y0476JMMR	M	41.914	76.466
Y0494WASG	M	38.760	60.789
Y0497DJHC	M	32.888	32.221
Y0499WADQ	M	37.191	48.835
Y0501EMST	M	99.230	55.065
Y0504AGGS	M	73.717	84.825

Y0476JMMR	M	50.061	41.914		Y0507FJGC	M	57.574	15.785
Y0486NJAC	M	24.859	25.811		Y0511JLGP	M	47.150	50.102
Y0494WASG	M	142.728	38.760		Y0515JDPA	M	151.874	14.056
Y0497DJHC	M	47.956	32.888		Y0560GAMS	M	35.100	43.393
Y0499WADQ	M	51.652	37.191		Y0589JRHA	M	47.961	27.241
Y0501EMST	M	39.055	99.230		Y0595NJCS	M	86.159	95.925
Y0502IJMS	M	18.614	23.406		Y0605DECS	M	20.678	26.880
Y0504AGGS	M	76.138	73.717		Y0607CAFC	M	60.999	159.546
Y0507FJGC	M	20.151	57.574		Y0629WSPX	M	22.276	85.943
Y0511JLGP	M	51.870	47.150		Y0631MAPB	M	75.448	172.519
Y0538HSAX	M	19.900	19.903		Y0661AJZA	M	51.477	23.098
Y0552DLRS	M	22.613	25.915		Y0662JGAC	M	19.903	123.947
Y0560GAMS	M	31.066	35.100		Y0674JAVL	M	60.896	31.501
Y0589JRHA	M	28.726	47.961		Y0680IJDP	M	19.903	82.353
Y0595NJCS	M	65.426	86.159		Y0686RAFM	M	75.133	56.984
Y0607CAFC	M	91.460	60.999		Y0707MAOC	M	69.075	112.491
Y0608JMRO	M	23.493	23.298		Y0714FRCX	M	22.559	23.916
Y0624JAPH	M	23.522	21.937		Y0764MPGG	M	46.707	70.610
Y0629WSPX	M	64.362	22.276		Y0790AERS	M	70.315	110.610
Y0649JDMF	M	21.543	62.906		Y0798BMGX	M	31.469	69.624
Y0661AJZA	M	24.439	51.477		Y0803MEAC	M	30.167	26.391
Y0662JGAC	M	41.342	19.903		Y0806IEMA	M	41.434	24.431
Y0668LAGM	M	31.619	21.114		Y0816UUTX	M	26.680	156.992
Y0677LMUV	M	96.925	33.172		Y0817SCGH	M	19.994	33.496
Y0694YARV	M	18.735	33.705		Y0830FACP	M	28.255	158.171
Y0707MAOC	M	79.196	69.075		Y0859TGBX	M	21.151	30.407
Y0714FRCX	M	62.530	22.559		Y0882MIEP	M	22.460	23.258
Y0716RMAX	M	21.217	27.730		Y0891AJLX	M	22.030	77.932
Y0723EJCS	M	19.651	43.850		Y0895MMSP	M	111.388	44.582
Y0764MPGG	M	104.204	46.707		Y0909JPGS	M	87.566	90.867
Y0776JGPV	M	51.537	74.028					
Y0779RARB	M	22.488	20.829					
Y0789MJSS	M	22.840	29.122					
Y0790AERS	M	83.423	70.315					
Y0797TGSP	M	21.517	41.675					
Y0798BMGX	M	97.624	31.469					
Y0803MEAC	M	49.973	30.167					
Y0806IEMA	M	35.421	41.434					
Y0816UUTX	M	94.789	26.680					
Y0830FACP	M	84.611	28.255					
Y0837ASGG	M	23.650	46.350					
Y0859TGBX	M	23.083	21.151					
Y0891AJLX	M	34.547	22.030					

| Y0895MMSP | M | 29.145 | 111.388 |

3.2.2 Recombinant proteins and SD1 peptide

PfMSP1 protein was purchased commercially (CTK Biotech, USA). Dr. Ali Salanti provided the recombinant full-length VAR2CSA , and I produced recombinant PvDBPII as described in Chapter II. The SD1 peptide used for the affinity-purifications was different from the one used to perform the ELISAs in Chapter II. The SD1 sequence presented in Chapter II (ASNTVMKN**C**NYKRKRRERDWD**C**NTKKDV**C**IADRRYQL**C**MK) has four cysteines which form the following disulphide bonds: Cys 1 binds to Cys 4, and Cys 2 binds to Cys 3. The peptide used in this Chapter, which we named SD1ss, has Cys 1 and 4 mutated to serine (ASNTVMKN**S**NYKRKRRERDWD**C**NTKKDV**C**IADRRYQL**S**MK). The reason for this was to guarantee that the internal cysteines form the correct disulfide bounds which is predicted to be important for the epitope recognized by 3D10. Mass spectrophotometry results showed that the four cysteines from the synthetic SD1 peptide tended to form random bonds which could affect the antibodies purified against this peptide.

3.2.3 Affinity-purification

To affinity-purify PvDBPII antibodies, I loaded 1000 µL of NHS-activated Sepharose (GE Healthcare Life Sciences) onto a plastic column and centrifuged it for two minutes at 500 x g. Next, I washed the NHS-activated Sepharose with 13 column volumes (13 mL) of ice-cold 1 mM HCl. The buffer was allowed to pass through the beads by gravity flow. Immediately, I coupled one ml of recombinant PvDBPII at 1.67 mg/mL to the beads and incubated it overnight at 4°C with rocking. To deactivate any excess of active groups that were not coupled to the ligand, one to three column volumes (one to three mL) of 0.5 M ethanolamine, 0.5 M NaCl pH 8.3 were added

to the Sepharose and incubated for two hours at room temperature. I measured the absorbance by

Nanodrop and calculated the coupling efficiency by applying the following formula:

Loaded coupling solution, A 280 × mL:
$A = A_{280} \times V$
where
A 280 = A_{280} of coupling solution after PD-10 run
V = loaded volume of coupling solution
Amount not coupled, A_{280} × mL:
$B = A_{280} \times V \times 2$
where
A280 = A_{280} of coupling solution after PD-10 run
V = loaded volume of coupling solution
2 = dilution when acidified
Coupling yield, %: (A-B)/A × 100

To remove non-specifically bound ligands, I washed with three volumes (3 mL) of buffer

A (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.3) and three volumes (3 mL) of buffer B (0.1 M sodium

acetate, 0.5 M NaCl, pH 4); I repeated these steps five times, and left the Sepharose at room

temperature for 20 minutes after the third wash with buffer A. Finally, I added five mL of binding

buffer (1x PBS, pH 7) to the Sepharose.

I added three mL of elution buffer (0.1 M glycine, pH 2.3) to the Sepharose followed by

the addition of 15 column volumes (15 mL) of binding buffer to equilibrate the column. To prepare

the sample, I adjusted the five mL pool of human sera to the composition of the binding buffer by

diluting it 1:2 (total volume 10 mL). I incubated the sample 30 minutes with the Sepharose at room

temperature by rocking, and the flow through was saved for downstream analysis. The Sepharose

was washed with nine mL of binding buffer. I eluted the purified antibodies with five mL of 0.1

M glycine, pH 2.3 and added to five mL of 1 M Tris-HCl, pH 9 to neutralize the eluted antibodies.

After the elution, I re-equilibrated the Sepharose by washing with 15 mL of binding buffer. I

concentrated the purified antibodies, and performed buffer exchange using an Amicon concentrator (10 kDa cut-off). I tested all the fractions by ELISA.

I performed the affinity-purification of the SD1ss antibodies following the above protocol but instead of using NHS-activated Sepharose, I used a 1 mL HiTrap NHS-Activated HP column (GE Healthcare Life Sciences). I coupled one mg of SD1ss to the column. Additionally, I purified total IgG from the unexposed Medellin population using a HiTrap Protein G HP column (GE Healthcare Life Sciences) and I used this sample as a negative control. I concentrated the elution fractions from the IgG purification and buffer exchanged it with 1x PBS, pH 7. I concentrated the total IgG using an Amicon concentrator and quantified it on the Nanodrop. I stored the solution with 0.02% sodium azide to prevent any bacterial growth.

3.2.4 *P. falciparum* culture

I cultured *P. falciparum* CS2 strain (FCR3 allele for VAR2CSA) in human red blood cells, incubated the cultures at 37°C in RPMI medium enriched with AlbuMAX^{TM}. The parasites completed their erythrocytic cycle approximately every 48 hours. I selected the parasites on CSA (C9819; Sigma-Aldrich) to increase expression of VAR2CSA. I coated a Petri dish with 15 mL of sterile CSA at 100 µg/mL diluted in 1x PBS and incubated it overnight at 4°C. I washed the plate once with 10 mL of sterile 1x PBS and blocked it with 15 mL of sterile BSA in RPMI at 3%. Next, I incubated the plate for one hour at 37°C and washed it three times with 10 mL of sterile 1x PBS. I added mid-late trophozoite parasite culture in RPC (ideally with 10% of trophozoites) to the plate. I incubated the plate for one hour at 37°C resuspending the parasites every 15 minutes by gently swirling the plate. I gently washed the plate three times with 10 mL of sterile 1x PBS and I checked parasite binding to the plate under the microscope. Next, I added 10 mL of sterile CSA at 500 µg/mL diluted in 1x PBS and incubated it 10 minutes at 37°C. I resuspended all the

cells in the CSA solution and collected it in a 15 mL Falcon tube. I centrifuged the cells at 400 x g for five minutes. I started a fresh culture using the cell pellet, with fresh uRBCs added to the new culture to reach 3% of haematocrit.
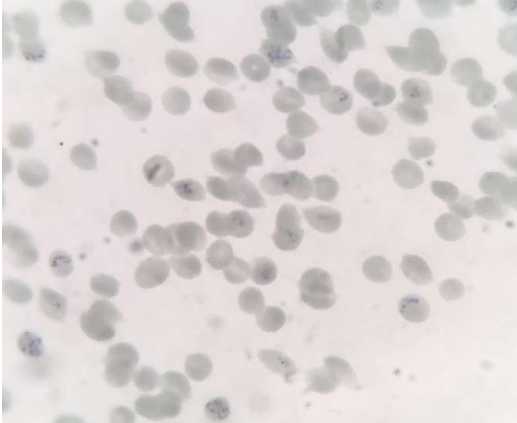
3.2.5 VarioMACS purification

I performed a sorbitol synchronization to eliminate the trophozoite and schizont stages and select the ring stage parasites. The purpose of this is to have the parasites at the same stage (Fig. 3.1A). For the synchronization, I centrifuged the parasite culture at 400 x g for five minutes. The volume of the cell pellet was estimated after aspirating the supernatant,. I added 5% sorbitol in a volume five times the pellet volume, and incubated it for five minutes at 37°C. Next, I filled the tube to 10 mL with RPMI and centrifuged for five minutes at 400 x g. Finally, I aspirated the supernatant, and started a new culture. I calculated the parasitemia of the cultures by dividing the iRBCs over 200 RBCs.

I purified late trophozoites from the culture using LD columns by VarioMACS (Miltenyil Biotec), following the manufacturer's instructions. After the magnet purification (Fig. 3.1B), I calculated the concentration of the cells in the pellet using a hemocytometer, counting five squares from the central square, taking the average of the cells and multiplying by 250,000 squares/mL. I calculated the purity by dividing the total iRBCs with trophozoites over the total number of cells on a thin smear stained with Giemsa. I conducted subsequent IBA experiments at 20% parasitemia. The percent parasitemia was adjusted using the following formula:

$$V_{iRBC} = \frac{(10 \times 10^6 \text{ cells/mL})(\text{volume of cells required})}{(\text{concentration of purified parasites})} \times \frac{(20\%)}{(\text{purity of parasites})}$$
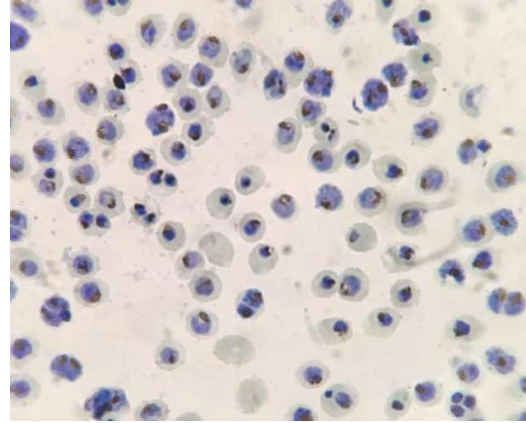
Figure 3.1 *P. falciparum* CS2 smears stained with Giemsa. (A) Ring stage parasites after sorbitol synchronization. (B) Purified trophozoites after a VarioMACS purification.

3.2.6 Inhibition of binding assay (IBA)

To test whether the PvDBPII and SD1ss purified antibodies were able to block the adhesion of iRBCs to CSA, I performed an inhibition of binding assay using 100 mm × 20 mm polystyrene Petri dishes (Falcon # 531029). I used a Petri dish-size piece of cardboard containing ten circles of 10 mm in diameter as a template to outline the spots on the back side of the plate. I only drawn the spots on half the plate, and each spot was separated in 18° increments around the perimeter of the dish (Fig. 3.2). The spots were designed to hold around 20 µL of liquid.

Figure 3.2 IBA plate design. Spots are drawn on half the plate, separated in 18° increments around the perimeter of the dish.

I spotted a 20 µL solution containing 50 µg/mL of CSA (diluted in sterile 1x PBS) onto the internal surface of the Petri dish within the edges of the drawn circles. I incubated the spots overnight at 4°C in a humidified chamber.

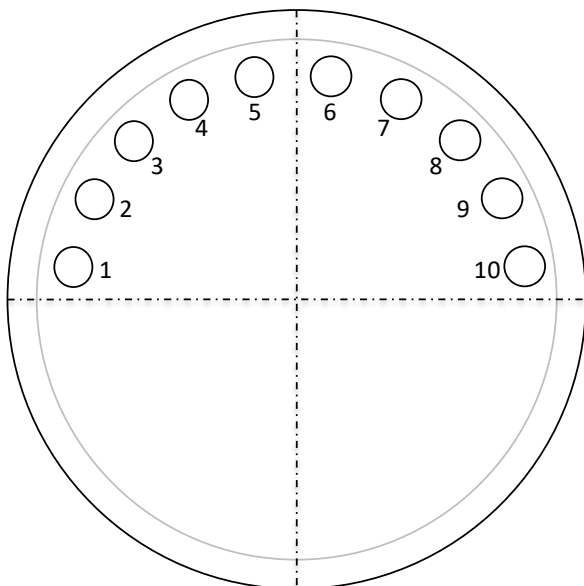After the overnight incubation of the spots with the CSA, I added 20 µL of 3% BSA/RPMI to each spot to block any non-specific cell attachment to the plate. I incubated the Petri dishes in a humidified chamber at 37°C for one hour. Next, I aliquoted 20 µL of parasites at 20% parasitemia in different 1.5 mL Eppendorf tubes (depending on the number of samples for testing). I centrifuged the cells at 400 x g for five minutes and aspirated the supernatant from the tubes. I resuspended the cell pellet in the proper dilution of antibody/serum in 1x PBS (see Table 3.1) and incubated the tubes at room temperature for 30 minutes.

Table 3.1 Dilution/concentrations of the different samples tested in the IBA

Sample	Dilution	Concentration
Controls		
Soluble CSA	-	200 µg/mL
Pre-immune rabbit sera	1:10	-
Immune anti-VAR2CSA rabbit sera	1:10	-
Test sera		
Pre-column serum pool	1:1.5	-
Flow-through serum pool	1:1.5	-
SD1ss purified antibodies	-	90 µg/mL
PvDBPII purified antibodies	-	100 µg/mL
IgG from unexposed population	-	90 µg/mL and 100 µg/mL

Next, I loaded 20 µL of the parasites plus sample mixtures onto each spot immediately after aspirating the 3% BSA/RPMI. I incubated the plates for 15 minutes at room temperature to allow parasites to bind to the CSA. To wash, I placed the plates on a 3D nutating rocker with the spots positioned at the top. Next, I added 16 mL of 1x PBS to the bottom of the plate without touching the spots. I started the rocker at the lowest speed for the machine and added an additional 9 mL of 1x PBS to the plates while the solution started to rotate. I slowly increased the speed of the rocker. After the wash, I aspirated the 1x PBS from the plate and slowly added 10 mL of 1.5% glutaraldehyde to the plates to fix the cells. I incubated the plates for 10 minutes at room temperature.

Once the cells were fixed to the plate, I stained them with 10 mL of 5% Giemsa for five minutes at room temperature. I washed the plates twice with 10 mL of distilled water. I left the plates upside down on the counter overnight making sure to lay them half on the lid so that the excess water drained to the end where there were no spots.

I imaged the spots using an EVOS FL Auto microscope (Invitrogen) using the 4x objective. I quantified the iRBCs that bound to CSA in each spot using ImageJ (FIJI). To calculate the percentage of inhibition, I divided the number of parasites bound per spot after incubation with the test antibodies, sera or IgG by the number of parasites bound in the negative control. I determined the inter-assay variation of FCR3-CSA with the mean binding on each plate to calculate a CV.

3.2.7 Statistical analysis

We used the Student's test (a=0.05; unpaired) to determine if the parasite binding inhibition was significantly different comparing the pre-column sera and post-column sera (flow through), and the affinity-purified antibodies (PvDBPII-specific IgG and SD1ss-specific IgG) compared to

total IgG from the unexposed group. I performed statistical analysis using Prism software (version 7; GraphPad).

3.3 Results

3.3.1 Affinity-purification

To test whether PvDBPII and SD1ss antibodies cross-react with VAR2CSA, we affinity-purified PvDBPII IgG and SD1ss IgG from pooled sera from men and children from Colombia that have a high level of antibodies to these three proteins.

3.3.1.1 PvDBPII affinity-purification

After coupling 1.67 mg of protein in the resign, the coupling efficiency was 92%, applying the formula mentioned previously. During the elution process, I added 5 mL of elution buffer to the Sepharose, and one drop was eluted sequentially from the column and collected into 50 µL of Tris-HCl to neutralize the antibodies. I collected around 100 fractions. I measured those fractions using the Nanodrop and I pooled them into 5 pools based on their concentration. Fractions 14 to 40 were collected in pool one; fractions 41 to 60 were collected in pool two, fractions 61 to 81 were collected in pool three, fractions 82 to 95 were collected in pool four and the remaining fractions were collected in pool five; I concentrated all pools, and buffer exchanged them using an Amicon column. I measured the concentration once more (Figure 3.3A), and pooled fractions one to three again (named hereafter as pool fraction one) and pooled fractions four and five (named hereafter as pool fraction two). The final concentration for the fraction pool one was 0.217 mg/mL and for fraction pool two was 88 µg/mL. I measured the reactivity of the different fractions (pre-column pool of sera, flow through, washes and elution fractions) against PvDBPII by ELISA (Fig. 3.3B). There was a slight reduction in the reactivity of the flow-through, consistent with partial depletion of these antibodies from the starting pool. Non-specific antibodies were washed from the column, as the concentration and reactivity decreased across the different washes (Figs. 3.3A-B). I eluted the PvDBPII affinity-purified antibodies from the column and tested them for reactivity

to PvDBPII, PfMSP1 and VAR2CSA (Fig. 3.3C). I used PfMSP1 to test the specificity of the

affinity-purified antibodies. The purified antibodies were negative against PfMSP1 and positive

against PvDBPII and VAR2CSA, showing that these antibodies are specific and cross-react with

VAR2CSA.

Figure 3.3 PvDBPII purified antibodies cross-react with VAR2CSA. (A) Different fractions from the PvDBPII affinity-purification, showing a slight decrease of the flow through concentration compare to the pre-column sera. The concentration of the non-specific antibodies decreased with more column washes. (B) ELISA results of the fractions showing a small depletion of the PvDBPII antibodies in the flow through compared to starting sera. There was no significant reactivity of the washes against PvDBPII. Eluted fraction 1 reacts to PvDBPII. (C) PvDBPII antibodies eluted from the column have reactivity to PvDBPII and VAR2CSA but not to PfMSP1 by ELISA. The antibodies were tested at 5 µg/mL.

3.3.1.2 SD1ss affinity-purification

The coupling efficiency for the SD1ss affinity-purification was 98%. I added 5 mL of elution to the HiTrap NHS-Activated HP column and I collected every eluted drop into 50 μL of Tris-HCl. I measured the concentration of collected fractions (around 100 fractions) using the Nanodrop and pooled them into four pools based on their concentrations; every pool contained around 25 fractions. The sample concentration was measured after concentration and buffer exchange of the pooled elution fractions (Fig. 3.4A); the final concentration for fraction pool one was 90 μg/mL. I measured the reactivity to SD1ss of the different fractions by ELISA. As in the PvDBPII affinity-purification, there was a slight reduction in the reactivity of the flow-through. Non-specific antibodies were removed during the washing (Fig. 3.5B) where the concentration and reactivity to SD1ss of the washes dropped to zero. Finally, the SD1ss purified antibodies were positive against SD1ss and VAR2CSA, but negative against PfMSP1 (Figure 3.5C).

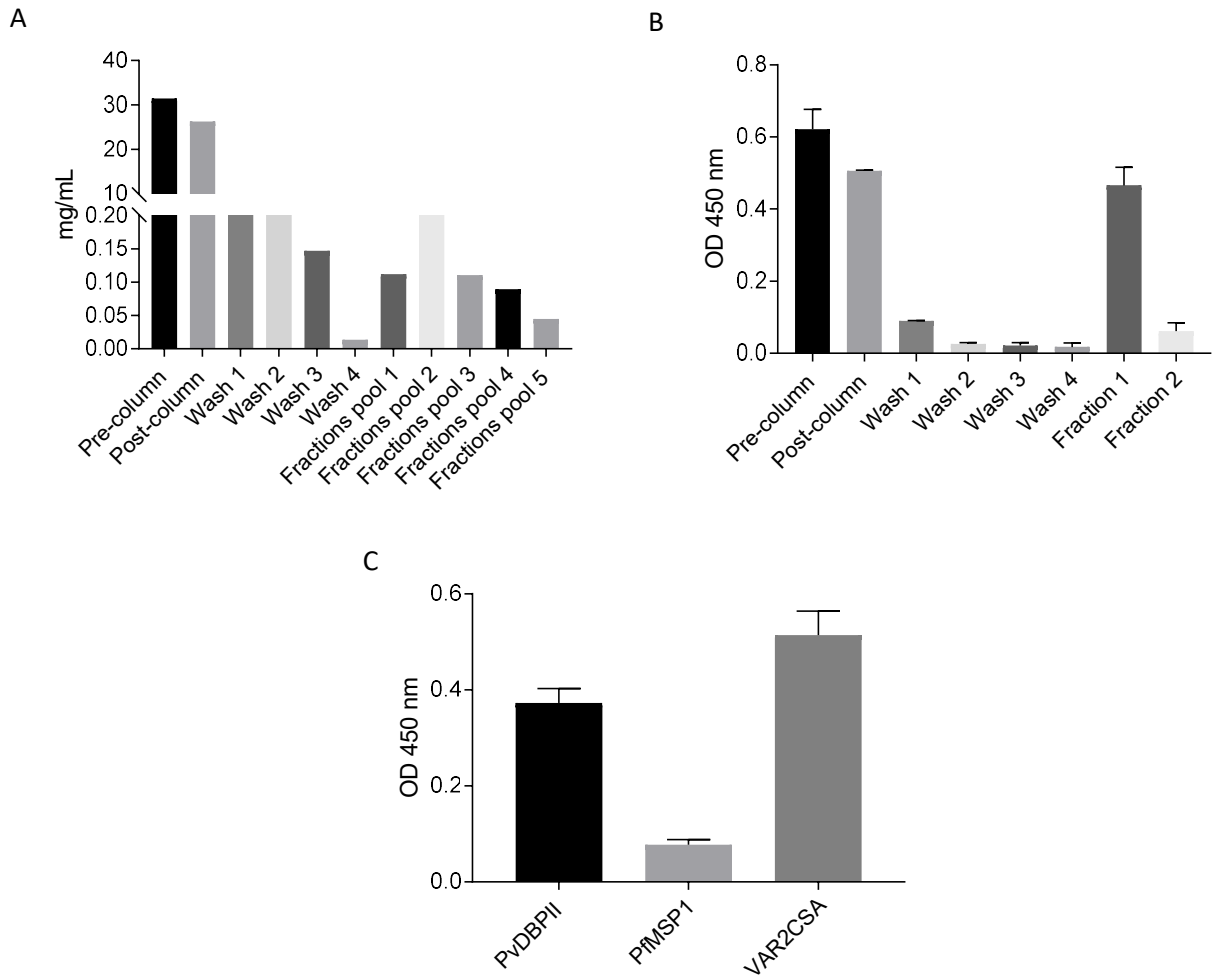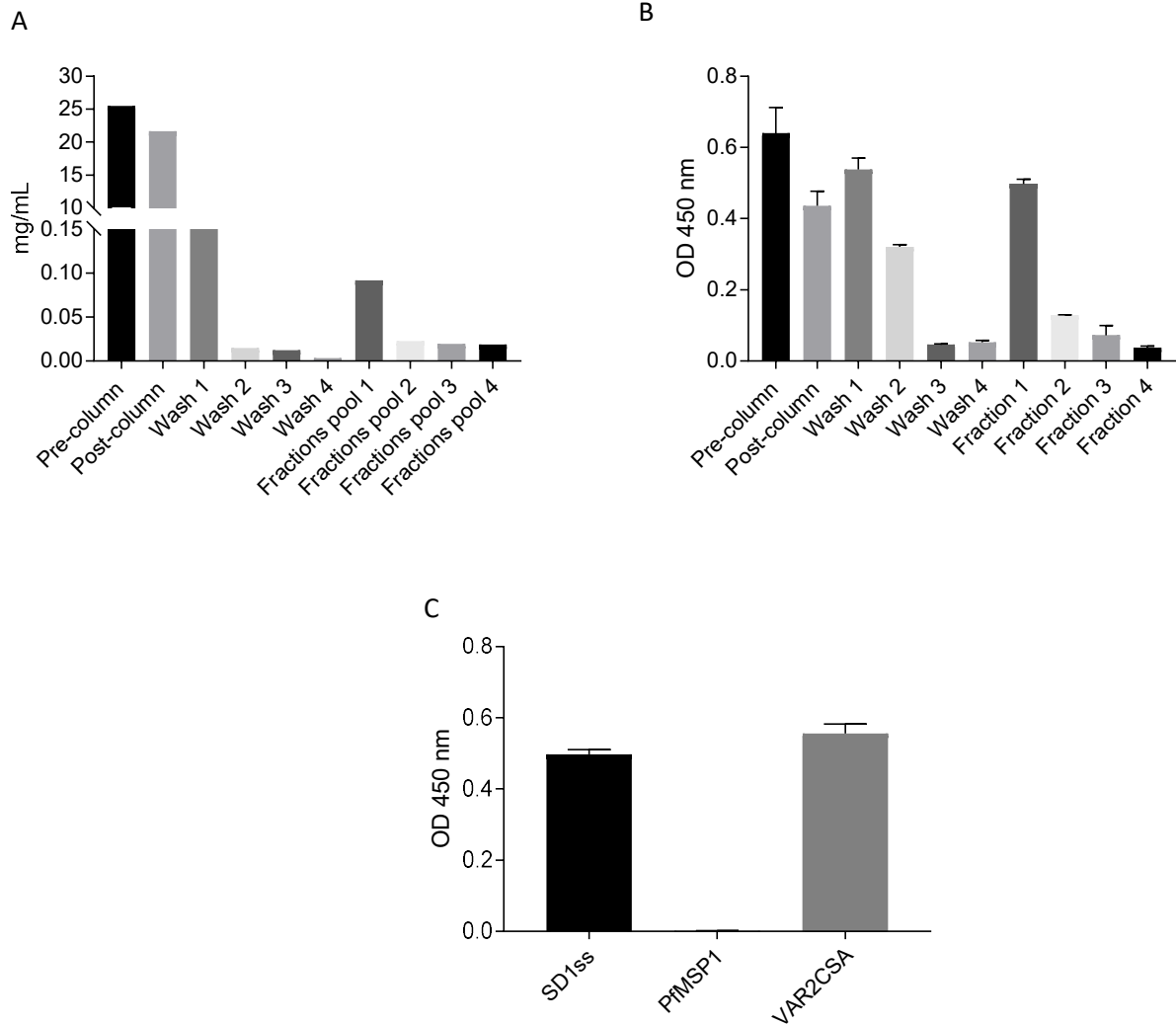**Figure 3.4 SD1ss purified antibodies cross-react with VAR2CSA.** (A) Different fractions from the SD1ss affinity-purification, showing a slight decrease of the flow through concentration compare to the pre-column sera. The concentration of the non-specific antibodies decreased with more column washes. (B) ELISA results of the fractions showing a small depletion of the SD1ss antibodies in the flow through compared to starting sera. There was no significant reactivity of the washes against SD1ss. Eluted fraction 1 reacts to SD1ss. (C) SD1ss antibodies eluted from the column have reactivity to SD1ss and VAR2CSA but not to PfMSP1 by ELISA The antibodies were tested at 5 µg/mL.

3.3.2 Inhibition of binding assay (IBA)

Finally, we were interested to know if the PvDBPII and SD1ss affinity-purified antibodies can block the interaction between VAR2CSA and CSA using the CS2 parasite strain. I used soluble CSA, pre-immune rabbit serum, and immune anti-VAR2CSA rabbit serum as controls to measure the inhibition of binding (Fig. 3.5A) per trial. I used total IgG from the unexposed Medellin group to compare the binding inhibition of the purified human antibodies.

The following results are from three different trials, each performed on a different day. Every trial included two plates with the controls, two plates with the sera pools and two plates with the PvDBPII and SD1ss affinity-purified antibodies; all samples were run in duplicate.

For the PvDBPII serum pools, the pre-column sera did not show significant binding inhibition relative to the post-column sera (flow through) in the first two trials but there was binding inhibition of 23% in the third trial, showing statistical significance (Figure 3.5B). The overall results for the human sera show that the antibodies in the pre-column sera did not effectively block the adhesion of the iRBC to CSA. The PvDBPII purified antibodies did not show significant binding inhibition across the three trials (Figure 3.5C).
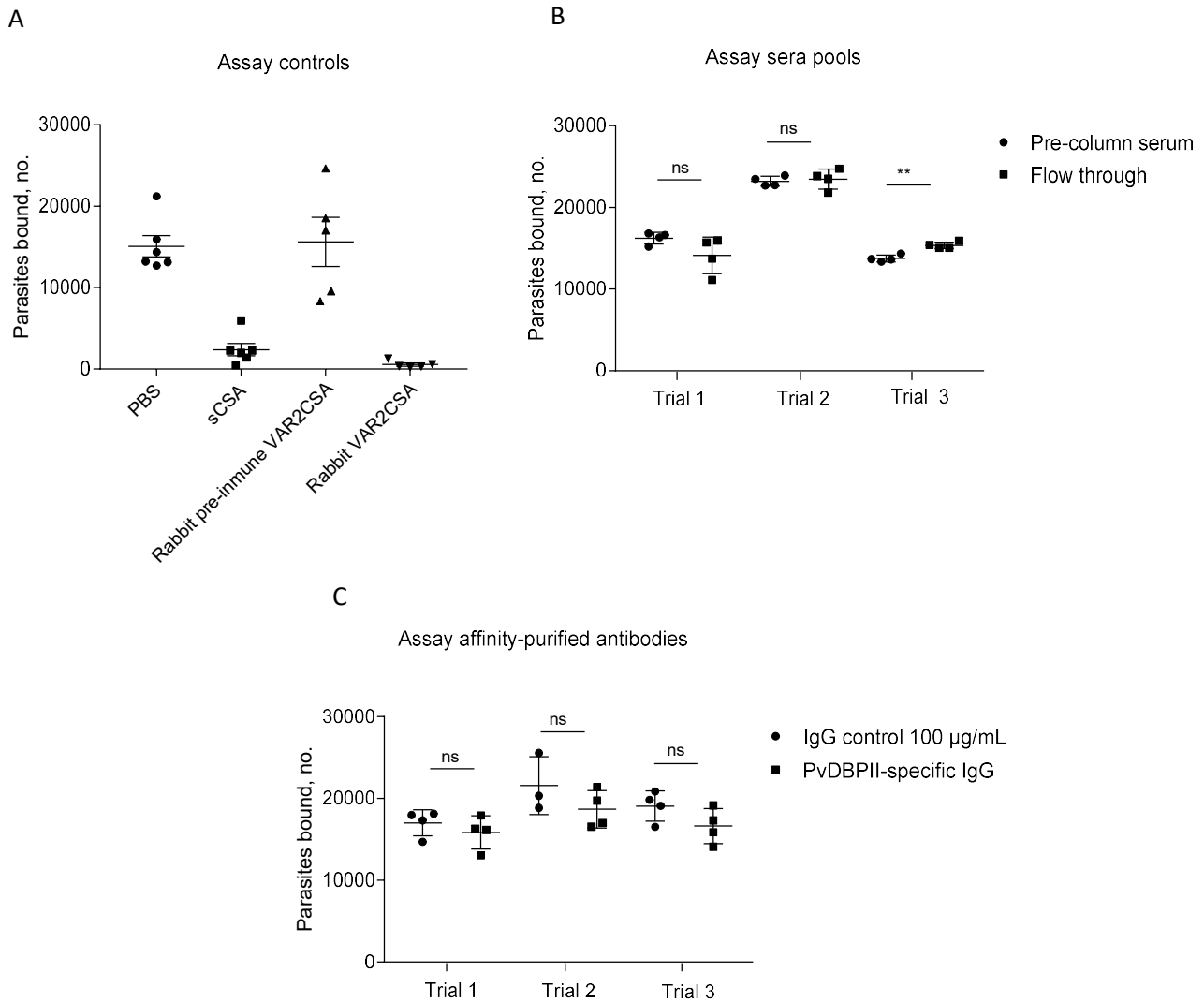
Figure 3.5 PvDBPII affinity-purified antibodies. (A) Controls for the inhibition of binding assay included *P. falciparum* strain-CS2 iRBCs incubated with PBS alone, soluble CSA and pre-immune and immune VAR2CSA rabbit sera. (B) Pre-column sera and flow-through from PvDBPII affinity-purifications did not show a significant binding inhibition. (C) PvDBPII affinity-purified antibodies and IgG from unexposed Colombians. Results are expressed as the number of parasites bound to CSA. There was not a significant inhibition of the affinity-purified antibodies.

For SD1ss, the results with the pre-column sera and flow through were similar to those seen for PvDBPII (Figure 3.6A). There was no significant binding inhibition for the two first trials, but there was significant binding inhibition for the third trial.

Unlike the results for PvDBPII affinity-purified antibodies, we observed an overall 28% binding inhibition of the SD1ss purified antibodies compared to unexposed total IgG across six plates run in duplicate (Figure 3.6B). The data point to SD1ss as the key epitope in PvDBPII responsible for the cross-reactivity with VAR2CSA. Although most plates had significant results, one plate in the second trial showed a non-significant inhibition (12.3%).

The immune anti-VAR2CSA rabbit sera and the soluble CSA showed high binding inhibition, 90% and 70% respectively, across the three trials.

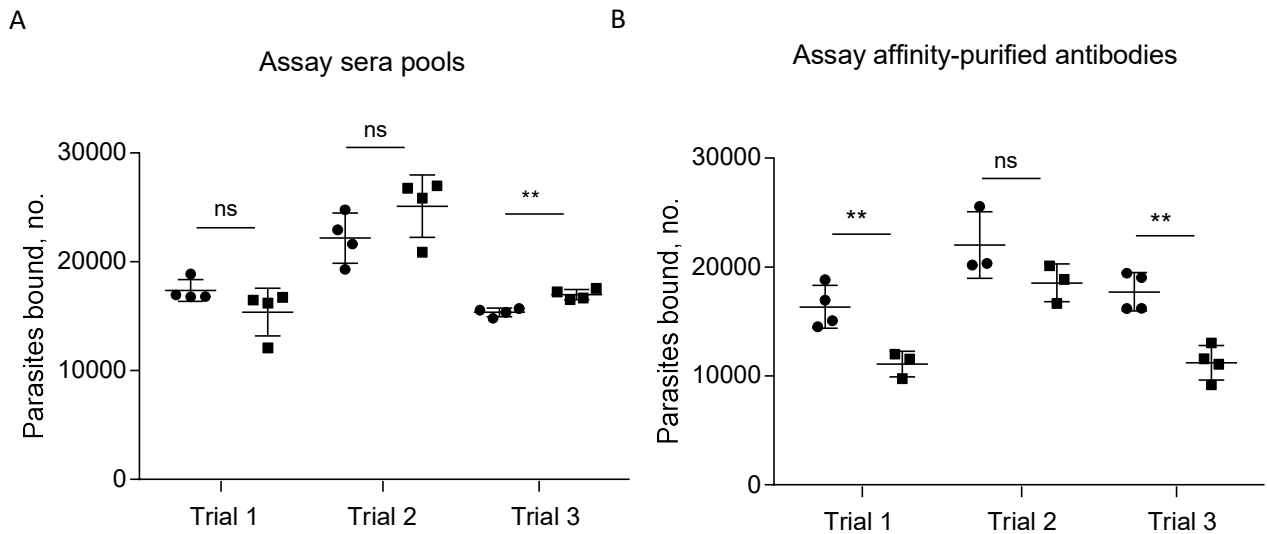**Figure 3.6 Affinity-purified antibodies of the subdomain 1 of PvDBPII block the adhesion of VAR2CSA-expressing iRBCs to CSA.** (A) Pre-column sera (●) and flow through (■) from the SD1ss affinity-purification. (B) SD1ss affinity-purified antibodies (■) and IgG from unexposed Colombians (●). Results are expressed as the number of parasites bound to CSA.

3.4 Conclusions

PvDBP and VAR2CSA are two non-orthologous proteins from different *Plasmodium* species involved in different pathological pathways: invasion and cytoadherence, respectively. However, they share structurally similar DBL domains. In this Chapter, I tested the hypothesis that the cross-reactive protective VAR2CSA antibodies in non-pregnant populations were coming from exposure to PvDBP and more specifically, the SD1 epitope within PvDBP. I tested this by affinity-purifying human PvDBPII and SD1 antibodies from pooled sera. Antibodies that I purified against either recognized VAR2CSA by ELISA, giving us strong evidence of cross-species reactivity coming from PvDBP. This is consistent with our data on the mouse mAb (3D10) that was raised against the DBL domain of PvDBP and recognized VAR2CSA (Gnidehou et al. 2019).

Comparing the IBA results for the PvDBPII and SD1 affinity-purified antibodies, I did not observe significant inhibition by the PvDBPII antibodies, whereas the percentage of inhibition by the SD1ss antibodies was 28%. One possible explanation for this is that SD1 represents a small region of the PvDBPII protein, and the proportion of antibodies to this specific epitope is low. Therefore most of the PvDBPII antibodies are directed at the immunodominant epitopes within the subdomains 2 and 3 (Chootong et al. 2010). However, in the SD1 affinity-purification, the antibodies are against the SD1 epitope, showing important binding inhibition in most of the trials. In this Chapter, I expand on this and theorize the potential origin of the protective VAR2CSA antibodies, identifying SD1 as the potential epitope responsible for the cross-reactivity with VAR2CSA.

Chapter 4: Discussion

In this research I explored the hypothesis that functional VAR2CSA-specific antibodies are acquired outside of pregnancy and originated from the exposure to the DBL antigens from *P. vivax*, PvDBPII and EBP2.

Most studies in Africa (where *P. falciparum* is the predominant parasite) associate MiP with a wide range of clinical manifestations which include maternal and infant death (Desai et al. 2007, Smith and Deitsch 2004, Rogerson et al. 2003, Duffy and Fried 2003). Opposite to the findings from Africa, in a previous study from our lab conducted in Colombia, we did not observe negative birth outcomes associated with malaria infection (Gavina et al. 2018). Most African studies have shown that acquisition of protective antibodies against VAR2CSA are restricted to multigravid women and that non-pregnant populations do not have high levels of these antibodies. Except for some studies in which a low frequency of these antibodies was found in African children, teenagers and men (Beeson et al. 2007, Fodjo et al. 2016). In Colombia, we found high levels (over 50%) of antibodies that recognized different domains of VAR2CSA in primigravid and non-pregnant Colombian populations with either exposure to *P. falciparum* or *P. vivax* (Gnidehou et al. 2014). We showed that men and children from Colombian and Brazil exposed only to *P. vivax* had antibodies that cross-reacted with VAR2CSA and blocked parasite adhesion to CSA *in vitro* (Gnidehou et al. 2019). Our findings demonstrated for the first time the cross-species functional immune recognition between two antigens from two divergent species of *Plasmodia*.

4.1 Cross-reactivity between *P. falciparum* and *P. vivax* species

The cross-reactivity between antigens from *P. falciparum* and *P. vivax* has being increasingly reported. Several antigens from *Plasmodium* species have partial sequence and

functional conservation, and this similarity could be the cause for cross-reactive immune responses that could lead to protection between species. For example, the apical membrane antigen 1 from *P. falciparum* and *P. vivax* had highly cross reactivity between them (Igonet et al. 2007), similar to PfMSP5 and PvMSP5, where people infected by *P. falciparum* had antibodies against *P. vivax* (Good et al. 2013). Antibodies from mice that were immunized with PfCLAG9 recognized iRBCs from *P. falciparum* and *P. vivax*. Additionally, sera from patients exposed to *P. vivax* recognized these peptides (Costa et al. 2013). Finally cross-boosting was shown between Pv48/45 and Pf48/45 in mice (Cao et al. 2016). However, our work is the first to identify an epitope in a *P. vivax* antigen (PvDBPII) responsible for functional immunity in placental malaria. The protective role of PvDBP antibodies is consistent with previous work (Requena et al. 2017) that naturally acquired binding-inhibitory antibodies to PvDBP from pregnant women from Brazil, Colombia, Guatemala, India, and Papua New Guinea (countries where *P. vivax* and *P. falciparum* co-exist) correlated with higher birth weight during MiP.

4.2 EBP2 as a possible source of cross-reactive antibodies

EBP2 posses the primary structure of members from the EBL superfamily, such as PvDBP, and the subdomain 1 from PvDBP and EBP2 are structurally similar (Hester et al. 2013a). This raised the question whether the cross-reactivity with VAR2CSA was mediated by both PvDBP and EBP2. A recent study showed that even though the two proteins are structurally similar, they are phylogenetically distant (suggesting they did not arise from a recent duplication event), and the genes are not similar (Ntumngia et al. 2016). The authors suggest EBP2 as an alternative ligand for invasion of reticulocytes when the main ligand, PvDBP is blocked by immune antibody (Ntumngia et al. 2016). In this research, I did not observe a correlation between SD1 antibodies from PvDBPII and antibodies to EBP2.

4.3 Limitations

Some of the limitations of my research are the sample size for the outbreak sample is very small and is not sufficient to draw solid conclusions. The volume of the individual samples from the Colombian cohort was not enough to perform individual affinity-purifications, that would allow more detailed characterization of the affinity-purified antibodies. Additionally, there was the technical challenge of getting purified antibodies that are in a very low concentration in the sera. Finally, the Petri dish IBA also represents a limitation in this research since intra and inter-assay variability is very high, and the method does not mimic the physiological conditions in which an infected erythrocyte binds to the placenta. However, due to the small concentration and volume obtained of affinity-purified antibodies, the IBA was a suitable option for the research, and yielded significant results.

4.4 Future directions

A future area of study stemming from this work is to determine whether cross-reactivity to these same antigens and the SD1 epitope occurs in populations from other regions endemic for both *P. falciparum* and *P. vivax*. The experiments presented in this thesis could be repeated with sera from men and children from Asia where *P. falciparum* and *P. vivax* co-circulate to evaluate the antibody levels of VAR2CSA and test for inhibition of adhesion. If functional VAR2CSA-specific antibodies are present in the non-pregnant population, the seroreactivity would be measured against PvDBPII, SD1 and EBP2. As described here, affinity-purification would allow us to test the function of these specific antibodies in the IBA.

Another important area of study is to determine whether the protective antibodies against VAR2CSA that arise from SD1 are directly associated with positive birth and pregnancy

outcomes. Additionally, the results from this work provide more evidence to develop SD1 as a cross-species vaccine candidate that can protect women against placental malaria.

4.5 Concluding remarks

In this study I performed a serological characterization of non-pregnant populations from Colombia (men, children, and women) and Brazil (men and children), finding high levels of antibodies against PvDBPII, SD1, VAR2CSA and EBP2. Despite the structural similarity with PvDBPII and the high level of antibodies against EBP2 in our study population, I did not find positive correlations at the antibody levels with VAR2CSA, excluding EBP2 as one source for the protective antibodies against VAR2CSA. To have more evidence that EBP2 is not involved in the cross-reactivity, EBP2 affinity-purified antibodies must be tested against VAR2CSA. I hypothesize that these antibodies would not have high reactivity to VAR2CSA.

PvDBPII and SD1ss affinity-purified antibodies recognized VAR2CSA by ELISA, showing cross-reactivity between PvDBPII, SD1 and VAR2CSA, and the purified antibodies were negative against PfMSP1, proving their specificity. More importantly, I showed that SD1 purified antibodies are functional because they can block the adhesion of iRBCs to CSA.

In conclusion, I identified SD1 as a key epitope from PvDBPII responsible for the protective antibodies against VAR2CSA. The findings from this work can be translated into a vaccine candidate against *P. falciparum* placental malaria, that mimics the natural protection observed in our Latin American populations.

References

Adams, J. H., B. K. Sim, S. A. Dolan, X. Fang, D. C. Kaslow, and L. H. Miller. 1992. "A family of erythrocyte binding proteins of malaria parasites." *Proc Natl Acad Sci U S A* 89 (15):7085-9.

Adegnika, A. A., J. J. Verweij, S. T. Agnandji, S. K. Chai, L. P. Breitling, M. Ramharter, M. Frolich, S. Issifou, P. G. Kremsner, and M. Yazdanbakhsh. 2006. "Microscopic and sub-microscopic Plasmodium falciparum infection, but not inflammation caused by infection, is associated with low birth weight." *Am J Trop Med Hyg* 75 (5):798-803.

Agboghoroma, C. O. 2014. "Current management and prevention of malaria in pregnancy: a review." *West Afr J Med* 33 (2):91-9.

Agudelo-García, O. M., E. M. Arango-Flórez, and J. Carmona-Fonseca. 2017. "Submicroscopic and Asymptomatic Congenital Infection by." *J Trop Med* 2017:3680758. doi: 10.1155/2017/3680758.

Arai, M., O. Billker, H. R. Morris, M. Panico, M. Delcroix, D. Dixon, S. V. Ley, and R. E. Sinden. 2001. "Both mosquito-derived xanthurenic acid and a host blood-derived factor regulate gametogenesis of Plasmodium in the midgut of the mosquito." *Mol Biochem Parasitol* 116 (1):17-24.

Arango, E., A. Maestre, and J. Carmona-Fonseca. 2010. "[Effect of submicroscopic or polyclonal Plasmodium falciparum infection on mother and gestation product: systematic review]." *Rev Bras Epidemiol* 13 (3):373-86.

Ataide, R., A. Mayor, and S. J. Rogerson. 2014. "Malaria, primigravidae, and antibodies: knowledge gained and future perspectives." *Trends Parasitol* 30 (2):85-94. doi: 10.1016/j.pt.2013.12.007.

Ataide, R., O. Murillo, J. G. Dombrowski, R. M. Souza, F. A. Lima, G. F. Lima, A. D. Hristov, S. C. Valle, S. M. Di Santi, S. Epiphanio, and C. R. Marinho. 2015. "Malaria in Pregnancy Interacts with and Alters the Angiogenic Profiles of the Placenta." *PLoS Negl Trop Dis* 9 (6):e0003824. doi: 10.1371/journal.pntd.0003824.

Babakhanyan, A., R. Fang, A. Wey, A. Salanti, G. Sama, C. Efundem, R. J. Leke, J. J. Chen, R. G. Leke, and D. W. Taylor. 2015. "Comparison of the specificity of antibodies to VAR2CSA in Cameroonian multigravidae with and without placental malaria: a retrospective case-control study." *Malar J* 14:480. doi: 10.1186/s12936-015-1023-6.

Batchelor, J. D., J. A. Zahm, and N. H. Tolia. 2011. "Dimerization of Plasmodium vivax DBP is induced upon receptor binding and drives recognition of DARC." *Nat Struct Mol Biol* 18 (8):908-14. doi: 10.1038/nsmb.2088.

Beeson, J. G., F. Ndungu, K. E. Persson, J. M. Chesson, G. L. Kelly, S. Uyoga, S. L. Hallamore, T. N. Williams, J. C. Reeder, G. V. Brown, and K. Marsh. 2007. "Antibodies among men and children to placental-binding Plasmodium falciparum-infected erythrocytes that express var2csa." *Am J Trop Med Hyg* 77 (1):22-8.

Beeson, J. G., S. J. Rogerson, B. M. Cooke, J. C. Reeder, W. Chai, A. M. Lawson, M. E. Molyneux, and G. V. Brown. 2000. "Adhesion of Plasmodium falciparum-infected erythrocytes to hyaluronic acid in placental malaria." *Nat Med* 6 (1):86-90. doi: 10.1038/71582.

Beri, D., B. Balan, and U. Tatu. 2018. "Commit, hide and escape: the story of Plasmodium gametocytes." *Parasitology*:1-11. doi: 10.1017/S0031182018000926.

Boeuf, P., A. Tan, C. Romagosa, J. Radford, V. Mwapasa, M. E. Molyneux, S. R. Meshnick, N. H. Hunt, and S. J. Rogerson. 2008. "Placental hypoxia during placental malaria." *J Infect Dis* 197 (5):757-65. doi: 10.1086/526521.

Botella Llusiá, J. 1992. *La Placenta. Fisiología y Patología.* Vol. 1. Madrid-España.

Cao, Y., G. P. Bansal, K. Merino, and N. Kumar. 2016. "Immunological Cross-Reactivity between Malaria Vaccine Target Antigen P48/45 in Plasmodium vivax and P. falciparum and Cross-Boosting of Immune Responses." *PLoS One* 11 (7):e0158212. doi: 10.1371/journal.pone.0158212.

Carmona-Fonseca, J., E. Arango, and A. Maestre. 2013. "Placental malaria in Colombia: histopathologic findings in Plasmodium vivax and P. falciparum infections." *Am J Trop Med Hyg* 88 (6):1093-101. doi: 10.4269/ajtmh.12-0363.

Carmona-Fonseca, Jaime., and Amanda. Maestre. 2009. "Incidencia de las malarias gestacional, congénita y placentaria en Urabá (Antioquia, Colombia), 2005-2007." *Revista Colombiana de Obstetricia y Ginecología* 60:19-33.

Carvalho, B. O., S. C. Lopes, P. A. Nogueira, P. P. Orlandi, D. Y. Bargieri, Y. C. Blanco, R. Mamoni, J. A. Leite, M. M. Rodrigues, I. S. Soares, T. R. Oliveira, G. Wunderlich, M. V. Lacerda, H. A. del Portillo, M. O. Araujo, B. Russell, R. Suwanarusk, G. Snounou, L. Renia, and F. T. Costa. 2010. "On the cytoadhesion of Plasmodium vivax-infected erythrocytes." *J Infect Dis* 202 (4):638-47. doi: 10.1086/654815.

Carvalho, B. O., J. S. Matsuda, S. L. Luz, F. E. Martinez-Espinosa, J. A. Leite, F. Franzin, P. P. Orlandi, G. B. Gregoracci, M. V. Lacerda, P. A. Nogueira, and F. T. Costa. 2011. "Gestational malaria associated to Plasmodium vivax and Plasmodium falciparum placental mixed-infection followed by foetal loss: a case report from an unstable transmission area in Brazil." *Malar J* 10:178. doi: 10.1186/1475-2875-10-178.

Cavasini, C. E., L. C. Mattos, A. A. Couto, C. R. Bonini-Domingos, S. H. Valencia, W. C. Neiras, R. T. Alves, A. R. Rossit, L. Castilho, and R. L. Machado. 2007. "Plasmodium vivax infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception?" *Trans R Soc Trop Med Hyg* 101 (10):1042-4. doi: 10.1016/j.trstmh.2007.04.011.

Ceravolo, I. P., B. A. Sanchez, T. N. Sousa, B. M. Guerra, I. S. Soares, E. M. Braga, A. M. McHenry, J. H. Adams, C. F. Brito, and L. H. Carvalho. 2009. "Naturally acquired inhibitory antibodies to Plasmodium vivax Duffy binding protein are short-lived and allele-specific following a single malaria infection." *Clin Exp Immunol* 156 (3):502-10. doi: 10.1111/j.1365-2249.2009.03931.x.

Chan, E. R., D. Menard, P. H. David, A. Ratsimbasoa, S. Kim, P. Chim, C. Do, B. Witkowski, O. Mercereau-Puijalon, P. A. Zimmerman, and D. Serre. 2012. "Whole genome sequencing of field isolates provides robust characterization of genetic diversity in Plasmodium vivax." *PLoS Negl Trop Dis* 6 (9):e1811. doi: 10.1371/journal.pntd.0001811.

Chen, E., N. D. Salinas, Y. Huang, F. Ntumngia, M. D. Plasencia, M. L. Gross, J. H. Adams, and N. H. Tolia. 2016. "Broadly neutralizing epitopes in the Plasmodium vivax vaccine candidate Duffy Binding Protein." *Proc Natl Acad Sci U S A* 113 (22):6277-82. doi: 10.1073/pnas.1600488113.

Chen, Q., V. Fernandez, A. Sundström, M. Schlichtherle, S. Datta, P. Hagblom, and M. Wahlgren. 1998. "Developmental selection of var gene expression in Plasmodium falciparum." *Nature* 394 (6691):392-5. doi: 10.1038/28660.

Cheng, J. Cunningham, and M. L. Gatton. 2015. "Systematic review of sub-microscopic P. vivax infections: prevalence and determining factors." *PLoS Negl Trop Dis* 9 (1):e3413. doi: 10.1371/journal.pntd.0003413.

Cheng, Y., Y. Wang, D. Ito, D. H. Kong, K. S. Ha, J. H. Chen, F. Lu, J. Li, B. Wang, E. Takashima, J. Sattabongkot, T. Tsuboi, and E. T. Han. 2013. "The Plasmodium vivax merozoite surface protein 1 paralog is a novel erythrocyte-binding ligand of P. vivax." *Infect Immun* 81 (5):1585-95. doi: 10.1128/IAI.01117-12.

Choe, H., M. J. Moore, C. M. Owens, P. L. Wright, N. Vasilieva, W. Li, A. P. Singh, R. Shakri, C. E. Chitnis, and M. Farzan. 2005. "Sulphated tyrosines mediate association of chemokines and Plasmodium vivax Duffy binding protein with the Duffy antigen/receptor for chemokines (DARC)." *Mol Microbiol* 55 (5):1413-22. doi: 10.1111/j.1365-2958.2004.04478.x.

Chootong, P., F. B. Ntumngia, K. M. VanBuskirk, J. Xainli, J. L. Cole-Tobian, C. O. Campbell, T. S. Fraser, C. L. King, and J. H. Adams. 2010. "Mapping Epitopes of the Plasmodium vivax Duffy Binding Protein with Naturally Acquired Inhibitory Antibodies ▽ †¶." In *Infect Immun*, 1089-95.

Chêne, Arnaud, Sophie Houard, Morten A. Nielsen, Sophia Hundt, Flavia D'Alessio, Sodiomon B. Sirima, Adrian J. F. Luty, Patrick Duffy, Odile Leroy, Benoit Gamain, and Nicola K. Viebig.

2016. "Clinical development of placental malaria vaccines and immunoassays harmonization: a workshop report." *Malaria Journal* 15 (1):476. doi: doi:10.1186/s12936-016-1527-8.

Cohee, L. M., L. Kalilani-Phiri, S. Boudova, S. Joshi, R. Mukadam, K. B. Seydel, P. Mawindo, P. Thesing, S. Kamiza, K. Makwakwa, A. Muehlenbachs, T. E. Taylor, and M. K. Laufer. 2014. "Submicroscopic malaria infection during pregnancy and the impact of intermittent preventive treatment." *Malar J* 13:274. doi: 10.1186/1475-2875-13-274.

Coleman, R. E., J. Sattabongkot, S. Promstaporm, N. Maneechai, B. Tippayachai, A. Kengluecha, N. Rachapaew, G. Zollner, R. S. Miller, J. A. Vaughan, K. Thimasarn, and B. Khuntirat. 2006. "Comparison of PCR and microscopy for the detection of asymptomatic malaria in a Plasmodium falciparum/vivax endemic area in Thailand." *Malar J* 5:121. doi: 10.1186/1475-2875-5-121.

CORDIS_EU. 2019. "Final Report Summary - PLACMALVAC (Clinical development of a VAR2CSA-based placental malaria vaccine)."

Costa, J. D., F. B. Zanchi, F. L. Rodrigues, E. R. Honda, T. H. Katsuragawa, D. B. Pereira, R. L. Taborda, M. S. Tada, R.e G Ferreira, and L. H. Pereira-da-Silva. 2013. "Cross-reactive anti-PfCLAG9 antibodies in the sera of asymptomatic parasite carriers of Plasmodium vivax." *Mem Inst Oswaldo Cruz* 108 (1):98-105.

Cottrell, G., A. Moussiliou, A. J. Luty, M. Cot, N. Fievet, A. Massougbodji, P. Deloron, and N. Tuikue Ndam. 2015. "Submicroscopic Plasmodium falciparum Infections Are Associated With Maternal Anemia, Premature Births, and Low Birth Weight." *Clin Infect Dis* 60 (10):1481-8. doi: 10.1093/cid/civ122.

Cowman, A. F., J. Healer, D. Marapana, and K. Marsh. 2016. "Malaria: Biology and Disease." *Cell* 167 (3):610-624. doi: 10.1016/j.cell.2016.07.055.

Dahlback, M., T. Lavstsen, A. Salanti, L. Hviid, D. E. Arnot, T. G. Theander, and M. A. Nielsen. 2007. "Changes in var gene mRNA levels during erythrocytic development in two phenotypically distinct Plasmodium falciparum parasites." *Malar J* 6:78. doi: 10.1186/1475-2875-6-78.

de Sousa, T. N., F. S. Kano, C. F. de Brito, and L. H. Carvalho. 2014. "The Duffy binding protein as a key target for a Plasmodium vivax vaccine: lessons from the Brazilian Amazon." *Mem Inst Oswaldo Cruz* 109 (5):608-17.

Deitsch, K. W., A. del Pinal, and T. E. Wellems. 1999. "Intra-cluster recombination and var transcription switches in the antigenic variation of Plasmodium falciparum." *Mol Biochem Parasitol* 101 (1-2):107-16.

del Portillo, H. A., C. Fernandez-Becerra, S. Bowman, K. Oliver, M. Preuss, C. P. Sanchez, N. K. Schneider, J. M. Villalobos, M. A. Rajandream, D. Harris, L. H. Pereira da Silva, B. Barrell, and M. Lanzer. 2001. "A superfamily of variant genes encoded in the subtelomeric region of Plasmodium vivax." *Nature* 410 (6830):839-42. doi: 10.1038/35071118.

Dellicour, S., A. J. Tatem, C. A. Guerra, R. W. Snow, and F. O. ter Kuile. 2010. "Quantifying the number of pregnancies at risk of malaria in 2007: a demographic study." *PLoS Med* 7 (1):e1000221. doi: 10.1371/journal.pmed.1000221.

Desai, M., F. O. ter Kuile, F. Nosten, R. McGready, K. Asamoa, B. Brabin, and R. D. Newman. 2007. "Epidemiology and burden of malaria in pregnancy." *Lancet Infect Dis* 7 (2):93-104. doi: 10.1016/s1473-3099(07)70021-x.

Dombrowski, J. G., R. M. Souza, N. R. M. Silva, A. Barateiro, S. Epiphanio, L. A. Gonçalves, and C. R. F. Marinho. 2018. "Malaria during pregnancy and newborn outcome in an unstable transmission area in Brazil: A population-based record linkage study." *PLoS One* 13 (6):e0199415. doi: 10.1371/journal.pone.0199415.

Dondorp, A. M., F. Nosten, P. Yi, D. Das, A. P. Phyo, J. Tarning, K. M. Lwin, F. Ariey, W. Hanpithakpong, S. J. Lee, P. Ringwald, K. Silamut, M. Imwong, K. Chotivanich, P. Lim, T. Herdman, S. S. An, S. Yeung, P. Singhasivanon, N. P. Day, N. Lindegardh, D. Socheat, and N. J. White. 2009. "Artemisinin resistance in Plasmodium falciparum malaria." *N Engl J Med* 361 (5):455-67. doi: 10.1056/NEJMoa0808859.

Doritchamou, J. Y., R. Herrera, J. A. Aebig, R. Morrison, V. Nguyen, K. Reiter, R. L. Shimp, N. J. MacDonald, D. L. Narum, M. Fried, and P. E. Duffy. 2016. "VAR2CSA Domain-Specific Analysis of Naturally Acquired Functional Antibodies to Plasmodium falciparum Placental Malaria." *J Infect Dis* 214 (4):577-86. doi: 10.1093/infdis/jiw197.

Doritchamou, Justin, Andrew Teo, Michal Fried, and Patrick E Duffy. 2017. "Malaria in pregnancy: the relevance of animal models for vaccine development." *Lab Animal* 46 (10):388. doi: doi:10.1038/laban.1349.

Duffy, M. F., A. G. Maier, T. J. Byrne, A. J. Marty, S. R. Elliott, M. T. O'Neill, P. D. Payne, S. J. Rogerson, A. F. Cowman, B. S. Crabb, and G. V. Brown. 2006. "VAR2CSA is the principal ligand for chondroitin sulfate A in two allogeneic isolates of Plasmodium falciparum." *Mol Biochem Parasitol* 148 (2):117-24. doi: 10.1016/j.molbiopara.2006.03.006.

Duffy, P. E., and M. Fried. 2003. "Antibodies that inhibit Plasmodium falciparum adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns." *Infect Immun* 71 (11):6620-3.

Dutta, S., J. R. Daugherty, L. A. Ware, D. E. Lanar, and C. F. Ockenhouse. 2000. "Expression, purification and characterization of a functional region of the Plasmodium vivax Duffy binding protein." *Mol Biochem Parasitol* 109 (2):179-84.

Eckstein-Ludwig, U., R. J. Webb, I. D. Van Goethem, J. M. East, A. G. Lee, M. Kimura, P. M. O'Neill, P. G. Bray, S. A. Ward, and S. Krishna. 2003. "Artemisinins target the SERCA of Plasmodium falciparum." *Nature* 424 (6951):957-61. doi: 10.1038/nature01813.

Fairhurst, R. M., and A. M. Dondorp. 2016. "Artemisinin-Resistant Plasmodium falciparum Malaria." *Microbiol Spectr* 4 (3). doi: 10.1128/microbiolspec.EI10-0013-2016.

Fernandez-Becerra, C., M. M. Yamamoto, R. Z. Vêncio, M. Lacerda, A. Rosanas-Urgell, and H. A. del Portillo. 2009. "Plasmodium vivax and the importance of the subtelomeric multigene vir superfamily." *Trends Parasitol* 25 (1):44-51. doi: 10.1016/j.pt.2008.09.012.

Ferrara, N. 2004. "Vascular endothelial growth factor: basic science and clinical progress." *Endocr Rev* 25 (4):581-611. doi: 10.1210/er.2003-0027.

Fodjo, B. A., N. Atemnkeng, L. Esemu, E. K. Yuosembom, I. A. Quakyi, V. H. Tchinda, J. Smith, A. Salanti, J. Bigoga, D. W. Taylor, R. G. Leke, and A. Babakhanyan. 2016. "Antibody responses to the full-length VAR2CSA and its DBL domains in Cameroonian children and teenagers." *Malar J* 15 (1):532. doi: 10.1186/s12936-016-1585-y.

Fraser, T., P. Michon, J. W. Barnwell, A. R. Noe, F. Al-Yaman, D. C. Kaslow, and J. H. Adams. 1997. "Expression and serologic activity of a soluble recombinant Plasmodium vivax Duffy binding protein." *Infect Immun* 65 (7):2772-7.

Fried, M., and P. E. Duffy. 1996. "Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta." *Science* 272 (5267):1502-4.

Fried, M., and P. E. Duffy. 2002. "Two DBLgamma subtypes are commonly expressed by placental isolates of Plasmodium falciparum." *Mol Biochem Parasitol* 122 (2):201-10.

Fried, M., and P. E. Duffy. 2015. "Designing a VAR2CSA-based vaccine to prevent placental malaria." *Vaccine* 33 (52):7483-8. doi: 10.1016/j.vaccine.2015.10.011.

Fried, M., R. O. Muga, A. O. Misore, and P. E. Duffy. 1998. "Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes." *J Immunol* 160 (5):2523-30.

Galatas, B., H. Marti-Soler, L. Nhamussua, P. Cistero, P. Aide, F. Saute, C. Menendez, N. R. Rabinovich, P. L. Alonso, Q. Bassat, and A. Mayor. 2018. "Dynamics of Afebrile Plasmodium falciparum Infections in Mozambican Men." *Clin Infect Dis* 67 (7):1045-1052. doi: 10.1093/cid/ciy219.

Garcia-Bustos, J. F., and F. J. Gamo. 2013. "Antimalarial drug resistance and early drug discovery." *Curr Pharm Des* 19 (2):270-81.

Gardner, M. J., N. Hall, E. Fung, O. White, M. Berriman, R. W. Hyman, J. M. Carlton, A. Pain, K. E. Nelson, S. Bowman, I. T. Paulsen, K. James, J. A. Eisen, K. Rutherford, S. L. Salzberg, A. Craig, S. Kyes, M. S. Chan, V. Nene, S. J. Shallom, B. Suh, J. Peterson, S. Angiuoli, M. Pertea, J. Allen,

J. Selengut, D. Haft, M. W. Mather, A. B. Vaidya, D. M. Martin, A. H. Fairlamb, M. J. Fraunholz, D. S. Roos, S. A. Ralph, G. I. McFadden, L. M. Cummings, G. M. Subramanian, C. Mungall, J. C. Venter, D. J. Carucci, S. L. Hoffman, C. Newbold, R. W. Davis, C. M. Fraser, and B. Barrell. 2002. "Genome sequence of the human malaria parasite Plasmodium falciparum." *Nature* 419 (6906):498-511. doi: 10.1038/nature01097.

Gavina, K., S. Gnidehou, E. Arango, C. Hamel-Martineau, C. Mitran, O. Agudelo, C. Lopez, A. Karidio, S. Banman, J. Carmona-Fonseca, A. Salanti, N. Tuikue Ndam, M. Hawkes, A. Maestre, and S. K. Yanow. 2018. "Clinical Outcomes of Submicroscopic Infections and Correlates of Protection of VAR2CSA Antibodies in a Longitudinal Study of Pregnant Women in Colombia." *Infect Immun* 86 (4). doi: 10.1128/IAI.00797-17.

Gnidehou, S., J. Doritchamou, E. M. Arango, A. Cabrera, M. I. Arroyo, K. C. Kain, N. T. Ndam, A. Maestre, and S. K. Yanow. 2014. "Functional Antibodies against VAR2CSA in Nonpregnant Populations from Colombia Exposed to Plasmodium falciparum and Plasmodium vivax." *Infect Immun* 82 (6):2565-73. doi: 10.1128/IAI.01594-14.

Gnidehou, S., C. J. Mitran, E. Arango, S. Banman, A. Mena, E. Medawar, B. A. S. Lima, J. Doritchamou, J. Rajwani, A. Jin, K. Gavina, F. Ntumngia, P. Duffy, D. Narum, N. T. Ndam, M. A. Nielsen, A. Salanti, F. S. Kano, L. H. Carvalho, J. H. Adams, A. Maestre, M. F. Good, and S. K. Yanow. 2019. "Cross-Species Immune Recognition Between Plasmodium vivax Duffy Binding Protein Antibodies and the Plasmodium falciparum Surface Antigen VAR2CSA." *J Infect Dis* 219 (1):110-120. doi: 10.1093/infdis/jiy467.

Good, M. F., J. M. Reiman, I. B. Rodriguez, K. Ito, S. K. Yanow, I. M. El-Deeb, M. R. Batzloff, D. I. Stanisic, C. Engwerda, T. Spithill, S. L. Hoffman, M. Lee, and V. McPhun. 2013. "Cross-species malaria immunity induced by chemically attenuated parasites." *J Clin Invest*. doi: 10.1172/JCI66634.

Grimberg, B. T., R. Udomsangpetch, J. Xainli, A. McHenry, T. Panichakul, J. Sattabongkot, L. Cui, M. Bockarie, C. Chitnis, J. Adams, P. A. Zimmerman, and C. L. King. 2007. "Plasmodium vivax invasion of human erythrocytes inhibited by antibodies directed against the Duffy binding protein." *PLoS Med* 4 (12):e337. doi: 10.1371/journal.pmed.0040337.

Hester, J., E. R. Chan, D. Menard, O. Mercereau-Puijalon, J. Barnwell, P. A. Zimmerman, and D. Serre. 2013a. "De Novo Assembly of a Field Isolate Genome Reveals Novel Plasmodium vivax Erythrocyte Invasion Genes." In *PLoS Negl Trop Dis*.

Hester, J., E. R. Chan, D. Menard, O. Mercereau-Puijalon, J. Barnwell, P. A. Zimmerman, and D. Serre. 2013b. "De novo assembly of a field isolate genome reveals novel Plasmodium vivax erythrocyte invasion genes." *PLoS Negl Trop Dis* 7 (12):e2569. doi: 10.1371/journal.pntd.0002569.

Howell, D. P., R. Samudrala, and J. D. Smith. 2006. "Disguising itself--insights into Plasmodium falciparum binding and immune evasion from the DBL crystal structure." *Mol Biochem Parasitol* 148 (1):1-9. doi: 10.1016/j.molbiopara.2006.03.004.

Hwang, J., E. Bitarakwate, M. Pai, A. Reingold, P. J. Rosenthal, and G. Dorsey. 2006. "Chloroquine or amodiaquine combined with sulfadoxine-pyrimethamine for uncomplicated malaria: a systematic review." *Trop Med Int Health* 11 (6):789-99. doi: 10.1111/j.1365-3156.2006.01571.x.

Igonet, S., B. Vulliez-Le Normand, G. Faure, M. M. Riottot, C. H. Kocken, A. W. Thomas, and G. A. Bentley. 2007. "Cross-reactivity studies of an anti-Plasmodium vivax apical membrane antigen 1 monoclonal antibody: binding and structural characterisation." *J Mol Biol* 366 (5):1523-37. doi: 10.1016/j.jmb.2006.12.028.

Ismail, M. R., J. Ordi, C. Menendez, P. J. Ventura, J. J. Aponte, E. Kahigwa, R. Hirt, A. Cardesa, and P. L. Alonso. 2000. "Placental pathology in malaria: a histological, immunohistochemical, and quantitative study." *Hum Pathol* 31 (1):85-93.

J, Stephens. 1927. "A new malaria parasite of man   " *Ann Trop Med Parasitol* 21:293-302. doi: 10.1080/00034983.1927.11684538.

Kasehagen, L. J., I. Mueller, B. Kiniboro, M. J. Bockarie, J. C. Reeder, J. W. Kazura, W. Kastens, D. T. McNamara, C. H. King, C. C. Whalen, and P. A. Zimmerman. 2007. "Reduced Plasmodium

vivax erythrocyte infection in PNG Duffy-negative heterozygotes." *PLoS One* 2 (3):e336. doi: 10.1371/journal.pone.0000336.

Khunrae, P., M. Dahlback, M. A. Nielsen, G. Andersen, S. B. Ditlev, M. Resende, V. V. Pinto, T. G. Theander, M. K. Higgins, and A. Salanti. 2010. "Full-length recombinant Plasmodium falciparum VAR2CSA binds specifically to CSPG and induces potent parasite adhesion-blocking antibodies." *J Mol Biol* 397 (3):826-34. doi: 10.1016/j.jmb.2010.01.040.

Kimura, M., H. Miyake, H. S. Kim, M. Tanabe, M. Arai, S. Kawai, A. Yamane, and Y. Wataya. 1995. "Species-specific PCR detection of malaria parasites by microtiter plate hybridization: clinical study with malaria patients." *J Clin Microbiol* 33 (9):2342-6.

Kirchner, S., B. J. Power, and A. P. Waters. 2016. "Recent advances in malaria genomics and epigenomics." *Genome Med* 8 (1):92. doi: 10.1186/s13073-016-0343-7.

Kyriacou, H. M., G. N. Stone, R. J. Challis, A. Raza, K. E. Lyke, M. A. Thera, A. K. Koné, O. K. Doumbo, C. V. Plowe, and J. A. Rowe. 2006. "Differential var gene transcription in Plasmodium falciparum isolates from patients with cerebral malaria compared to hyperparasitaemia." *Mol Biochem Parasitol* 150 (2):211-8. doi: 10.1016/j.molbiopara.2006.08.005.

Lindsay, S., J. Ansell, C. Selman, V. Cox, K. Hamilton, and G. Walraven. 2000. "Effect of pregnancy on exposure to malaria mosquitoes." *Lancet* 355 (9219):1972. doi: 10.1016/S0140-6736(00)02334-5.

Lopez-Perez, M., M. A. Pacheco, L. Buritica, A. A. Escalante, S. Herrera, and M. Arevalo-Herrera. 2016. "Malaria in pregnancy: a passive surveillance study of pregnant women in low transmission areas of Colombia, Latin America." *Malar J* 15:66. doi: 10.1186/s12936-016-1125-9.

Luttun, A., M. Tjwa, L. Moons, Y. Wu, A. Angelillo-Scherrer, F. Liao, J. A. Nagy, A. Hooper, J. Priller, B. De Klerck, V. Compernolle, E. Daci, P. Bohlen, M. Dewerchin, J. M. Herbert, R. Fava, P. Matthys, G. Carmeliet, D. Collen, H. F. Dvorak, D. J. Hicklin, and P. Carmeliet. 2002. "Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1." *Nat Med* 8 (8):831-40. doi: 10.1038/nm731.

Maguire, J. D., I. W. Sumawinata, S. Masbar, B. Laksana, P. Prodjodipuro, I. Susanti, P. Sismadi, N. Mahmud, M. J. Bangs, and J. K. Baird. 2002. "Chloroquine-resistant Plasmodium malariae in south Sumatra, Indonesia." *Lancet* 360 (9326):58-60. doi: 10.1016/s0140-6736(02)09336-4.

Marchesini, P., F. T. Costa, and C. R. Marinho. 2014. "A decade of malaria during pregnancy in Brazil: what has been done concerning prevention and management." *Mem Inst Oswaldo Cruz* 109 (5):706-8.

Mbengue, A., S. Bhattacharjee, T. Pandharkar, H. Liu, G. Estiu, R. V. Stahelin, S. S. Rizk, D. L. Njimoh, Y. Ryan, K. Chotivanich, C. Nguon, M. Ghorbal, J. J. Lopez-Rubio, M. Pfrender, S. Emrich, N. Mohandas, A. M. Dondorp, O. Wiest, and K. Haldar. 2015. "A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria." *Nature* 520 (7549):683-7. doi: 10.1038/nature14412.

McGready, R., B. B. Davison, K. Stepniewska, T. Cho, H. Shee, A. Brockman, R. Udomsangpetch, S. Looareesuwan, N. J. White, S. R. Meshnick, and F. Nosten. 2004. "The effects of Plasmodium falciparum and P. vivax infections on placental histopathology in an area of low malaria transmission." *Am J Trop Med Hyg* 70 (4):398-407.

McMorrow, M. L., M. Aidoo, and S. P. Kachur. 2011. "Malaria rapid diagnostic tests in elimination settings--can they find the last parasite?" *Clin Microbiol Infect* 17 (11):1624-31. doi: 10.1111/j.1469-0691.2011.03639.x.

Menard, D., C. Barnadas, C. Bouchier, C. Henry-Halldin, L. R. Gray, A. Ratsimbasoa, V. Thonier, J. F. Carod, O. Domarle, Y. Colin, O. Bertrand, J. Picot, C. L. King, B. T. Grimberg, O. Mercereau-Puijalon, and P. A. Zimmerman. 2010. "Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people." *Proc Natl Acad Sci U S A* 107 (13):5967-71. doi: 10.1073/pnas.0912496107.

Mendes, C., F. Dias, J. Figueiredo, V. G. Mora, J. Cano, B. de Sousa, V. E. do Rosário, A. Benito, P. Berzosa, and A. P. Arez. 2011. "Duffy negative antigen is no longer a barrier to Plasmodium

vivax--molecular evidences from the African West Coast (Angola and Equatorial Guinea)." *PLoS Negl Trop Dis* 5 (6):e1192. doi: 10.1371/journal.pntd.0001192.

Miller, L. H., D. I. Baruch, K. Marsh, and O. K. Doumbo. 2002. "The pathogenic basis of malaria." *Nature* 415 (6872):673-9. doi: 10.1038/415673a.

Miroux, B., and J. E. Walker. 1996. "Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels." *J Mol Biol* 260 (3):289-98. doi: 10.1006/jmbi.1996.0399.

Moraga C, Javier, Santiago Universidad Autónoma de Chile, Chile, Ricardo Cartes-Velásquez, Concepción Universidad de Concepción, Chile, and Temuco Universidad de La Frontera, Chile. 2015. "PAUTAS DE CHEQUEO, PARTE II: QUOROM Y PRISMA." *Rev Chil Cir* 67 (3):325-330. doi: 10.4067/S0718-40262015000300015.

Murphy, S. C., J. P. Shott, S. Parikh, P. Etter, W. R. Prescott, and V. A. Stewart. 2013. "Malaria diagnostics in clinical trials." *Am J Trop Med Hyg* 89 (5):824-39. doi: 10.4269/ajtmh.12-0675.

MYCDCGP. 2019. *Management Guideline of Malaria in Malaysia*.

Nguyen, T. N., L. von Seidlein, T. V. Nguyen, P. N. Truong, S. D. Hung, H. T. Pham, T. U. Nguyen, T. D. Le, V. H. Dao, M. Mukaka, N. P. Day, N. J. White, A. M. Dondorp, G. E. Thwaites, and T. T. Hien. 2018. "The persistence and oscillations of submicroscopic Plasmodium falciparum and Plasmodium vivax infections over time in Vietnam: an open cohort study." *Lancet Infect Dis* 18 (5):565-572. doi: 10.1016/s1473-3099(18)30046-x.

Nielsen, M. A., and A. Salanti. 2015. "High-Throughput Testing of Antibody-Dependent Binding Inhibition of Placental Malaria Parasites." *Methods Mol Biol* 1325:241-53. doi: 10.1007/978-1-4939-2815-6_20.

Ntumngia, F. B., R. Thomson-Luque, L.e M Torres, K. Gunalan, L. H. Carvalho, and J. H. Adams. 2016. "A Novel Erythrocyte Binding Protein of Plasmodium vivax Suggests an Alternate Invasion Pathway into Duffy-Positive Reticulocytes." *MBio* 7 (4). doi: 10.1128/mBio.01261-16.

Ohrt, C., W. P. O'Meara, S. Remich, P. McEvoy, B. Ogutu, R. Mtalib, and J. S. Odera. 2008. "Pilot assessment of the sensitivity of the malaria thin film." *Malar J* 7:22. doi: 10.1186/1475-2875-7-22.

Oleinikov, A. V., E. Rossnagle, S. Francis, T. K. Mutabingwa, M. Fried, and P. E. Duffy. 2007. "Effects of sex, parity, and sequence variation on seroreactivity to candidate pregnancy malaria vaccine antigens." *J Infect Dis* 196 (1):155-64. doi: 10.1086/518513.

Oleinikov, A. V., V. V. Voronkova, I. T. Frye, E. Amos, R. Morrison, M. Fried, and P. E. Duffy. 2012. "A plasma survey using 38 PfEMP1 domains reveals frequent recognition of the Plasmodium falciparum antigen VAR2CSA among young Tanzanian children." *PLoS One* 7 (1):e31011. doi: 10.1371/journal.pone.0031011.

Parekh, F. K., J. N. Hernandez, D. J. Krogstad, W. M. Casapia, and O. H. Branch. 2007. "Prevalence and risk of Plasmodium falciparum and P. vivax malaria among pregnant women living in the hypoendemic communities of the Peruvian Amazon." *Am J Trop Med Hyg* 77 (3):451-7.

Patel, J., K. Landers, R. H. Mortimer, and K. Richard. 2010. "Regulation of hypoxia inducible factors (HIF) in hypoxia and normoxia during placental development." *Placenta* 31 (11):951-7. doi: 10.1016/j.placenta.2010.08.008.

Payne, R. O., S. E. Silk, S. C. Elias, K. H. Milne, T. A. Rawlinson, D. Llewellyn, A. R. Shakri, J. Jin, G. M. Labbé, N. J. Edwards, I. D. Poulton, R. Roberts, R. Farid, T. Jørgensen, D. G. Alanine, S. C. de Cassan, M. K. Higgins, T. D. Otto, J. S. McCarthy, W. A. de Jongh, A. Nicosia, S. Moyle, A. V. Hill, E. Berrie, C. E. Chitnis, A. M. Lawrie, and S. J. Draper. 2017. "Human vaccination against Plasmodium vivax Duffy-binding protein induces strain-transcending antibodies." *JCI Insight* 2 (12). doi: 10.1172/jci.insight.93683.

Pehrson, C., K. K. Heno, Y. Adams, M. Resende, L. Mathiesen, M. Soegaard, W. A. de Jongh, T. G. Theander, A. Salanti, and M. A. Nielsen. 2017. "Comparison of functional assays used in the clinical development of a placental malaria vaccine." *Vaccine* 35 (4):610-618. doi: 10.1016/j.vaccine.2016.12.028.

Pehrson, Caroline, Line Mathiesen, Kristine K. Heno, Ali Salanti, Mafalda Resende, Ron Dzikowski, Peter Damm, Stefan R. Hansson, Christopher L. King, Henning Schneider, Christian W. Wang, Thomas Lavstsen, Thor G. Theander, Lisbeth E. Knudsen, and Morten A. Nielsen. 2016. "Adhesion of Plasmodium falciparum infected erythrocytes in ex vivo perfused placental tissue: a novel model of placental malaria." *Malaria Journal* 15 (1):292. doi: doi:10.1186/s12936-016-1342-2.

Pineros-Jimenez, J. G., G. Alvarez, A. Tobon, M. Arboleda, S. Carrero, and S. Blair. 2011. "Congenital malaria in Uraba, Colombia." *Malar J* 10:239. doi: 10.1186/1475-2875-10-239.

Prevention, CDC - Centers for Disease Control and. 2018. "CDC - Malaria - About Malaria - Disease." Last Modified 2018-04-11T12:45:28Z. https://www.cdc.gov/malaria/about/disease.html.

Quadt, K. A., L. Barfod, D. Andersen, J. Bruun, B. Gyan, T. Hassenkam, M. F. Ofori, and L. Hviid. 2012. "The density of knobs on Plasmodium falciparum-infected erythrocytes depends on developmental age and varies among isolates." *PLoS One* 7 (9):e45658. doi: 10.1371/journal.pone.0045658.

Ralph, S. A., C. Scheidig-Benatar, and A. Scherf. 2005. "Antigenic variation in Plasmodium falciparum is associated with movement of var loci between subnuclear locations." *Proc Natl Acad Sci U S A* 102 (15):5414-9. doi: 10.1073/pnas.0408883102.

Rask, T. S., D. A. Hansen, T. G. Theander, A. Gorm Pedersen, and T. Lavstsen. 2010. "Plasmodium falciparum erythrocyte membrane protein 1 diversity in seven genomes--divide and conquer." *PLoS Comput Biol* 6 (9). doi: 10.1371/journal.pcbi.1000933.

Requena, P., M. Arevalo-Herrera, M. Menegon, F. E. Martinez-Espinosa, N. Padilla, C. Botto-Menezes, A. Malheiro, D. Hans, M. E. Castellanos, L. Robinson, P. Samol, S. Kochar, S. K. Kochar, D. K. Kochar, M. Desai, S. Sanz, L. Quinto, A. Mayor, S. Rogerson, I. Mueller, C. Severini, H. A. Del Portillo, A. Bardaji, C. C. Chitnis, C. Menendez, and C. Dobano. 2017. "Naturally Acquired Binding-Inhibitory Antibodies to Plasmodium vivax Duffy Binding Protein in Pregnant Women Are Associated with Higher Birth Weight in a Multicenter Study." *Front Immunol* 8:163. doi: 10.3389/fimmu.2017.00163.

Rijken, M. J., R. McGready, M. E. Boel, R. Poespoprodjo, N. Singh, D. Syafruddin, S. Rogerson, and F. Nosten. 2012. "Malaria in pregnancy in the Asia-Pacific region." *Lancet Infect Dis* 12 (1):75-88. doi: 10.1016/S1473-3099(11)70315-2.

Rogerson, S. J., L. Hviid, P. E. Duffy, R. F. Leke, and D. W. Taylor. 2007. "Malaria in pregnancy: pathogenesis and immunity." *Lancet Infect Dis* 7 (2):105-17. doi: 10.1016/S1473-3099(07)70022-1.

Rogerson, S. J., E. Pollina, A. Getachew, E. Tadesse, V. M. Lema, and M. E. Molyneux. 2003. "Placental monocyte infiltrates in response to Plasmodium falciparum malaria infection and their association with adverse pregnancy outcomes." *Am J Trop Med Hyg* 68 (1):115-9.

Rosenthal, P. J. 2018. "Artemisinin Resistance Outside of Southeast Asia." *Am J Trop Med Hyg* 99 (6):1357-1359. doi: 10.4269/ajtmh.18-0845.

Rota, C., D. Carles, V. Schaeffer, F. Guyon, R. Saura, and J. Horovitz. 2006. "[Perinatal prognosis of pregnancies complicated by placental chronic intervillitis]." *J Gynecol Obstet Biol Reprod (Paris)* 35 (7):711-9.

Rowe, J. A., A. Claessens, R. A. Corrigan, and M. Arman. 2009. "Adhesion of Plasmodium falciparum-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications." *Expert Rev Mol Med* 11:e16. doi: 10.1017/S1462399409001082.

Rowe, J. A., and S. A. Kyes. 2004. "The role of Plasmodium falciparum var genes in malaria in pregnancy." *Mol Microbiol* 53 (4):1011-9. doi: 10.1111/j.1365-2958.2004.04256.x.

Salanti, A., M. Dahlback, L. Turner, M. A. Nielsen, L. Barfod, P. Magistrado, A. T. Jensen, T. Lavstsen, M. F. Ofori, K. Marsh, L. Hviid, and T. G. Theander. 2004. "Evidence for the involvement of VAR2CSA in pregnancy-associated malaria." *J Exp Med* 200 (9):1197-203. doi: 10.1084/jem.20041579.

Salanti, A., T. Staalsoe, T. Lavstsen, A. T. Jensen, M. P. Sowa, D. E. Arnot, L. Hviid, and T. G. Theander. 2003. "Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria." *Mol Microbiol* 49 (1):179-91. doi: 3570 [pii].

Satchwell, T. J. 2016. "Erythrocyte invasion receptors for Plasmodium falciparum: new and old." *Transfus Med* 26 (2):77-88. doi: 10.1111/tme.12280.

Saveria, T., P. E. Duffy, and M. Fried. 2015. "Evaluation of Pregnancy Malaria Vaccine Candidates: The Binding Inhibition Assay." *Methods Mol Biol* 1325:231-9. doi: 10.1007/978-1-4939-2815-6_19.

Schantz-Dunn, J., and N. M. Nour. 2009. "Malaria and pregnancy: a global health perspective." *Rev Obstet Gynecol* 2 (3):186-92.

Scherf, A., R. Hernandez-Rivas, P. Buffet, E. Bottius, C. Benatar, B. Pouvelle, J. Gysin, and M. Lanzer. 1998. "Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum." *EMBO J* 17 (18):5418-26. doi: 10.1093/emboj/17.18.5418.

Singh, S., K. Pandey, R. Chattopadhayay, S. S. Yazdani, A. Lynn, A. Bharadwaj, A. Ranjan, and C. Chitnis. 2001. "Biochemical, biophysical, and functional characterization of bacterially expressed and refolded receptor binding domain of Plasmodium vivax duffy-binding protein." *J Biol Chem* 276 (20):17111-6. doi: 10.1074/jbc.M101531200.

Singh, V., P. Gupta, and V. Pande. 2014. "Revisiting the multigene families: Plasmodium var and vir genes." *J Vector Borne Dis* 51 (2):75-81.

Sinha, S., B. Medhi, and R. Sehgal. 2014. "Challenges of drug-resistant malaria." *Parasite* 21:61. doi: 10.1051/parasite/2014059.

Slater, H. C., A. Ross, I. Felger, N. E. Hofmann, L. Robinson, J. Cook, B. P. Gonçalves, A. Björkman, A. L. Ouedraogo, U. Morris, M. Msellem, C. Koepfli, I. Mueller, F. Tadesse, E. Gadisa, S. Das, G. Domingo, M. Kapulu, J. Midega, S. Owusu-Agyei, C. Nabet, R. Piarroux, O. Doumbo, S. N. Doumbo, K. Koram, N. Lucchi, V. Udhayakumar, J. Mosha, A. Tiono, D. Chandramohan, R. Gosling, F. Mwingira, R. Sauerwein, E. M. Riley, N. J. White, F. Nosten, M. Imwong, T. Bousema, C. Drakeley, and L. C. Okell. 2019. "The temporal dynamics and infectiousness of subpatent Plasmodium falciparum infections in relation to parasite density." *Nat Commun* 10 (1):1433. doi: 10.1038/s41467-019-09441-1.

Smith, J. D. 2014. "The role of PfEMP1 adhesion domain classification in Plasmodium falciparum pathogenesis research." *Mol Biochem Parasitol* 195 (2):82-7. doi: 10.1016/j.molbiopara.2014.07.006.

Smith, J. D., and K. W. Deitsch. 2004. "Pregnancy-associated malaria and the prospects for syndrome-specific antimalaria vaccines." *J Exp Med* 200 (9):1093-7. doi: 10.1084/jem.20041974.

Smith, J. D., G. Subramanian, B. Gamain, D. I. Baruch, and L. H. Miller. 2000. "Classification of adhesive domains in the Plasmodium falciparum erythrocyte membrane protein 1 family." *Mol Biochem Parasitol* 110 (2):293-310.

Souza, R. M., R. Ataíde, J. G. Dombrowski, V. Ippólito, E. H. Aitken, S. N. Valle, J. M. Álvarez, S. Epiphanio, S. Epiphânio, and C. R. Marinho. 2013. "Placental histopathological changes associated with Plasmodium vivax infection during pregnancy." *PLoS Negl Trop Dis* 7 (2):e2071. doi: 10.1371/journal.pntd.0002071.

Steketee, R. W., B. L. Nahlen, M. E. Parise, and C. Menendez. 2001. "The burden of malaria in pregnancy in malaria-endemic areas." *Am J Trop Med Hyg* 64 (1-2 Suppl):28-35.

Suguitan, A. L., R. G. Leke, G. Fouda, A. Zhou, L. Thuita, S. Metenou, J. Fogako, R. Megnekou, and D. W. Taylor. 2003. "Changes in the levels of chemokines and cytokines in the placentas of women with Plasmodium falciparum malaria." *J Infect Dis* 188 (7):1074-82. doi: 10.1086/378500.

Sutherland, C. J., N. Tanomsing, D. Nolder, M. Oguike, C. Jennison, S. Pukrittayakamee, C. Dolecek, T. T. Hien, V. E. do Rosario, A. P. Arez, J. Pinto, P. Michon, A. A. Escalante, F. Nosten, M. Burke, R. Lee, M. Blaze, T. D. Otto, J. W. Barnwell, A. Pain, J. Williams, N. J. White, N. P. Day, G. Snounou, P. J. Lockhart, P. L. Chiodini, M. Imwong, and S. D. Polley. 2010. "Two

nonrecombining sympatric forms of the human malaria parasite Plasmodium ovale occur globally." *J Infect Dis* 201 (10):1544-50. doi: 10.1086/652240.

Tarning, Joel. 2016. "Treatment of Malaria in Pregnancy." *The new england journal o f medicine* 374;10:891-982.

Taylor, H. M., S. A. Kyes, and C. I. Newbold. 2000. "Var gene diversity in Plasmodium falciparum is generated by frequent recombination events." *Mol Biochem Parasitol* 110 (2):391-7.

Trampuz, A., M. Jereb, I. Muzlovic, and R. M. Prabhu. 2003. "Clinical review: Severe malaria." In *Crit Care*, 315-23.

Tran, T. M., J. Oliveira-Ferreira, A. Moreno, F. Santos, S. S. Yazdani, C. E. Chitnis, J. D. Altman, E. V. Meyer, J. W. Barnwell, and M. R. Galinski. 2005. "Comparison of IgG reactivities to Plasmodium vivax merozoite invasion antigens in a Brazilian Amazon population." *Am J Trop Med Hyg* 73 (2):244-55.

Tshibola Mbuyi, M. L., M. K. Bouyou-Akotet, and D. P. Mawili-Mboumba. 2014. "Molecular Detection of Plasmodium falciparum Infection in Matched Peripheral and Placental Blood Samples from Delivering Women in Libreville, Gabon." *Malar Res Treat* 2014:486042. doi: 10.1155/2014/486042.

Tun, K. M., M. Imwong, K. M. Lwin, A. A. Win, T. M. Hlaing, T. Hlaing, K. Lin, M. P. Kyaw, K. Plewes, M. A. Faiz, M. Dhorda, P. Y. Cheah, S. Pukrittayakamee, E. A. Ashley, T. J. Anderson, S. Nair, M. McDew-White, J. A. Flegg, E. P. Grist, P. Guerin, R. J. Maude, F. Smithuis, A. M. Dondorp, N. P. Day, F. Nosten, N. J. White, and C. J. Woodrow. 2015. "Spread of artemisinin-resistant Plasmodium falciparum in Myanmar: a cross-sectional survey of the K13 molecular marker." *Lancet Infect Dis* 15 (4):415-21. doi: 10.1016/S1473-3099(15)70032-0.

Udeinya, I. J., J. A. Schmidt, M. Aikawa, L. H. Miller, and I. Green. 1981. "Falciparum malaria-infected erythrocytes specifically bind to cultured human endothelial cells." *Science* 213 (4507):555-7.

Udomsangpetch, R., B. Wåhlin, J. Carlson, K. Berzins, M. Torii, M. Aikawa, P. Perlmann, and M. Wahlgren. 1989. "Plasmodium falciparum-infected erythrocytes form spontaneous erythrocyte rosettes." *J Exp Med* 169 (5):1835-40.

Watermeyer, J. M., V. L. Hale, F. Hackett, D. K. Clare, E. E. Cutts, I. Vakonakis, R. A. Fleck, M. J. Blackman, and H. R. Saibil. 2016. "A spiral scaffold underlies cytoadherent knobs in Plasmodium falciparum-infected erythrocytes." *Blood* 127 (3):343-51. doi: 10.1182/blood-2015-10-674002.

Welch, S. G., I. A. McGregor, and K. Williams. 1977. "The Duffy blood group and malaria prevalence in Gambian West Africans." *Trans R Soc Trop Med Hyg* 71 (4):295-6.

White, N. J. 2008. "Plasmodium knowlesi: the fifth human malaria parasite." *Clin Infect Dis* 46 (2):172-3. doi: 10.1086/524889.

White, N. J. 2011. "Determinants of relapse periodicity in Plasmodium vivax malaria." *Malar J* 10:297. doi: 10.1186/1475-2875-10-297.

White, N. J., S. Pukrittayakamee, T. T. Hien, M. A. Faiz, O. A. Mokuolu, and A. M. Dondorp. 2014. "Malaria." *Lancet* 383 (9918):723-35. doi: 10.1016/s0140-6736(13)60024-0.

WHO. 2000. The use of antimalarial drugs : report of a WHO informal consultation. Geneva : World Health Organization. .

WHO. 2010. "Guidelines for the treatment of malaria. 2nd edition." https://www.paho.org/hq/dmdocuments/2011/TreatmentGuidelines-2nd-ed-2010-eng.pdf.

WHO. 2013. "WHO | T3: Test. Treat. Track. Scaling up diagnostic testing, treatment and surveillance for malaria." World Health Organization, Last Modified 2013-09-04 15:25:01. https://www.who.int/malaria/publications/atoz/t3_brochure/en/.

WHO. 2016. Countries, epidemiological profile. Colombia.

WHO. 2017. World Malaria Report 2017. Geneva, World Health Organization.

WHO. 2018. "WHO | Overview of malaria treatment." World Health Organization, Last Modified 2018-02-07 17:55:18. https://www.who.int/malaria/areas/treatment/overview/en/.

WHO. 2019. "Malaria diagnostics: now and the future | Parasitology | Cambridge Core." doi: doi:10.1017/S0031182014001371.

WHO. 2015. Guidelines for the treatment of malaria. 3rd edition. edited by Third edition Geneva: WHO.

WHO. 2017. Malaria in pregnant women. High risk groups.

WHO. 2018. World Malaria Report 2018. Geneva, World Health Organization.

Xainli, J., J. H. Adams, and C. L. King. 2000. "The erythrocyte binding motif of plasmodium vivax duffy binding protein is highly polymorphic and functionally conserved in isolates from Papua New Guinea." *Mol Biochem Parasitol* 111 (2):253-60.

Zimmerman, P. A., I. Woolley, G. L. Masinde, S. M. Miller, D. T. McNamara, F. Hazlett, C. S. Mgone, M. P. Alpers, B. Genton, B. A. Boatin, and J. W. Kazura. 1999. "Emergence of FY*A(null) in a Plasmodium vivax-endemic region of Papua New Guinea." *Proc Natl Acad Sci U S A* 96 (24):13973-7.