

THE KINETICS OF ANTIBODY RESPONSES TO PLASMODIUM VIVAX
VACCINE CANDIDATE ANTIGENS IN BRAZILIANS
WITH ACUTE VIVAX MALARIA

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Master of Science
in the Department of Microbiology and Immunology,
Indiana University

May 2022

Accepted by the Graduate Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Master of Science.

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DEDICATION

To my mentor Professor Tuan M. Tran and my friends in the Tran Laboratory. It has been a privilege to learn from you and work with you over the past two and a half years.

To my friends in John Laboratory and Schmidt Laboratory. Thank you so much for your great advice and support.

To my family. Thank you for all the love and support.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my committee members, Prof. Tuan M. Tran (mentor), Prof. Margaret E. Bauer (academic advisor), and Prof. Quyen Q. Hoang, for their guidance and encouragement over the course of my time in the master's degree program.

I would like to thank Dr. Josué Lima Junior (IOC, Fundação Oswaldo Cruz, Brazil), Dr. Julian Rayner (CIMR, UK), and Dr. Wai-Hong Tham (WEHI, AU) for providing reagents and guidance for this study.

I would like to thank all the lab members: Aditi Sachin Upadhye for processing data and generating figures and Jyoti Bhardwaj and Erik L. Gaskin for their technical guidance and careful review of this thesis.

I would like to thank Cindy Booth for helping me register for classes and arrange the virtual thesis committee meetings.

Finally, I would like to thank the Microbiology and Immunology Department and the IU School of Medicine Infectious Disease Laboratory for providing all the resources required to complete this project and their staff for their critical work on and around this study.

Tenzin Tashi

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Plasmodium vivax malaria is geographically widespread and remains a significant public health burden in the Americas, Southeast Asia, and the western Pacific. In order to achieve the end goal of malaria eradication, a highly effective vaccine targeting *P. vivax* is urgently needed. Unlike pre-erythrocytic vaccines that aim to confer sterile immunity that prevents malaria infection all together, *Plasmodium vivax* blood-stage vaccines aim to confer clinical immunity that protects against malarial disease by controlling parasitemia and mitigating the symptomatic manifestations of malaria after infection. To design an effective *P. vivax* blood-stage vaccine, it is essential to understand the acquisition and longevity of natural humoral immune responses against promising *P. vivax* blood-stage vaccine candidate antigens. We hypothesize that acute vivax malaria induces differential humoral immune responses against *P. vivax* antigens that exhibit antigen-specific kinetic and compositional profiles, which can be used to identify vaccine candidates that elicit durable humoral responses. Therefore, we compared the kinetic profiles and half-lives of naturally acquired IgG antibodies reactive against nine promising *P. vivax* blood-stage vaccine candidate antigens up to 180 days post-infection in Brazilians with acute vivax malaria. Naturally acquired IgG antibodies against these antigens have previously been associated with a reduced risk of vivax malaria. Among the *P. vivax* antigens evaluated, the merozoite antigen Pv12 elicited the most durable IgG antibodies, whereas the DBP-FL elicited the most short-lived responses. Neither patient

age nor prior malaria exposure significantly correlated with the magnitude and durability of IgG responses to any *P. vivax* antigen. Seropositivity, against Pv12, was generally maintained for at least 30 days after acute vivax malaria. These findings suggest that a blood-stage vaccine targeting Pv12 may benefit from boosting IgG antibodies against this antigen after natural vivax “breakthrough” infections. Further studies will be needed to determine the Pv12-specific memory B cell response as well as the functional role for naturally acquired Pv12-specific antibodies in reducing parasitemia and/or clinical disease. In summary, the current study has provided insight into the longevity of IgG antibody responses to important *P. vivax* antigens after an acute malaria episode.

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Introduction

1.1 Malaria

Malaria is a mosquito-borne disease caused by protozoan parasites of the genus *Plasmodium*. *Anopheles* mosquitoes are the vectors for transmission of malaria. There are five *Plasmodium* parasite species that have been recognized to cause malaria in humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*.

Malaria is a global disease that is widely distributed across tropical and subtropical regions. In 2020, the World health organization (WHO) reported 241 million cases of malaria globally, resulting in 627,000 deaths. Unfortunately, both malaria cases and related deaths rose by 5% and 12% comparing 2019, respectively, most likely due to the disruption of malaria prevention and treatment programs caused by the COVID-19 pandemic [3]. Most malaria-attributable deaths occurred in children under five years old living in sub-Saharan Africa [3]. Among the *Plasmodium* parasite species that can infect humans, *P. falciparum* and *P. vivax* are the most common, with *P. falciparum* being the most prevalent in Africa and *P. vivax* predominating in Southeast Asia and the Americas [3, 4], (Figure 1).

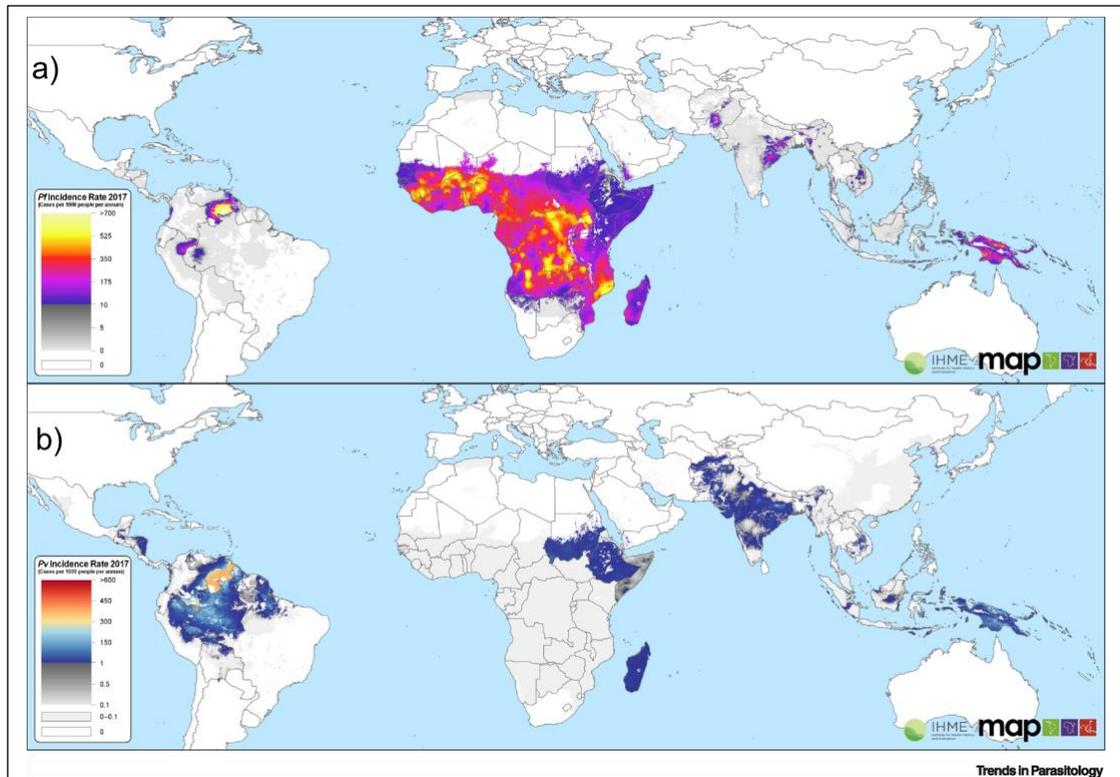


Figure 1. Distribution of two common human malaria parasites worldwide

a) *Plasmodium falciparum* is predominant in Africa, while b) *P. vivax* is prevalent in Southeast Asia and the Americas. Reproduced from Price et al. *Trends in Parasitology*, 2020 [1].

Plasmodium falciparum is responsible for most severe malaria cases, including cerebral malaria and severe malaria anemia, both of which have high mortality if left untreated. However, *P. vivax* has increasingly been reported to cause severe disease that sometimes leads to death [5, 6].

1.2 History of Malaria

The word malaria comes from the Italian *mal' aria*, meaning bad air, owing to the observation that the disease seemed to be associated with vapors emanating from swamps. One of the earliest evidence of the malaria parasite was the discovery of *Plasmodium dominicana*, an avian malaria species, within a female *Culex* mosquito preserved in the amber of Mid - Tertiary Dominican Republic [7]. The presence of malaria parasites during the ancient Egyptian period was confirmed via molecular DNA-based method in the Egyptian mummies [8, 9].

Competing hypotheses exist on how malaria parasites came to infect humans. One hypothesis suggests that malaria parasites coevolved with their mosquito and vertebrate hosts, diverging into lineages specific to their obligate hosts, with one being ancient humans [10]. However, a phylogenetic analysis of *Plasmodium* spp. deoxyribonucleic acid (DNA) obtained from fecal samples of African apes living in the wild suggested that the human malaria parasite *P. falciparum* originated from a gorilla-to-human cross-species transmission event rather than co-evolution with ancient to modern humans (Liu et al. Nature 2010). A *P. vivax*-like parasite was subsequently identified in fecal specimens of gorillas and chimpanzees in sub-Saharan Africa, further supporting the notion that modern human malaria parasites originated from primates [11]. However, the discovery of parasites closely related to *P. falciparum* and *P. vivax* in wild-living chimpanzees, bonobos, and western gorillas have contradicted these theories, indicating that both human pathogens emerged much more recently from parasites infecting African apes [12, 13]. *Plasmodium falciparum* resulted from a recent cross-species transmission of a parasite from a gorilla, while *P. vivax* emerged from an ancestral stock of parasites

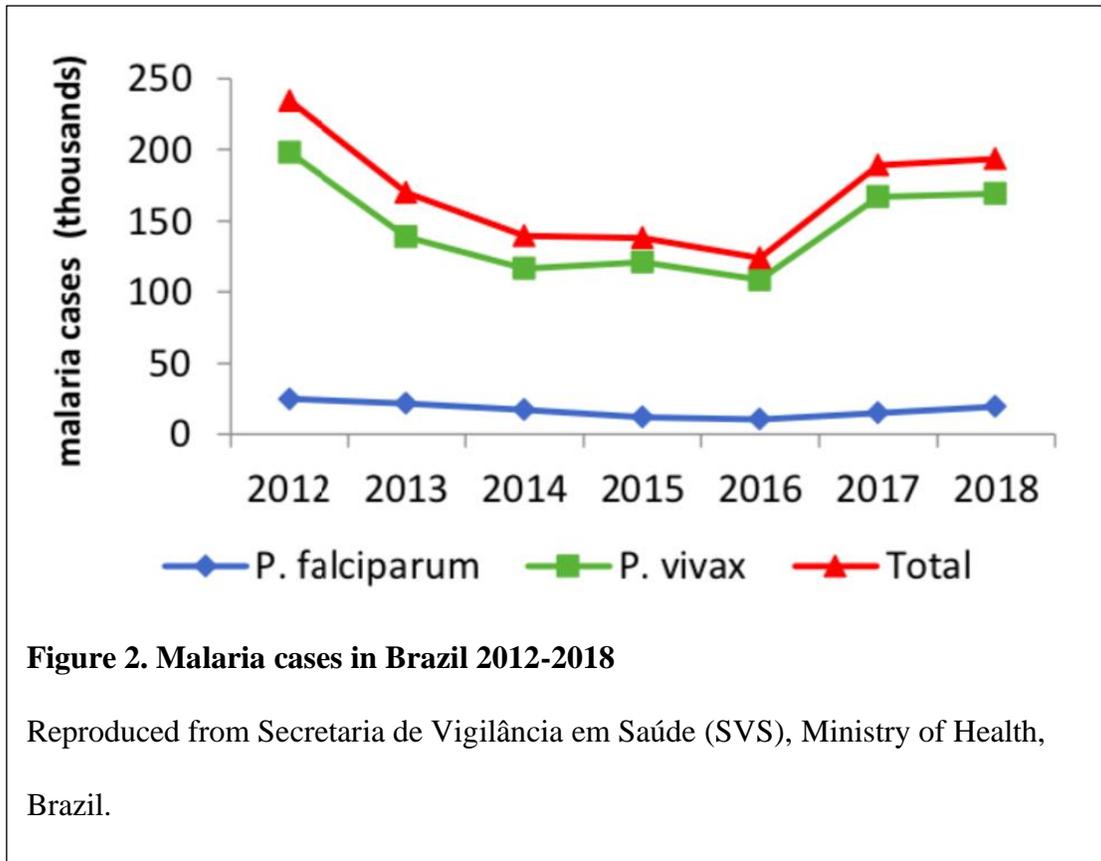
that infected chimpanzees, gorillas, and humans in Africa until the spread of the protective Duffy-negative mutation eliminated *P. vivax* from human populations there [12]. Duffy-negative mutation or Duffy-negative antigen generally confers protection from *P. vivax* infection, given that the Duffy antigen is an essential receptor for *P. vivax* invasion [14, 15].

During the late 19th century, French military doctor Alphonse Laveran discovered protozoan parasites in the blood of a patient who died of malaria in an Algerian military hospital [16], identifying *Plasmodium* as the causative agent of malaria. In 1897, Sir Ronald Ross British officer in the Indian Medical Service, identified a malaria parasite in the stomach of a mosquito that fed on the blood of a patient suffering from the disease, confirming a hypothesis previously proposed by Patrick Manson that mosquitoes transmitted human malaria. Ross later identified these mosquitoes to be of the genus *Anopheles* [17]. Both Laveran and Ross were awarded the Nobel Prize for Medicine in 1902 and 1907, respectively, for their discoveries.

1.3 *Plasmodium vivax* Background and Epidemiology

It was previously thought that *P. vivax* crossed from non-human primates into humans in Southeast Asia, given the high prevalence of *vivax* infections and diverse reservoir of *Plasmodium* parasites in primates in this region [18]. However, an African-origin theory for *P. vivax* has gained acceptance and is supported by the previously mentioned discovery of *P. vivax*-like parasites in African apes [19] and the high frequency of the Duffy-negative mutation through much of central Africa.

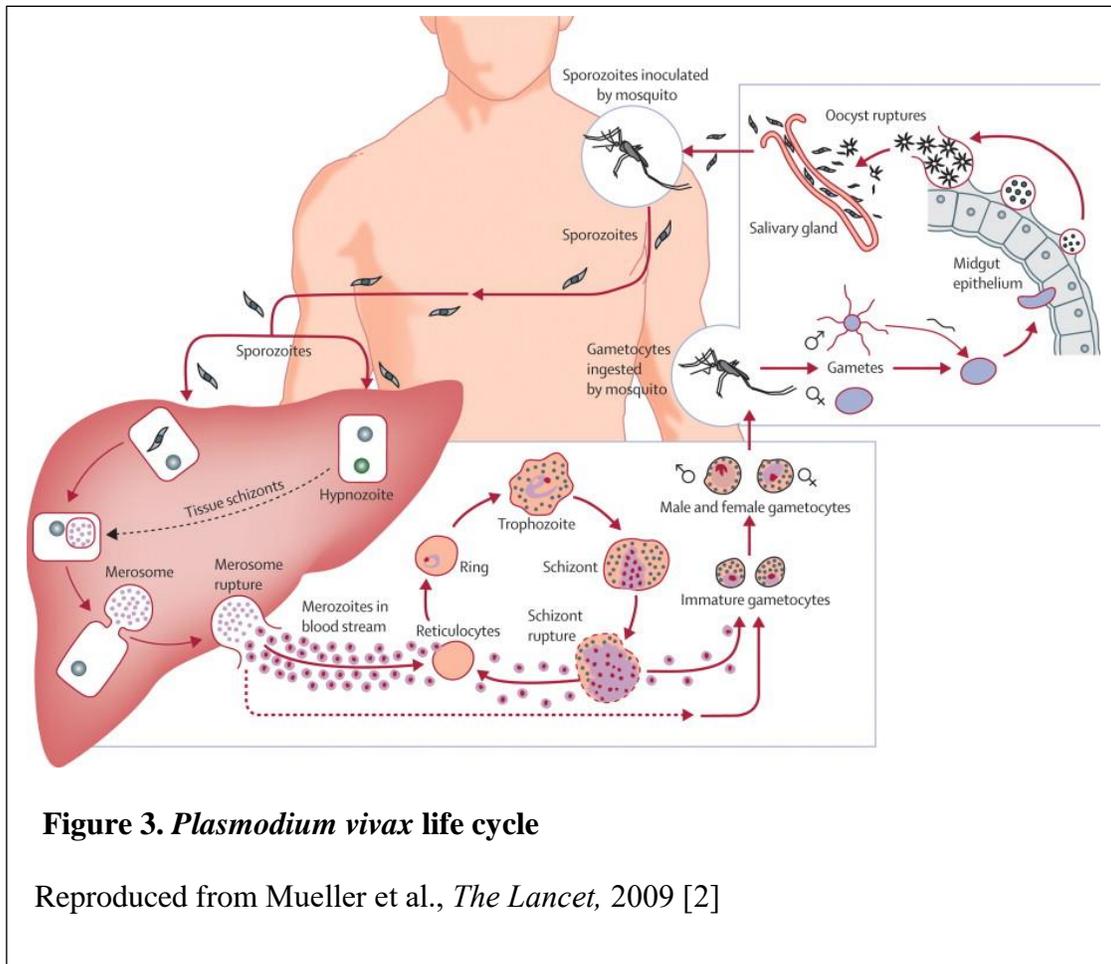
Plasmodium vivax species remains geographically widespread and is the dominant malaria species in Asia and South America [20, 21] (Figure 1). Although the total number of *P. vivax* malaria cases worldwide fell from 18.5 million in 2000 to 4.5 million in 2020, the WHO region of the Americas has more recently suffered from increases in Venezuela, where the number of malaria cases increased from 35,500 in 2000 to 467,000 by 2019. However, in 2020, the cases dropped to 232,000 due to travel restrictions during the COVID-19 pandemic and fuel shortages impacting the mining industry, which is the main contributor to malaria in the region [3]. Brazil, the largest country in South America in both population and land area, accounted for the majority of *P. vivax* malaria cases in the Americas [22, 23]. Based on data from the Brazil Ministry of Health, *P. vivax* malaria cases began to increase in 2017 after five years of decline (Figure 2). Despite its increasing prevalence in regions of the Americas, *P. vivax* remains understudied.



1.4 *Plasmodium vivax* Life Cycle

The malaria life cycle begins when an infected female *Anopheles* mosquito injects sporozoites into the human host during a blood meal. The sporozoites traverse through the skin and quickly migrate to the liver via the bloodstream to infect hepatocytes. For *P. vivax* and *P. ovale*, liver-stage infections can have two different fates. The parasite can 1) remain dormant within hepatocytes as hypnozoites that can later re-activate, or 2) develop immediately within hepatocytes, forming mature liver schizonts that eventually rupture from their host cells as nascent merozoites. *Plasmodium vivax* merozoites then proceed to invade young, immature erythrocytes called reticulocytes via specific receptors present on these cells [24, 25]. During this asexual blood-stage stage within the infected reticulocyte, the parasite develops initially into an early trophozoite, often called

“ring” form, progress to the trophozoite phase, and matures into schizonts that contain multiple invasive merozoites [26, 27] (Figure 3). All the clinical symptoms appear during the blood-stage. Some merozoites will develop into male and female gametocytes. Male and female gametocytes, when ingested by female anopheline mosquitoes during a bloodmeal, can undergo sexual development within the mosquito, eventually forming sporozoites that migrate to their invertebrate host’s salivary glands. The transmission cycle continues when the infected mosquito bites another human host (Figure 3).



1.5 Challenges to Studying *Plasmodium vivax*

Despite the ongoing effort of malaria elimination programs in a few endemic countries, *P. vivax* malaria eradication has been hindered by several factors. First, *P. vivax* is understudied relative to the more lethal human malaria parasite *P. falciparum*. Second, unlike *P. falciparum*, which can be continuously cultured in human erythrocytes *in vitro* [28], *P. vivax* is difficult to maintain and grow in the lab due to the requirement for reticulocyte enrichment [29]. Several unsuccessful attempts have been made to continuously culture *P. vivax in vitro* using various culture media and erythropoietic cells [28, 30]. However, one research group was able to sustain up to 26 months but with very low parasitemia (Roobsoong et al., 2015). In addition, the biology of *P. vivax* in human

infection has presented several challenges to the malaria eradication program. One characteristic of *Plasmodium vivax* is that it can differentiate into gametocytes during the pre-symptomatic period of infection, allowing infected individuals to unknowingly transmit the parasite to capable mosquito vectors [31]. *Plasmodium vivax* parasites have also displayed resistance to a wider temperature range that allowed survival at broader regions and diverse climates compared to *P. falciparum* [31, 32]. Additionally, *P. vivax* hypnozoite form (inactive latent stage of *P. vivax* resides in the malaria-infected human liver) has the ability to relapse and reactivate to cause a new blood-stage infection weeks to months after initial exposure [33]. Hypnozoite poses a challenge, given there is no diagnostic tool to identify these latent liver-stage infections. Moreover, the currently available treatments targeting hypnozoites, primaquine, and tafenoquine, require testing for glucose-6-phosphate dehydrogenase deficiency owing to the drug-induced hemolytic anemia in individuals lacking this enzyme [34]. In addition, resistance to chloroquine, the conventional first line of treatment for *P. vivax*, and other antimalarial drugs (mefloquine, sulfadoxine, and pyrimethamine) have been reported since the late 1990s in Indonesia [35] and several other regions [36-38].

Although the WHO recently approved a malaria vaccine for wide use [3] this vaccine, called RTS, S, is effective only against *P. falciparum*. Currently, there is no licensed vaccine against *P. vivax* malaria. In order to achieve the elimination and eradication of all human malaria, a highly effective vaccine against *P. vivax* malaria will be needed.

1.6 Blood-stage Antigens as Malaria Vaccine Targets

In general, there are three main types of malaria vaccines, each targeting a specific stage of the parasite within the human host (Figure 4).

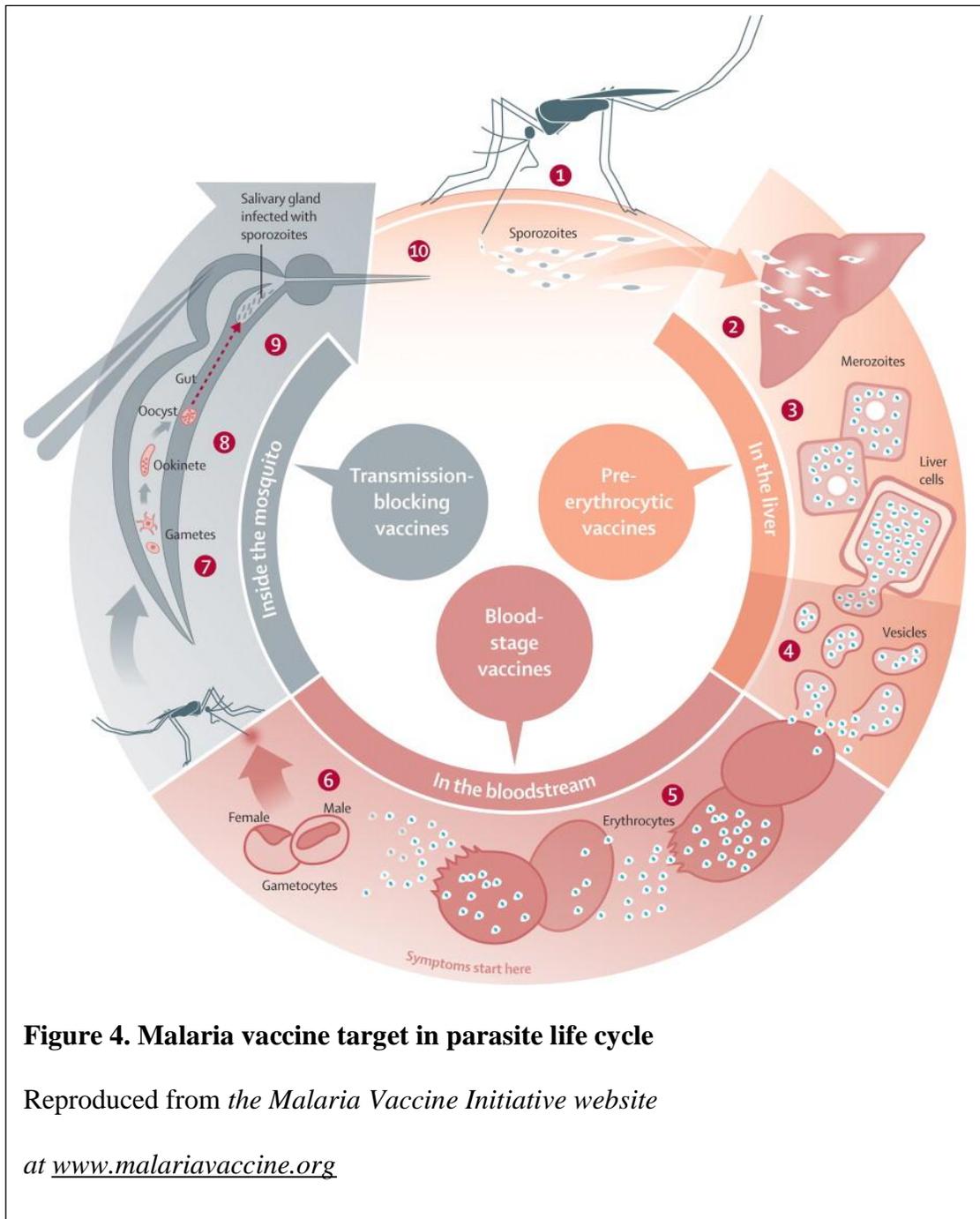


Figure 4. Malaria vaccine target in parasite life cycle

Reproduced from *the Malaria Vaccine Initiative website*

at www.malariavaccine.org

Pre-erythrocytic vaccines aim to induce sterile protection by preventing liver-stage infection or progression to blood-stage infection. Blood-stage vaccines target the blood stage and aim to reduce asexual parasitemia and, the clinical symptoms of malaria. Lastly, transmission-blocking vaccines are generally referred to as “altruistic” vaccines as they target the sexual-stage gametocytes within the blood to prevent effective parasite transmission to mosquitoes but confer no immediate benefit to the vaccinee.

To understand malaria vaccine targets, it is necessary to understand how the parasite invades the host erythrocyte during the blood stage. *Plasmodium vivax* merozoites use multiple parasite ligand-host receptor interactions to invade reticulocytes. Several *P. vivax* asexual blood-stage antigens have been considered for vaccine development based on their essential role in erythrocyte invasion.

One of the first *P. vivax* merozoite invasion antigens was discovered following the observation that Duffy-negative individuals were resistant to *P. vivax* infection [15, 39, 40]. In this seminal study, Africans who lacked the Duffy blood group antigen due to a genetic mutation were challenged with the bites of *P. vivax*-infected mosquitoes. After observing the absence of *P. vivax* parasitemia by blood smear in these individuals over six months of surveillance, they concluded that the Duffy-negative genotype common in the West African population conferred resistance to *P. vivax* infection [15]. A *P. vivax* receptor-like protein that interacted with the human Duffy blood group antigen was identified and subsequently named Duffy binding protein [40]. The Duffy-antigen receptor for chemokines (DARC) was later confirmed as a critical host erythrocyte receptor for *P. vivax* [41]. Follow-up studies revealed that the erythrocyte binding domain of DBP is a conserved, cysteine-rich region that was named region II (PvDBPII) [42].

More recently, cases of *P. vivax* infections have been reported in Duffy-negative individuals throughout mainland Africa and Madagascar [43-46], suggesting that certain *P. vivax* strains have developed an alternative, Duffy-independent erythrocyte invasion pathways or low-level Duffy blood group expression occurs in Duffy-negative Africans [44]. In 1992, Galinski and colleagues discovered two novel *P. vivax* merozoite proteins that demonstrated specific binding to reticulocytes, originally naming them *P. vivax* reticulocyte-binding proteins 1 and 2 (PvRBP1 and PvRBP2) [25]. Later, with the arrival of the *P. vivax* genome sequence, more members were identified and categorized into two groups, one with the predicted protein-encoding genes (*Pvrbp1b*, *Pvrbp2a*, *Pvrbp2b*, *Pvrbp1p*, *Pvrbp2p1*, and *Pvrbp2p2*), and others as pseudogenes (*Pvrbp2d*, *Pvrbp2e*, and *Pvrbp3*) [47, 48]. The original *Pvrbp1* and *Pvrbp2* of the Belem strain discovered by Galinski et al. were renamed *Pvrbp1a* and *Pvrbp2c*, respectively. More than two decades later, elegant experiments performed by Wai-Hong Tham's research group identified the interaction between host transferrin receptor 1 (TfR1) and PvRBP2b as essential for the invasion of reticulocytes by *P. vivax* merozoites [49].

1.7 *Plasmodium vivax* Blood-stage Vaccine Candidate Antigens

Identifying blood-stage vaccine targets requires an understanding of the mechanisms underlying protective immunity in humans, which can be facilitated by evaluating the effectiveness of antibodies targeting specific malaria antigens via parasite invasion assays *in vitro*. Such evaluations have been challenging given *P. vivax*'s requirement for reticulocytes, which limits the ability to maintain the blood-stage parasites in long-term continuous culture as is routinely done for *P. falciparum* [28]. Therefore, much of what we have learned about *P. vivax* has been inferred and applied from *P. falciparum* or other *Plasmodium* species such as *P. knowlesi* [50, 51]. Two general approaches have been used to identify potential blood-stage vivax vaccine candidate antigens. Seroepidemiological analyses of prospective cohort studies in malaria-endemic regions have helped identify antigen-specific antibody responses that associate with subsequently reduced malaria risk. Functional studies have been used to determine whether recombinant versions of *P. vivax* merozoite proteins can bind to reticulocytes. However, one of the major obstacles for identifying an ideal blood-stage vaccine antigen for *P. vivax* is that the genetic diversity of *P. vivax* is far greater than that of *P. falciparum* [52].

The ideal vaccine targets are antigens that are both essential for the survival of the infectious pathogen and antigenically conserved across the global isolates. An optimal vaccine would theoretically induce a specific and durable immune response that broadly and effectively neutralizes multiple strains of the parasite.

1.8 Naturally Acquired Immune Responses to *P. vivax* Antigens

Protective immunity against blood-stage parasites aims to prevent *P. vivax* merozoites from invading the reticulocytes or properly exiting invaded erythrocytes, therefore reducing parasite replication and survival within the host. For malaria, this protective immunity can be achieved naturally to a large extent by IgG antibodies [53]. Thus, individuals who acquire such protective blood-stage immunity can effectively control parasitemia and exhibit minimal clinical symptoms when infected [54, 55]. An effective blood-stage vaccine would elicit antibodies specific for parasite proteins necessary for reticulocyte invasion or parasite egress by neutralizing parasite antigens essential for these processes. For example, IgG antibodies that target the erythrocyte binding antigens PvDBPII or PvRBP2b can effectively block the essential interactions with their respective cognate host receptors Duffy antigens and Tfr1, thereby inhibiting reticulocyte invasion by *P. vivax* (Wen-Qiang He, 2019).

A study of antibody responses to a panel of *P. vivax* antigens found that IgG responses against Pv12, Pv41, and PVX_081550 were strongly associated with clinical malaria disease in Papua New Guinea [56]. The same research group subsequently carried out serological studies using 28 *P. vivax* antigens and identified additional proteins (EBP, MSP3a, RBP1a, RBP2b, DBPII) that associated with reduced prospective risk of *P. vivax* malaria episodes in a naturally exposed cohort in Papua New Guinea [57].

1.9 Rationale, Hypothesis, and Goal of the Study

Given the prior literature supporting a select number of vivax antigens as promising vaccine candidates, further characterization of natural IgG responses to these antigens in other malaria-transmission settings may inform how their natural immunogenicity may influence vaccine responses to these antigens. For instance, pre-existing antibodies generated against an antigen from prior infections can shape and potentially interfere with the antibody response generated by a vaccine targeting that antigen [58, 59] or, in the case of viral vector vaccines, antigens that displayed by the modified viral vector [60]. On the other hand, identifying antigenic targets that naturally elicit robust, durable antibody responses can help focus vaccine development on candidate antigens that would benefit from boosting during natural exposure to the parasite.

To this end, we conducted a study to evaluate naturally acquired IgG antibody responses against nine *P. vivax* vaccine candidate antigens in Brazilian patients with acute vivax malaria who were longitudinally followed for a period of up to 180 days. We chose Brazil as a study site given that *P. vivax* in South America has been consistently understudied, yet any *P. vivax* vaccine must be able to be applied across Asia and the Americas. Furthermore, the recent resurgence of *P. vivax* in Brazil underscores the need to investigate acquired malaria immunity to promising malaria vaccine candidates in this region. By measuring the IgG reactivity to each of the antigens by multiplex immunoassay during and after acute vivax episode for each patient, we were able to compare the longevity of antibody responses between *P. vivax* antigens. Lastly, we

examined associations between patient age and prior malaria exposure and the magnitude and durability of IgG responses to each vivax antigen.

Materials and Methods

2.1 Ethical Statement

The human subjects research protocol for the collection of plasma samples for this study was originally approved by the Institutional Review Board (IRB) of the Federal University of Juiz de Fora, Brazil (Protocol# 262.875/2013). Written informed consent was obtained from all the study participants. The use of de-identified samples and subject metadata from this original study was approved as exempt human subjects research by the Indiana University IRB (Protocol# 2001093209).

2.2 Study Site and Participants

Details of the study site and participants were previously described [61]. Briefly, adult study participants were recruited from the farming settlements near four cities (Acrelândia, Califônia, Jéssica, Plácido) in northwestern Brazil, located just north of the border with Bolivia (Figure 5)

a)



b)



Figure 5. Location of study sites

Location of study sites shown a) on map of South America and b) on detailed map of border between Brazil and Bolivia. Images are from Google Maps data, ©2021 Google, INEGI.

These settlements are in an area of the Amazon Basin where *P. vivax* transmission occurs at a low-level year-round with no reported cases of *P. falciparum* malaria since early 2012 [44, 62].

2.3 Study Design

Between May 2013 and August 2014, 47 study participants with acute, uncomplicated *P. vivax* malaria were enrolled and longitudinally followed up to 180 days. The study inclusion criteria are children and adults aged 12 to 64 years with acute, febrile vivax malaria diagnosed first by thin smear with further *P. vivax* parasite species-specific confirmation via PCR vivax positive. The exclusion criteria for the study are chronic illness, history of immunodeficiency or use of immunosuppressive medications, recent malaria or use of anti-malarial medication in the 30 days prior to acute malaria presentation, and pregnancy.

Venous blood samples were collected into heparinized tubes from all study participants at the time of admission for acute vivax malaria (Day 0) and on post-admission days 30, 60, and 180 for those participants who were able to complete follow up. At the time of enrollment, all acute vivax cases were treated with anti-malaria drugs (25 mg/kg of chloroquine over 3 days and 0.5 mg/kg/d of primaquine for 7 days) in accordance with the Ministry of Health of Brazil's guidelines. Study participants remained malaria-free at the time of following blood collections (confirmed by microscopy and PCR test) and between the time points of the blood collections (confirmed by microscopy). Plasma was separated from blood cells by centrifugation within six hours of collection, aliquoted, and stored at -20° C in Dr. Kézia Scopel's

laboratory in Juiz de Fora, Brazil. Aliquots were shipped to Indiana University School of Medicine on dry ice in November 2021. For this study, we received a total of 127 plasma samples of 47 participants. Only eight participants were able to complete all 4 time points (Tables 1).

Donor ID	Day 0	Day 30	Day 60	Day 180
001		X	X	X
002		X	X	
003	X	X	X	X
004	X	X		
005	X	X	X	
006	X	X	X	
007	X	X		
008	X	X	X	X
009	X	X		
010	X	X	X	
011	X	X	X	
012	X	X	X	X
013	X	X	X	X
014	X	X	X	X
015	X	X	X	
016	X	X	X	
017	X		X	X
018	X	X	X	
019	X	X		
020	X	X		
021	X	X	X	
022	X	X	X	
023	X	X	X	X
025	X	X	X	
026	X	X	X	
027	X	X	X	X
029	X	X		
030	X	X		X
031	X	X		X
032	X	X	X	X
033		X		X
034	X	X		
035	X	X		
036	X	X		
039	X	X		X
040	X	X		X
041	X	X		
042	X	X		
043	X	X		
044	X	X		X
046	X	X		
047	X			X
049	X			
051	X			X
056	X			X
057	X			X
062	X			X
Total	44	40	22	21

Table 1. Available plasma samples by participant and timepoint

X indicates presence of plasma samples.

2.4 Study Proteins

Recombinant proteins were kindly provided by Dr. Julian C. Rayner (Cambridge Institute, United Kingdom) and Dr. Wai-Hong Tham [Walter and Eliza Hall Institute (WEHI), Australia] (Table 2).

PlasmoDB ID	Protein	Description	Protein MW	bio-linker His tag (4.2 kDa)	rat CD4 tag (20.4 kDa)	Total MW (kDa)	Source
PVX_097720	MSP3a	Merozoite Surface Protein 3a	90.6	Yes	No	~95	Rayner Lab
PVX_088910	PvGAMA	<i>Plasmodium vivax</i> GPI- anchored Micronemal antigen	80.8	Yes	No	~85	Rayner Lab
PVX_113775	Pv12	<i>Plasmodium vivax</i> 12	38.6	Yes	No	~43	Rayner Lab
PVX_110810	DBP (full length)	Duffy binding protein ectodomain	117.1	Yes	Yes	~142	Rayner Lab
PVX_000995	Pv41	<i>Plasmodium vivax</i> 41	44.1	Yes	Yes	~68	Rayner Lab
PVP01_0102300	EBP (DBP 2)	Erythrocyte Binding Proteins	95.1	Yes	Yes	~120	Rayner Lab
PVX_081550	START protein putative	StAR-related lipid transfer protein putative	56.9	Yes	Yes	~81	Rayner Lab
-	CD4	rat Cd4 domain 3 and 4 tag (Cd4d3+d4)	20.3	No	No	~24.5	Rayner Lab
PVX_094255	PvRBP2b	<i>Plasmodium vivax</i> reticulocyte binding protein 2b	152.8	No	No	~153	Tham Lab
PVX_098585	PvRBP1a	<i>Plasmodium vivax</i> reticulocyte binding protein 1a	117.9	No	No	~118	Tham Lab

Table 2. Description of proteins used in study including source

MW = molecular weight, kDa = kiloDaltons

Seven *P. vivax* proteins and one CD4 protein were expressed and purified by the Rayner lab as previously described [63]. Recombinant proteins were expressed with relevant tags as indicated in Table 3. All *P. vivax* recombinant proteins were expressed using the Salvador I reference genome [48]. The purity and integrity of the expressed *P. vivax* recombinant proteins were confirmed by SDS PAGE. Additional PvRBP2b recombinant protein was produced at IUSM with assistance from Dr. Quyen Hoang's laboratory for teaching purposes and for use in optimization experiments. The method to express PvRBP2b recombinant proteins is described in Appendix B.

2.5 SDS-PAGE and western Immunoblot Assay

To verify protein integrity prior to coupling to beads, approximately 2 µg of each recombinant protein was resolved by sodium dodecyl sulphate-polyacrylamide polyacrylamide gel electrophoresis (SDS PAGE) and detected by western immunoblotting using Bio-Rad's standard immunoblotting protocol. Briefly, protein samples with or without the reducing agent β-mercaptoethanol (Catalog: 161-0710, Bio-Rad) were diluted in Laemmli sample buffer (Catalog: 161-0737, Bio-Rad) and incubated at 90°C for 10 mins. Protein preparations were loaded onto duplicate 4-20% polyacrylamide gels (Catalog: 4568096, BIO-RAD) alongside a molecular weight marker (Catalog: 1610374S, Bio-Rad) and resolved on a Mini-PROTEAN Tetra cell system (BIO-RAD) in 1x SDS-PAGE running buffer at 100 V for 90 mins. To visualize the band, one gel was stained with Coomassie G-250 (Catalog: 24620, ThermoFisher Scientific) overnight at room temperature (RT). Gel images were captured using the ChemiDoc Touch Imaging system (Catalog: 732BR1564, Bio-Rad). The second duplicate

gel was washed with distilled water and incubated in 70% ethanol for 5 mins. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using iBlot 2 Gel Transfer Device (Catalog: IB21001, ThermoFisher Scientific) for 12 mins per the manufacturer's instructions. To confirm the protein transfer, the PVDF membrane was stained with Ponceau S (Catalog: 6226-79-5, TOCRISA bioscience) for 20 mins and de-stained in Tris-buffered saline with 1% Tween (TBST) overnight at 4° C. The membrane was washed for 10 mins with TBST thrice prior to incubation with horseradish peroxidase (HRP)-conjugated 6xHis-Tag mouse monoclonal antibody (Catalog: 29-091596, HRP-66005, Proteintech) at RT with gentle agitation for 30 mins. The membrane was washed an additional three times, and His-tagged proteins were detected by chemiluminescence using the Super Signal West Dure Extended Duration Substrate per the manufacturer's instructions (Catalog: 34075, ThermoFisher Scientific) and documented using the ChemiDoc Touch Imaging system.

2.6 Flow Cytometry-Based Erythrocyte Binding Assays

2.6.1 Preparation of Washed Red Blood Cells from the Whole Blood

Whole blood was collected intravenously from healthy volunteers in the K3 EDTA BD vacutainer tube (Catalog: 366450, Becton-Dickinson) and centrifuged at 500 G for 15 mins. The supernatant plasma and the buffy coat were discarded, and the remaining packed red blood cells (pRBCs) were washed with 2X volume of prewarmed (~37°C) RPMI Medium 1640 (Catalog: 11875-093, Gibco) 3x for 5 mins each with 500 G at 22°C with a slow break setting. The pRBCs were further washed with 6 ml of 1% BSA-PBS (1 mg BSA in 100 ml PBS, PH: 7.4) 3x at 500 G for 5 mins each, and the

supernatant was discarded after each wash. Packed RBCs were used in downstream flow-binding assays at a working concentration of 1×10^6 red blood cells per 50 μ l reaction diluted in 1% BSA-PBS.

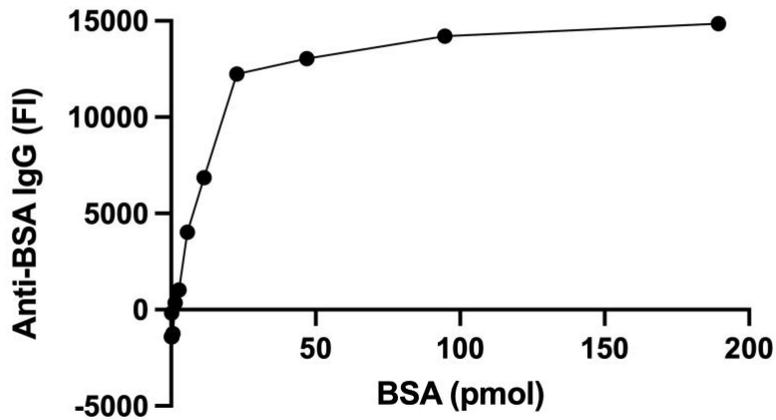
2.6.2 Flow-Binding Assay

We used a previously described flow-binding assay [64] to evaluate the ability of recombinant proteins to differentially adhere to the surface of reticulocytes and mature RBCs. Thioredoxin (TxA) was used as non-specific RBC binding control. We did not have positive control for this assay. Approximately 1×10^6 pRBCs were incubated with each protein million diluted one of three final protein concentrations (5, 15, or 25 μ g/ml) in 1% BSA-PBS for 4 hours at RT. Cells were washed 3x with 200 μ l of 1% BSA-PBS and incubated with Alexa Fluor 647 conjugated anti-penta-His monoclonal antibody (Catalog: 1019252, Qiagen) diluted to a final concentration of 1:25 in 1% BSA-PBS for 1 hour at 4°C in the dark. Cells were washed and incubated with 200 μ l/reaction of thiazole orange, an RNA-intercalating reagent (Catalog: 349204, BD Retic-count™ Reagent, BD Biosciences), for 30 mins at RT in the dark. Cells were again washed 3x with 1% BSA-PBS. After the final wash, the stained cells were resuspended in 1% BSA-PBS, and 100,000 cells/sample were acquired on a flow cytometer (Catalog: 337175, Becton-Dickinson). Flow cytometry data were analyzed using FlowJo software (v10.6.2, Becton Dickinson).

2.7 Determination of Optimal Protein Concentration

We used bovine serum albumin (BSA, 66 KDa) to determine the optimum protein concentration for coupling using the monoplex immunoassay method. Details of general coupling techniques are described under section 2.8; however, we used two-fold BSA serial dilution [500 µg/ml (757.58 pmol) to 0.05 µg/ml (0.07 pmol)] to couple with ~12,000 magnetic beads for each dilution. Anti-BSA biotin tagged antibody was used to detect BSA, followed by PE-streptavidin to detect the anti-BSA biotin. The light emitted by PE-Streptavidin was captured by the Luminex machine to generate MFI. We have chosen 40 pmol as an optimal protein concentration for each study protein to couple with a million magnetic beads after optimization of antigen-bead coupling assay using BSA (Figure 6).

a)



b)

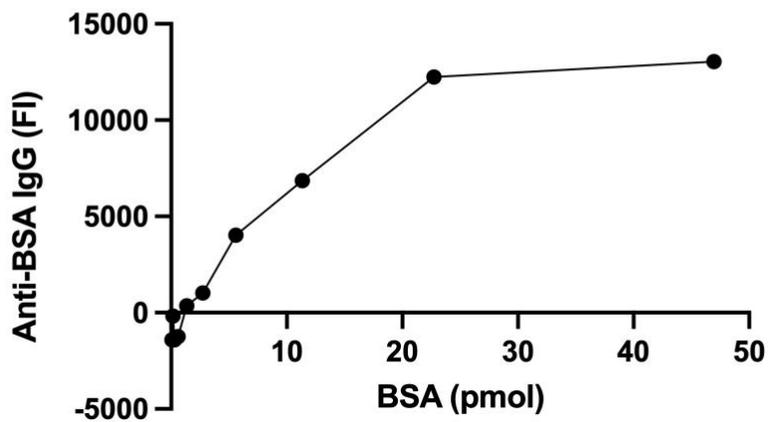


Figure 6. Optimization of antigen-bead coupling using bovine serum albumin (BSA)

Each plot represents two-fold BSA serial dilutions and anti-BSA antibody FI readings. Each anti-BSA IgG FI values represents mean of the duplicates. Both figures represent same data to demonstrate the saturation point.

2.8 Coupling of Proteins to Magnetic Beads

Each *P. vivax* antigen and control protein was coupled to one color-coded magnetic Luminex® microsphere bead regions (Table 3).

Index	Bead Region	Protein	Molecular Weight (kDa)
1	020	<i>Plasmodium vivax</i> reticulocyte binding protein 2b (PvRBP2b)	152
2	021	<i>Plasmodium vivax</i> reticulocyte binding protein 1a (PvRBP1a)	117
3	025	Thioredoxin (TxA)	16
4	026	StAR-related lipid transfer protein putative (PVX_081550)	81
5	027	Erythrocyte Binding Proteins (EBP)	120
6	028	<i>Plasmodium vivax</i> 41 (Pv41)	68
7	029	Duffy binding protein ectodomain (DBP FL)	142
8	030	rat Cd4 domain 3 and 4 tag (Cd4d3+d4)	24.5
9	033	<i>Plasmodium vivax</i> 12 (Pv12)	43
10	034	<i>Plasmodium vivax</i> GPI- anchored Micronemal antigen (PvGAMA)	85
11	035	Merozoite Surface Protein 3a (MSP3a)	95
12	036	Tetanus Toxoid	150
13	037	Bovine Serum Albumin (BSA)	66.5

Table 3. Assigned bead region for each protein in study

The coupling procedure was followed as per the manufacturer's recommended protocol (xMAP® Antibody Coupling Kit, catalog: 40-50016, Luminex Corporation). For this coupling procedure, a few important washing steps were followed. After adding distilled water or buffer, the vials were vortex and sonicated for 10 seconds each and stand it on the magnetic stand for 2 mins, followed by supernatant discard using the disposable transfer plastic pipette. Approximately 1×10^6 of each of the 13 different color-coded magnetic beads were transferred to amber color vials and washed 3x with double-distilled water followed by the addition of 500 μ L activation buffer (catalog: 11-25171, xMAP® Abc Activation buffer, Luminex Corporation). After the final wash, 480 μ L of activation buffer, 10 μ L (50 mg/ml) of Sulfo-NHS (catalog: 11-25168, xMAP® Abc S-NHS, Luminex Corporation) and 10 μ L (50 mg/ml) of EDC, hydrochloride (catalog: 11-40144, Luminex Corporation) were added, mix, and incubated at the RT for 20 minutes under constant agitation (15 to 20 RPM). After the final wash, the proteins were added to their corresponding activated color-coded magnetic beads labeled and assigned in Table 3. The samples were incubated at RT for 2 hours in the dark with constant agitation (15 - 30 RPM). After the last wash with washing buffer (catalog: 11-25167, xMAP® Abc Wash buffer, Luminex Corporation), 1 mL was added. The protein-coupled magnetic beads were counted under the Neubauer chamber and preserved under the storage buffer (wash buffer) at 4°C in the dark until use.

2.9 Determination of Optimal Plasma Dilution

To determine an optimum plasma dilution that would operate within the linear range across our study antigen-specific antibody, we performed two-fold plasma serial

dilution (1:50 to 1:3200) multiplex immunoassay using immune plasma from five individuals with recent acute vivax malaria within 60 days of blood collection [Sample ID (Timepoints): 001 (day 30), 003 (day 30), 006 (day 60), 013 (day 30), 043 (day 30)]. Similar multiplex immunoassay techniques were applied as described in Section 2.10.3. After considering the range between the lowest and highest IgG antibody responses against all *P. vivax* antigens, we chose 1:400 plasma dilution as an optimal plasma dilution for the multiplex immunoassay and downstream analysis (Figure 7).

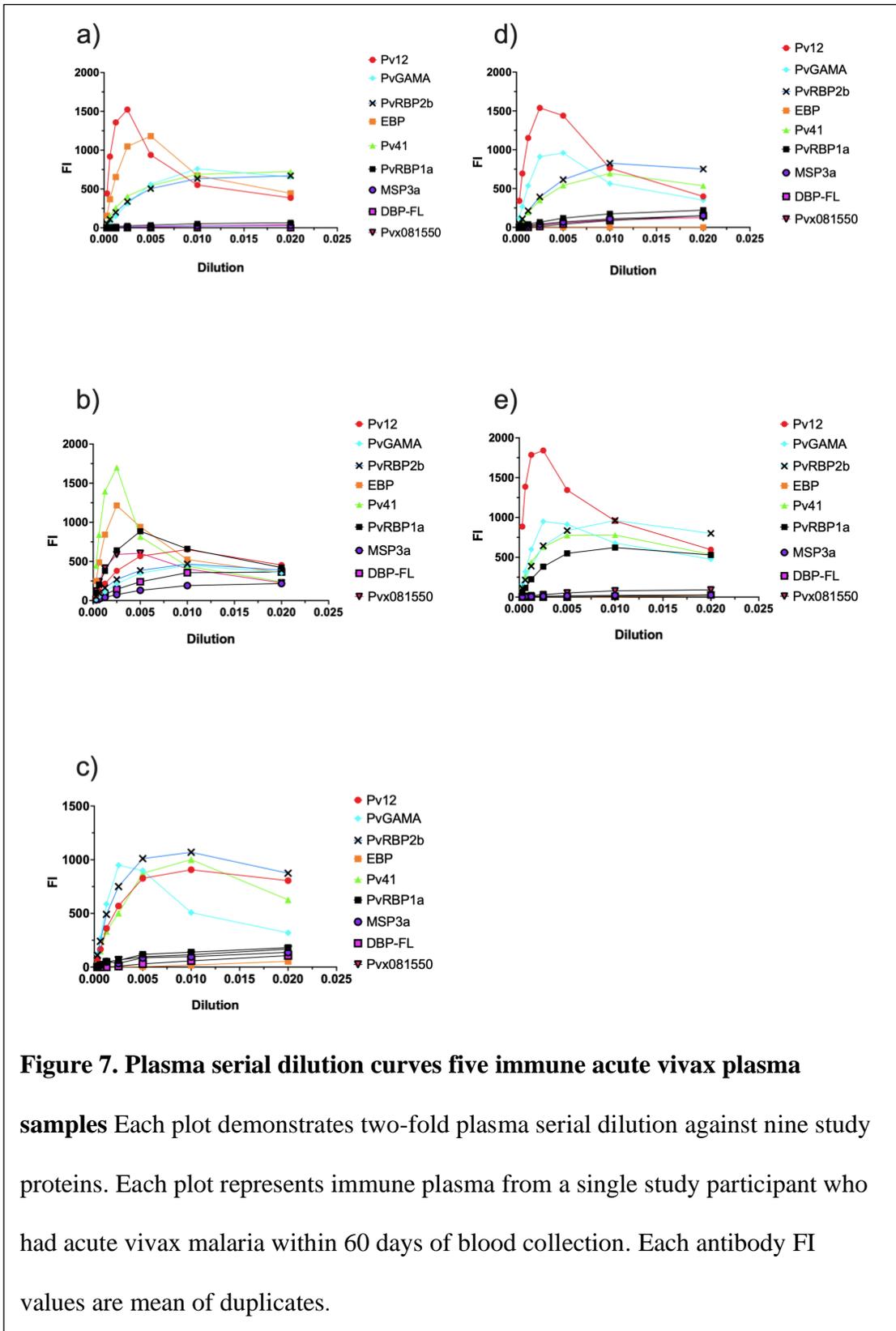


Figure 7. Plasma serial dilution curves five immune acute vivax plasma

samples Each plot demonstrates two-fold plasma serial dilution against nine study proteins. Each plot represents immune plasma from a single study participant who had acute vivax malaria within 60 days of blood collection. Each antibody FI values are mean of duplicates.

2.10 Multiplex Immunoassay Method

2.10.1 Preparation of Study Plasma

Twenty plasma samples were randomly selected from the Brazilian participants with acute vivax malaria at day 0 and day 30 time points and pooled to use as a “positive” control for vivax reactivity. Nine malaria-naïve, North American plasma samples were used as negative controls for vivax reactivity and for establishing a seropositivity threshold for reactivity against vivax antigens. To perform multiplex immunoassay, a clear supernatant plasma was used. Eighty-seven microliters volume of each plasma sample was diluted at 1:400 was prepared.

2.10.2 Preparation of Protein-Coupled Magnetic Beads

One microliter containing 500 to 800 of each protein-coupled magnetic beads were considered for one reaction. Thus, a total of 13 μ l of 13 different protein-coupled magnetic beads (13 μ l/well) was prepared.

2.10.3 Multiplex Immunoassay

We used the reagent company’s protocol and the previously published papers techniques [65, 66] to perform a multiplex immunoassay. A flat bottom 96 well dark microplate (Catalog: 655097, Greiner bio-one) was washed with 200 μ l of PBS-TN (PBS 7.4, Tween 20, NA azide) on the agitator for 15 minutes at RT. After wash, a diluted plasma (two-fold serial dilution or 1/400) in PBST (PBS 7.4, 0.01% Tween20, 1% BSA) was added to each well, followed by the addition of each protein-coupled beads (1 μ l has ~500 to 800 beads). The plasma sample and protein-coupled beads were mixed and

incubated at RT on the microplate shaker at 550 RPM for 45 mins. After washing with PBS-TN, 100 μ L of 1:500 dilution of phycoerythrin-labeled donkey anti-human IgG (catalog: ab7005, Abcam) in PBST was added to each well and incubated for 45 minutes in the dark with constant shaking. The beads were then re-suspended in 150 μ L PBS-TN and analyzed on a Multiplex MAGPIX system (Millipore) using the xPONENT 4.1 software for data acquisition.

2.11 Determination of *P. vivax* Antigen IgG Antibody Half-lives

In order to quantitatively measure each study *P. vivax* IgG antibody half-lives, we used linear regression method and half-lives equation (detail protocol is explained under statistical method: Section 2.12). One example to estimate PvRBP2b IgGs half-lives is demonstrated using three time points (day 30, 60, 180) plasma samples of one study participant (ID: 001) (Table 4, Figure 8).

Donor ID	PvRBP2b (Actual FI)	Timepoint (days)	Actual FI converted to natural log	Antilog FI (Predicted)	Residual (Actual-Predicted)
001(day 30)	1031.3	0	6.93858	1019.125311	12.17468915
001(day 60)	724.5	30	6.58548	734.8746938	-10.3746938
001(day180)	200	150	5.29832	198.680896	1.319103999

Table 4. Determination of PvRBP2b IgG antibody half-lives by linear regression

The study participants (001) actual timepoint day 30 was taken as a new timepoint (day 0) because it does not have day 0 timepoint plasma sample, and a minimum of three timepoints that follows exponential decay pattern was used to estimate *P. vivax* IgG antibody half-lives.

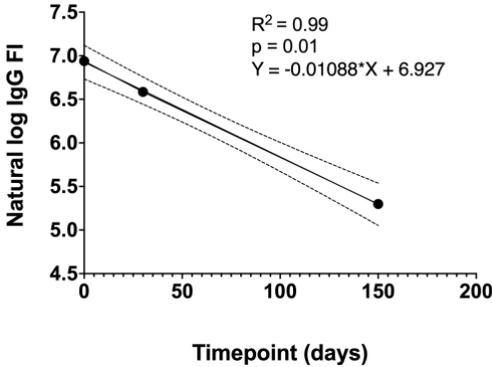


Figure 8. Linear regression plot for PvRBP2b IgG antibody kinetic profile using one study participant. IgG antibody response (FI) in three timepoint plasma samples were converted to natural log and best fit linear regression model was applied. The predicted PvRBP2b IgG antibody FI values were used to determine total IgG antibodies half-lives using half-lives equation.

Dependent variables are PvRBP2b antibody response (IgG, FI) and independent variables are the three timepoints (day 30, 60, and 180). Each *P. vivax* antigen-specific IgGs FI values were converted to natural log to get the linear regression equation. Using this equation, predicted values were generated for each timepoint. The antilog predicted FI values were then applied in the half-life's equation to determine the half-lives. PvRBP2b antibody IgGs half-lives of one acute vivax study participant (01) is 63 days (Table 4, Figure 8). Similar techniques were applied to estimate other *P. vivax* antigens-specific antibody IgGs half-lives (Table 5).

Donor ID	PvRBP2b	PvRBP1a	Pv12	PvGAMA	MSP3a	Pv41	PVX_081550	EBP	DBP-FL
001	64	267	169	68	61	73	31	107	ND
003	128	42	56	39	39	34	25	ND	27
008	75	462	224	33	54	75	55	ND	48
012	193	37	462	40	71	42	20	433	ND
013	96	87	100	28	116	99	89	ND	ND
014	98	139	90	39	87	56	35	29	ND
023	72	60	34	42	39	42	ND	ND	ND
025	26	21	51	16	17	41	ND	28	9
032	51	60	40	39	37	48	ND	ND	22

Table 5. *P. vivax* IgG antibody half-lives (days) in nine study participants

IgG antibody half-life were estimated in indicated antigen (columns) for nine study participants (rows). ND = Half-life estimate not determined due to seronegative status.

2.12 Statistical Methods

To estimate *P. vivax* IgG antibodies half-lives exponential decay curve model and half-lives equation was used. For the linear regression, dependent variables are *P. vivax* antigens antibody response (IgG) and independent variables are the four time points (day 0, 30, 60, 180). For each antigen, Median Fluorescent Intensity (MFIs) from the ‘hyperimmune’ pool control plasma were adjusted MFI of test samples to reduce the effects of plate-to-variation using a previously described normalization procedure developed for multiplex assays to measure antibodies to *P. falciparum* antigens [67]. Background reactivity was subtracted for each antigen, any negative MFIs after backgrounds were set to 0. CD4 MFI were subtracted from the four *P. vivax* antigens that have CD4 tag with them (Table 2). To estimate the longevity of antigen-specific antibody responses, we applied a simplified model in which antibodies are generated in a single boost generated by acute malaria (% MFI_{max}). Time verses percent of maximum median fluorescent intensity (MFI) during or after acute malaria (% MFI_{max}) were plotted for each *P. vivax* antigen using exponential decay curve equation:

$$[\% \text{ MFI}_{\text{maximum}}]_{\text{time}} = [\% \text{ MFI}_{\text{max}}]_t 0e^{-kt} + \text{Plateau}$$

The MFI IgG values were fitted to all downward sloping data points (decay phase) where [% MFI_{max}]₀ and [% MFI_{max}]_t are concentrations at time 0 and t respectively, *k* is a rate constant and plateau is the % MFI_{max} at the end of the decay. Antibody half-lives are estimated using the simple formula:

$$T1/2 = \log (2)/ \text{Decay constant } (\lambda)$$

To determine associations between patient age and prior malaria exposure and the magnitude and durability of IgG responses to each vivax antigen, a simple linear

regression method was applied. Each patient age and prior malaria episodes are independent variable respectively against each dependent variable *P. vivax* IgG antibody response and its half-lives. For each relationship assessment, a significance threshold was $p < 0.05$. p values were adjusted with Bonferroni correction.

To compare *P. vivax* antibody IgGs half-lives mean-rank, Friedman nonparametric statistical method was used, followed by post-hoc test: Dunn's non-parametric method, for the pairwise multiple comparisons. *P. vivax* antigens' antibodies IgG half-lives were first ranked within study participant and then mean of ranks of each study *P. vivax* were compared using a significance level of $\alpha = 0.05$. All statistical tests were performed using R software and GraphPad prism 9.

Results

3.1 Study Site and Participant Characteristics

The current project made use of plasma samples collected from 47 adults (median age 32 years, interquartile range (IQR) 23.5 – 45.5) who participated in a longitudinal cohort study of acute vivax malaria conducted from May 2013 through August 2014. Participants were residents of farming settlements in the cities of Acrelândia, Califônia, Jéssica, and Plácido, which are located in northwestern Brazil just north of the Bolivian border (Figure 5). Most participants were males (72.3%) (Table 6). All participants had symptomatic, PCR-confirmed *P. vivax* malaria with *P. vivax* parasite density (parasites/ μ l) of median 1200, IQR 1980 (300 - 2280) by peripheral blood smear at acute presentation. Self-reported histories of previous malaria exposure and the time since the last infection are shown in Table 1. Due to the migratory nature of the study population, retention was poor, with only 8 of 47 participants providing samples at all four time points through 180 days of follow-up.

Characteristics	City				All
	Acrelândia	Califónia	Jéssica	Plácido	
Total participants (%)	10 (21.3)	6 (12.3)	19 (40.4)	12 (25.5)	47 (100)
Male (%)	7 (20.6)	3 (8.8)	15 (44.1)	9 (26.4)	34 (100)
Female (%)	3 (23.1)	3 (23.1)	4 (30.8)	3 (23.1)	13 (100)
Median age in years (interquartile range)	31 (22.5-44.2)	41 (33.7 - 52)	30 (25.5 - 45)	23 (16.7 – 40.7)	32 (23.5 – 45.5)
Median <i>P. vivax</i> parasite/ mm ³ at diagnosis (range)	1605 (90 - 24000)	1140 (120 - 1920)	690 (90 -5700)	1980 (120 -28500)	120 (90 - 28500)
Median years residing in Amazonian state (range)	21 (8 - 43)	33 (7 - 54)	30 (18 - 54)	19 (7 - 64)	26 (7 - 64)
Median number of prior malaria episodes (range)	1 (0 - 6)	3 (0 - 15)	3 (0 - 30)	2 (0 - 10)	3 (0 - 30)
Median years since last malaria episode (range)	1.5 (0 - 20)	5 (0 - 12)	4.5 (0 - 28)	1 (0 - 17)	2.5 (0 - 28)

Table 6. Characteristics of study participants by city

3.2 Description and Characterization of Antigens used for this Study

For this study, nine *P. vivax* vaccine candidate antigens were used (Table 2). All the proteins were previously associated with reduced risk of clinical vivax malaria and considered as highly potential blood stage vaccine candidate antigen. We have included one member of merozoite surface antigen family (MSP3a), two members of PvRBP family (PvRBP1a and PvRBP2b), two members of Duffy binding protein family (DBP-FL and EBP), two members of 6-cysteine family (Pv12 and Pv41), *P. vivax* GPI-anchored micronemal antigen (PvGAMA) and a novel hypothetical proteins PVX_081550 also called StAR-related lipid transfer protein. Six antigens are involved in erythrocyte binding and invasion (PvDBP-FL, PvEBP, PvGAMA, PvMSP3a, PvRBP1a, PvRBP2b). These antigens have been strongly associated with a reduced risk of clinical vivax malaria along with Pv12 and Pv41 [56]. Notably, IgG antibodies against PvRBP2b, PvRBP1a, EBP, DBPFL, RBP1a, and PVX_081550 were previously found to be protective against *P. vivax* malaria in prospective cohort studies [68, 69]]. Tetanus toxoid was chosen as a positive control for antibody reactivity against a vaccine that is universally administered in this population. We used three additional proteins to control for background reactivity (BSA, thioredoxin, and rat CD4) given that some of the antigens were expressed as fusion proteins (Table 2).

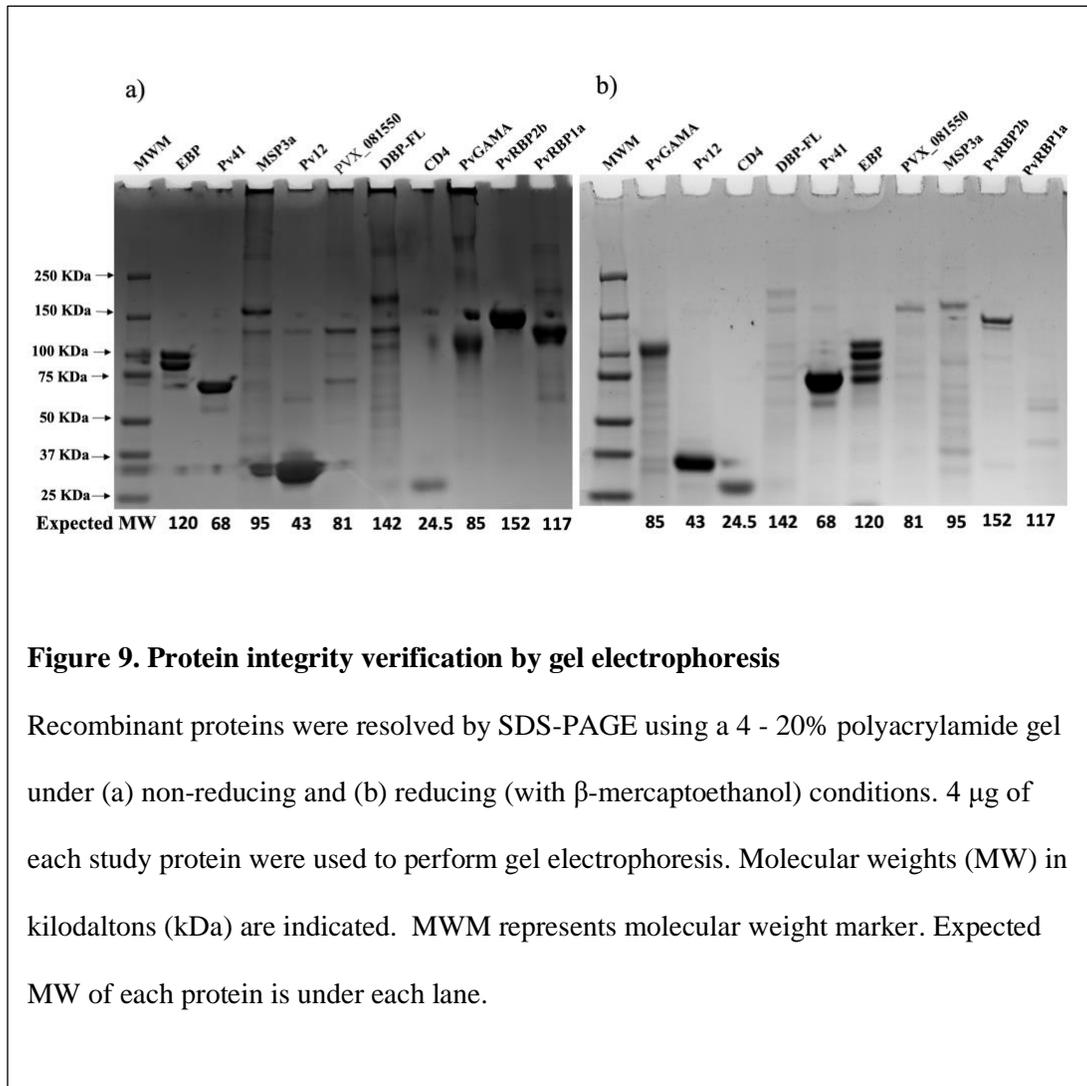
3.2.1 Verification of Protein Integrity

The interpretability of serological results obtained from bead-based immunoassays is dependent on the ability to covalently couple intact, soluble, monomeric antigens onto microspheres. Therefore, reducing and non-reducing sodium dodecyl-

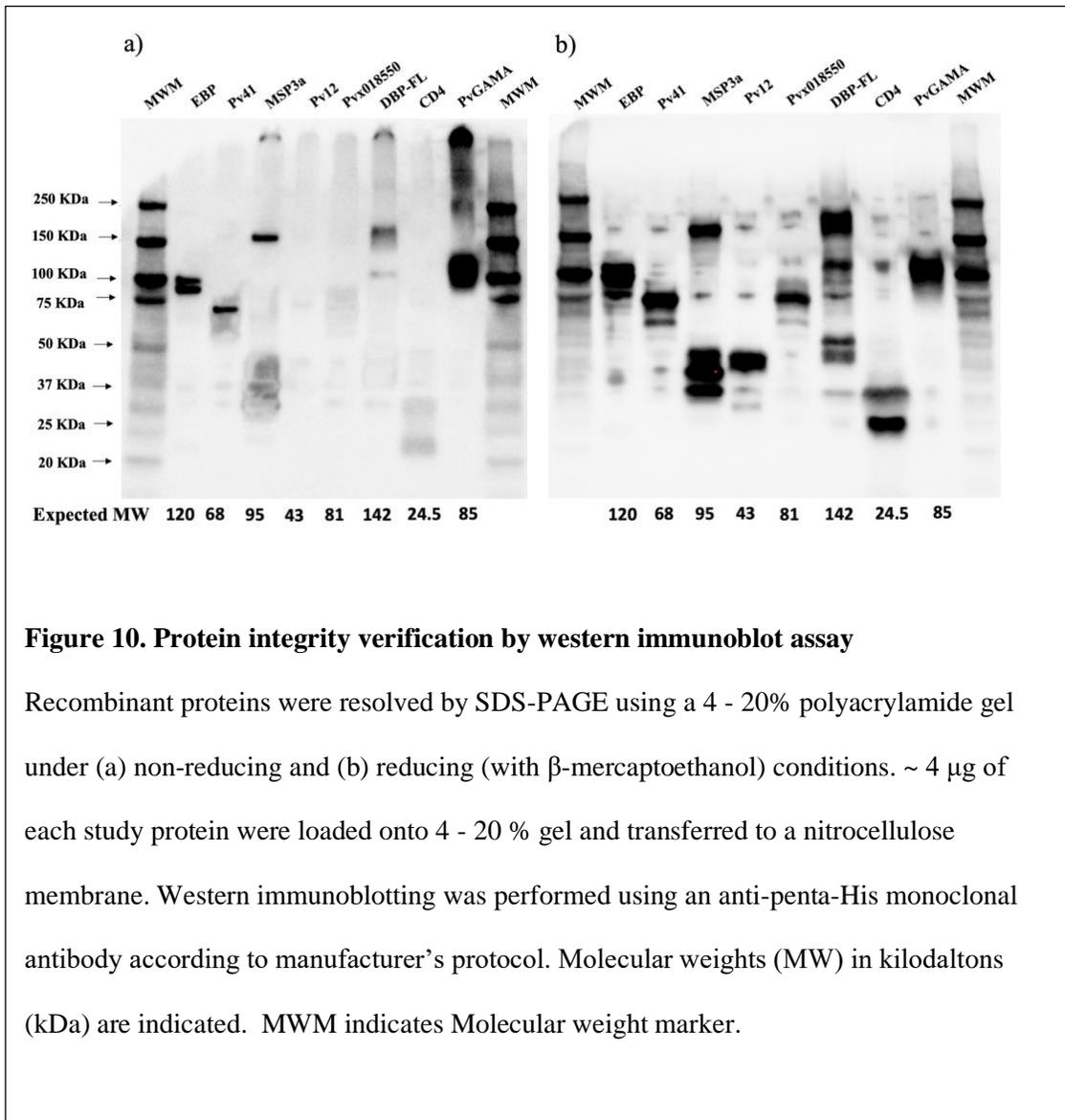
sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblotting were performed to determine the integrity and aggregation potential of the purified recombinant *P. vivax* antigens used immediately before coupling.

Under non-reducing conditions, EBP, Pv41, Pv12, PvRBP2b and PvRBP1a, demonstrated predominant single bands near the expected MW by SDS-PAGE, suggesting that these antigens were intact monomers at the time of coupling. Under the same conditions, PvMSP3a, Pv_x018550, DBP-FL, PvGAMA demonstrated bands near their respective MWs but also additional high MW bands (>250 kDa), the latter suggesting protein aggregation (Figure 9a).

To determine if aggregates were due to the formation of disulfide-linked multimers, we resolved the proteins by SDS-PAGE under reducing conditions with β -mercaptoethanol. Reducing the disulfide bonds eliminated the high MW bands for these four proteins (Figure 9b). Notably, both reducing and non-reducing gels revealed that PvMSP3a, Pv_x018550, DBP-FL, and PvGAMA demonstrated evidence of aggregation as noted with bands of the respective proteins. EBP under reducing conditions demonstrated double bands that were both less than the expected MW of 120 kDa, suggesting degradation products. PvRBP1a showed weak bands which could be due to either less concentration or defect caused by reducing agent or freeze thaw prior to running a reducing SDS-PAGE (Figure 9b).



For the recombinant proteins with a poly-His-tag (Table 2), protein aggregation and integrity were confirmed by western immunoblot using an anti-His monoclonal antibody. Large MW bands were confirmed to be disulfide-linked multimers (Figure 10a). Bands below the expected MW were confirmed to be degradation products still containing the C-terminal His-tags, which was particularly notable for PvMSP3a (Figure 10b).



3.2.2 *Plasmodium vivax* Recombinant Proteins Functional Characterization

Plasmodium vivax recombinant proteins containing the 6xHis tag (Table 2) were evaluated in flow cytometry-based erythrocyte binding assays (F-EBA) [64] to determine their ability to bind specifically to reticulocytes (Figure 11).

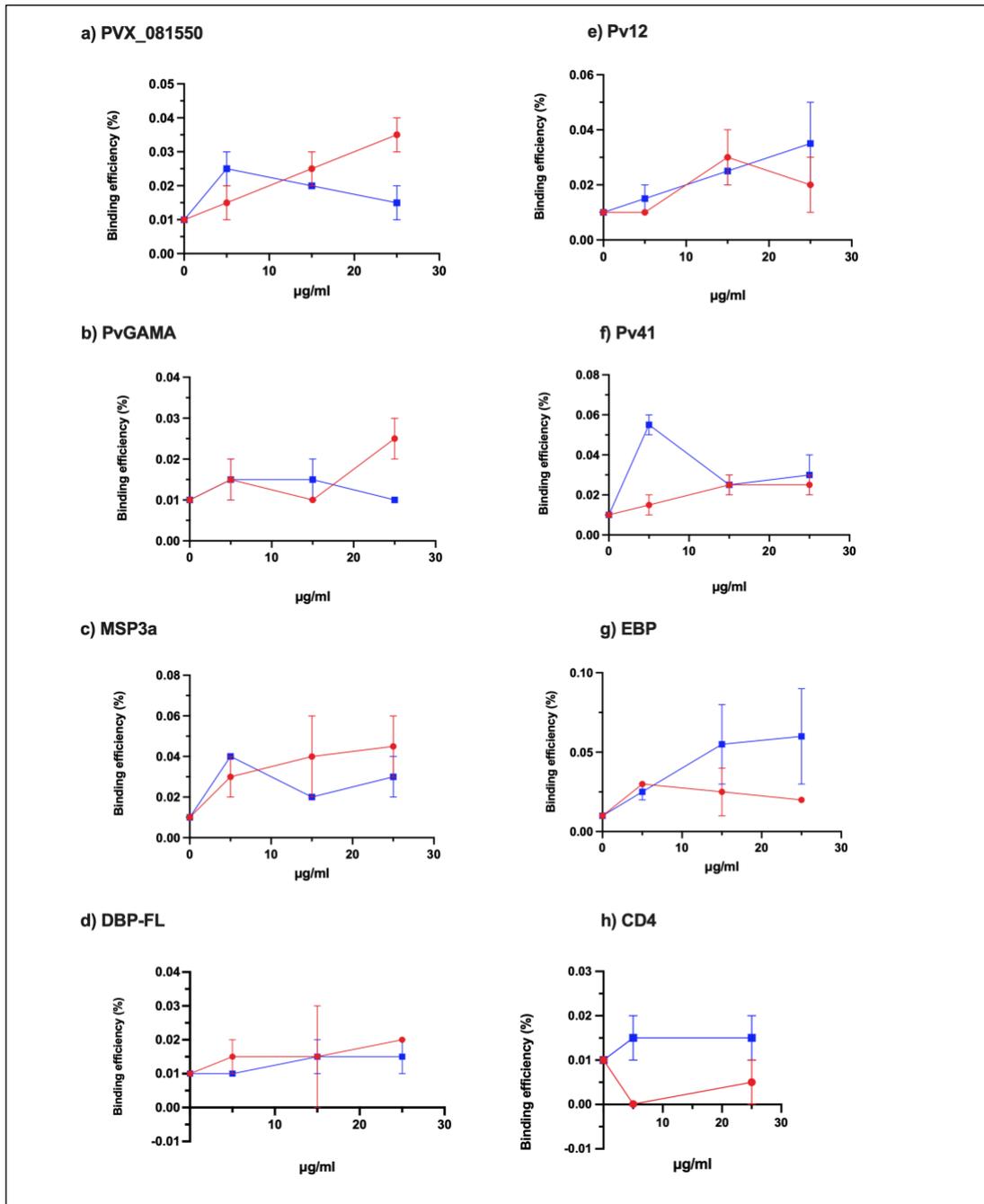


Figure 11. Dose response of *P. vivax* recombinant proteins in flow cytometric erythrocyte binding assay. Erythrocytes were incubated with increasing concentrations of the indicated *P. vivax* proteins (red) and same pmol of thioredoxin control (blue). The x axis is shown on pmol scale to facilitate comparisons between test antigen with thioredoxin. Reticulocyte-specific binding was assessed using the flow cytometry-based erythrocyte binding assay (described in Methods). Binding efficiency was calculated as percent of antigen-positive, thiazole-orange-positive reticulocytes over total antigen-positive erythrocytes. Shown are median values of two technical replicates. Error bars represent 95% confidence interval. Data represents one experiment per antigen.

We evaluated the binding ability of DBP, PvGAMA and EBP, which have previously been shown to bind to erythrocytes [40, 49, 70-73], as well as the novel hypothetical protein PVX_081550 and the merozoite antigens Pv12 and Pv4, which, to our knowledge, have not previously been tested in erythrocyte binding assays.

Although PvRBP1a and PvRBP2b were originally expressed as His-tag fusion proteins, their His-tags were cleaved by our collaborators prior to our receipt of these two proteins. Thus, PvRBP2b and PvRBP1a were excluded from F-EBAs.

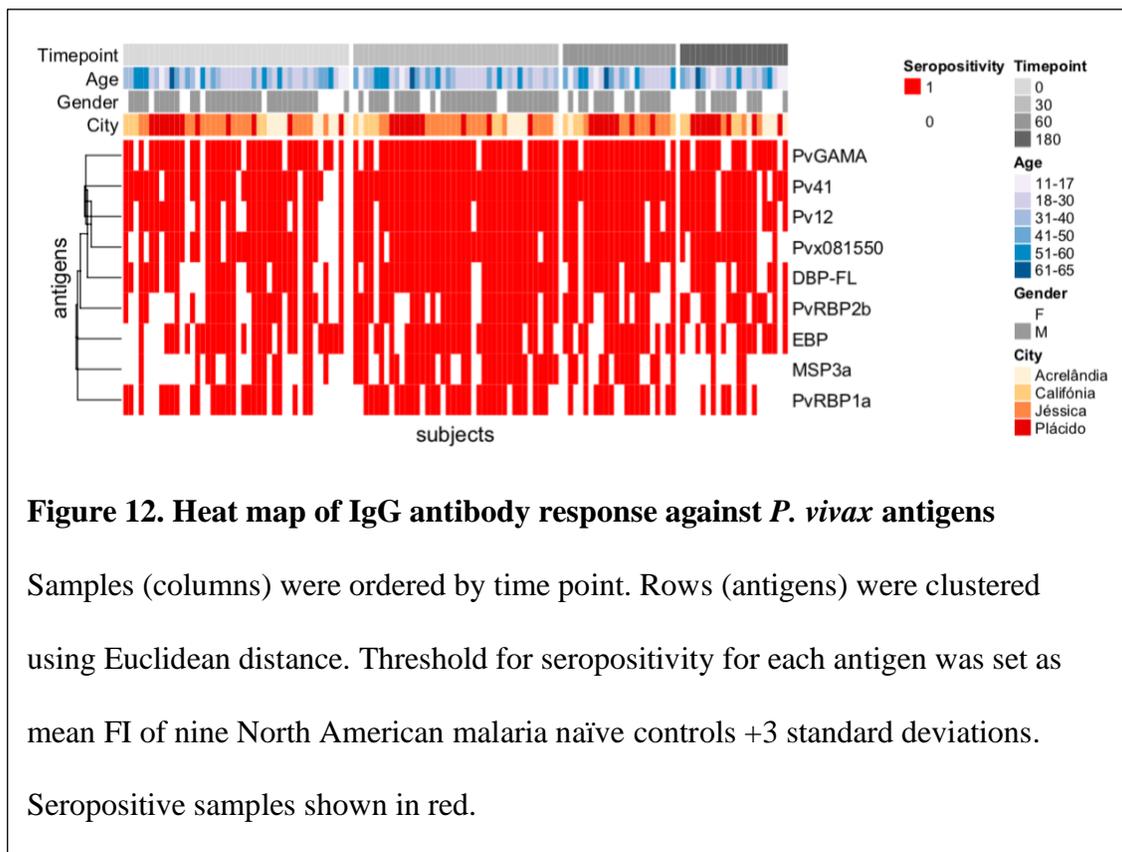
Erythrocyte binding assessed by F-EBA was evaluated at only three concentrations (5 µg/ml, 15 µg/ml, 25 µg/ml) due to the limited availability of each test protein. For each test antigen, we compared binding against the same pmol of the non-specific binding control thioredoxin (at each concentration of test antigen) to provide a fair comparison in terms of absolute numbers of molecules given the large differences in molecular weight.

A major limitation of our experiment is the lack of positive control for erythrocyte binding. However, we are able to evaluate binding over background by comparing against the non-specific binding control thioredoxin. PVX_081550, PvGAMA, MSP3a, and PvDBP-FL each demonstrated modest binding at the highest concentrations, whereas Pv12, Pv41, EBP and CD4 did not (Figure 11). Our results were consistent with the earlier studies that demonstrated PvGAMA and DBP (PvDBP II) binding to reticulocytes (Baquero et al., 2017; Wertheimer & Barnwell, 1989). In contrast to previous studies, we did not observe reticulocyte binding by EBP, which has been shown to be an alternative parasite ligand for reticulocyte invasion [73]. The important findings of this preliminary assay are the reticulocyte binding ability demonstrated by novel hypothetical protein PVX_081550 that was not tested before.

3.3 Multiplex Immunoassay Serological Data

3.3.1 Seropositivity Across All Study Participants and Four Timepoints

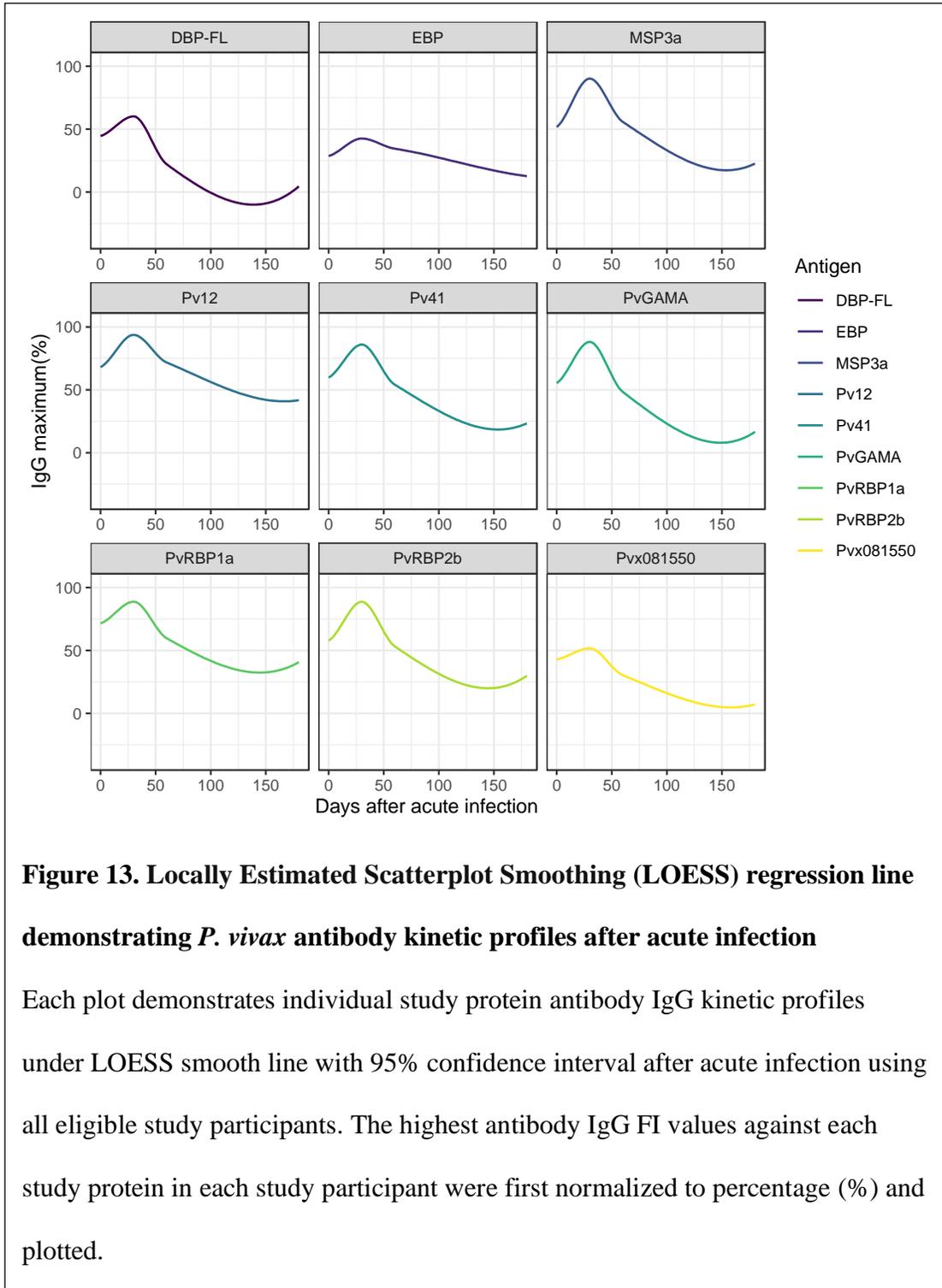
A broad assessment of IgG seropositivity against all antigens revealed that PvGAMA, Pv41, and Pv12 were highly immunogenic and clustered together. PVX_081550, DBP-FL, PvRBP2b and EBP formed an intermediate reactivity, MSP3a and PvRBP1a constituted a lowest reactivity cluster. Regarding seroconversion and seroreversion, highly immunogenic antigens PvGAMA, Pv41 and Pv12 demonstrated early seroconversion and delayed seroreversion compared to MSP3a and PvRBP1a. To further analyze, we performed kinetic profile analysis with each study *P. vivax* antigens and seroreactivity ranking among nine study *P. vivax* antigens (Figure 12).



3.3.2 Longitudinal Profiling of IgG Reactivity Against Multiple *P. vivax* Antigens after Naturally Acquired Acute Vivax Malaria

We determined longitudinal profiles of *P. vivax* antigen-specific IgG reactivity for 37 participants with plasma samples available at ≥ 2 timepoints (Table 1), of which the first is either day 0 or day 30. Here, we assumed a model in which *P. vivax*-specific IgG antibodies rapidly rise during acute *P. vivax* malaria with the maximum observed antibody response occurring at either Day 0 or Day 30 then decay exponentially after this peak.

We used locally estimated scatterplot smoothing (LOESS) to help visualize how antigen-specific IgG reactivity varies over time. We first determined which among the two earliest time points (day 0 and day 30) demonstrate the highest antibody responses after acute vivax malaria infection. Day 30 timepoint plasma samples showed the highest IgG (%) across nine *P. vivax* study antigens. After that, we analyzed the individual proteins boosting and decaying pattern after maximum antibody IgG (%). All study *P. vivax* antigens' IgG (%) demonstrated peak boosting at day 30 timepoint (Figure 13).



When all nine *P. vivax* antigens IgG antibody kinetics were compared, we observed EBP, PVX_081550, and DBP-FL as weak boosting among which DBP-FL decayed fastest (around day 60 to 100) whereas Pv12 IgG displayed best boosting with reaching the peak at day 30 and remained last one to decay, followed by PvRBP1a and other proteins (Figure 14).

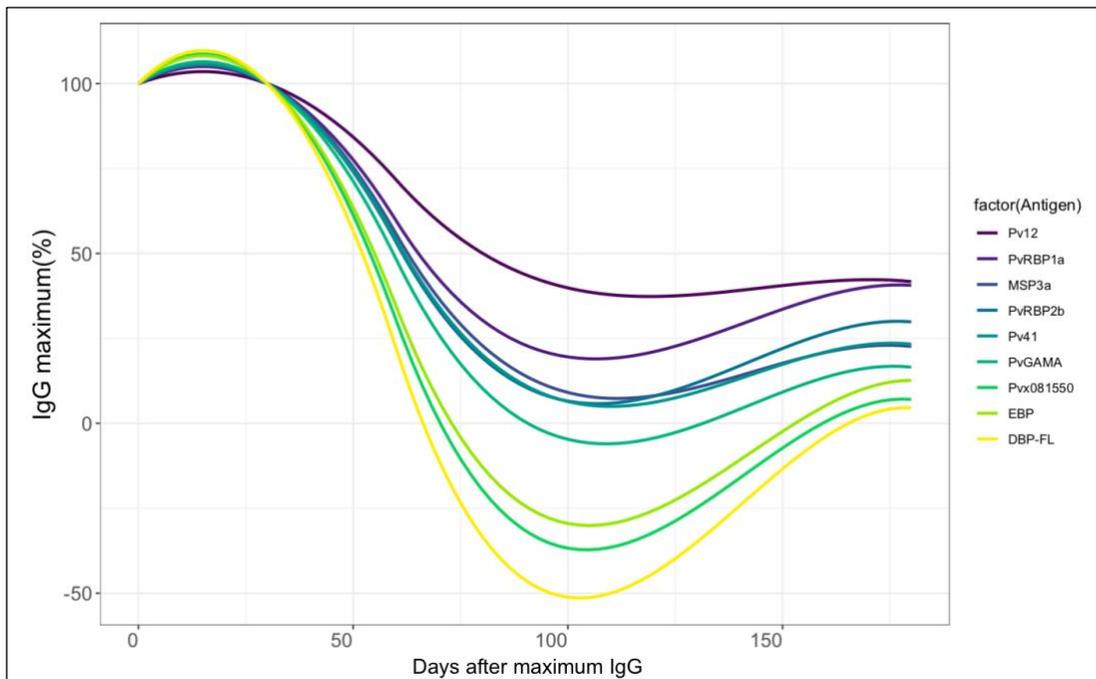


Figure 14. Locally Estimated Scatterplot Smoothing (LOESS) regression line demonstrating *P. vivax* antibody kinetic profiles across nine study proteins.

The plot compared individual study protein antibody IgG kinetic profiles under LOESS smooth line after maximum antibody response IgG using 37 study participants. The highest antibody IgG FI values against each study protein in each study participant were normalized to percentage (%). The estimates below 0 were due to few individuals who have day 180 values more than day60 values.

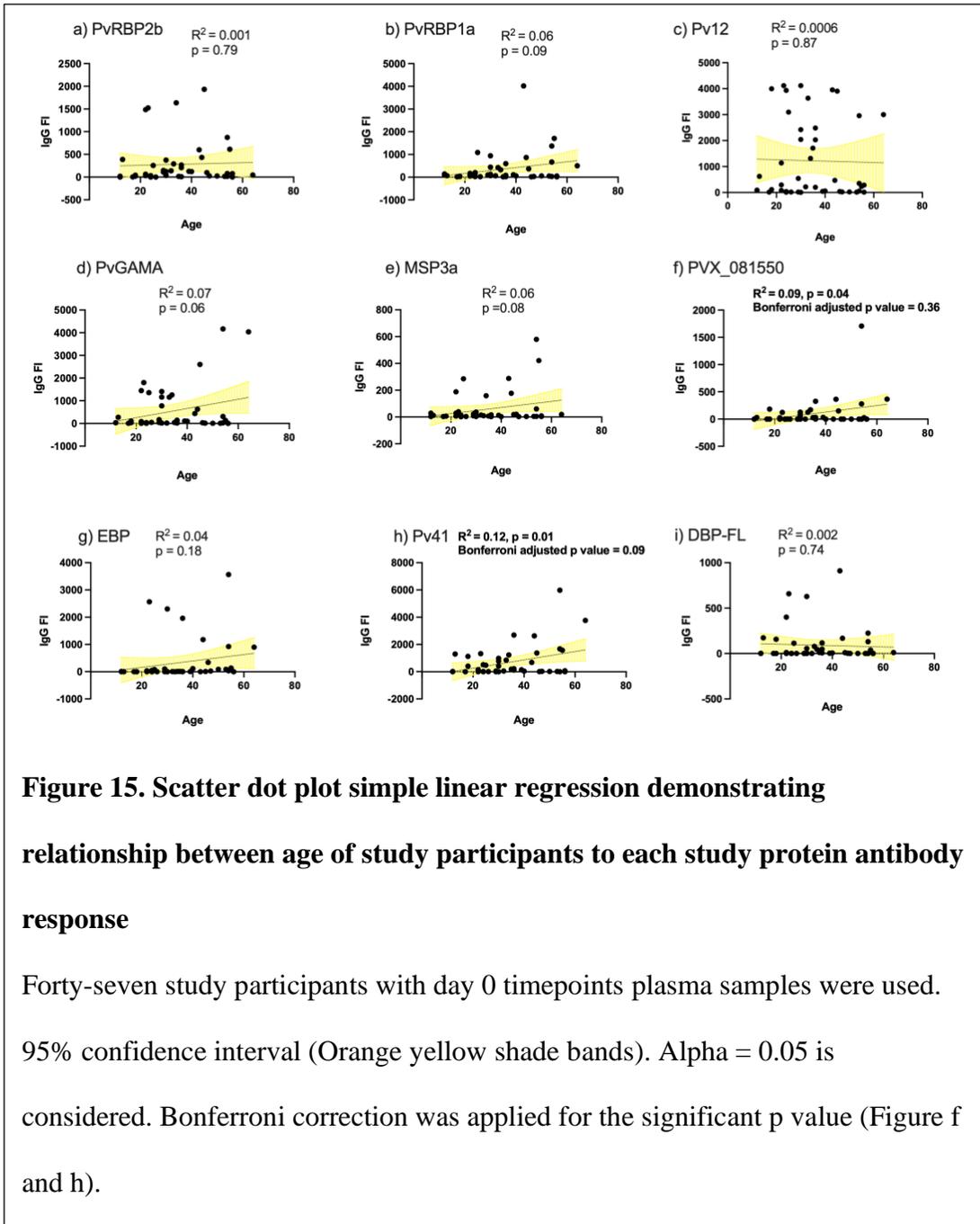
From the LOESS regression smooth line pattern (Figure 13, 14), Pv12 demonstrated highest antibody IgG boosting and the last one to decay whereas DBP-FL was the first one to decay (< 100 days) after acute infection (day 0). However, to confirm IgG antibody durability statistically, we estimated IgG antibody half-lives using exponential decay model and the half-lives equation (section 2.11), followed by mean-rank comparison of IgG antibody half-lives using Friedman statistical method (section 3.3.4).

3.3.3 Determination of Association Between Study Participants Age and Prior Malaria Exposure and the Magnitude and Durability of IgG Responses to each Vivax Antigen

Several studies have reported an association between study participants' age, prior malaria episodes to the humoral antibody response against *P. vivax* antigens, and IgG antibody longevity [74-76]. Thus, we examined an association between patients' age and prior malaria exposure and the magnitude and durability of IgG responses to each vivax antigen.

To analyze the association between study participants' age and prior malaria episodes to each vivax antigen IgG response, we used 47 and 40 study participants' acute vivax plasma samples (day 0 timepoint), respectively. We used nine study participants' IgG antibody half-lives data to analyze the correlation between prior malaria episodes and the magnitude and durability of IgG responses against each vivax antigen. Among the nine study participants, two did not have any previous malaria episodes, one had 15 times, and others had 3 to 7 previous malaria episodes. All cases were self-reported.

A simple linear regression model with 95% confidence interval was applied to assess the relationship. Based on our findings, neither patient age nor prior malaria exposure significantly correlated with the magnitude and durability of IgG responses to any *P. vivax* antigen after adjusted with Bonferroni p-value correction (Figure 15, 16, 17).



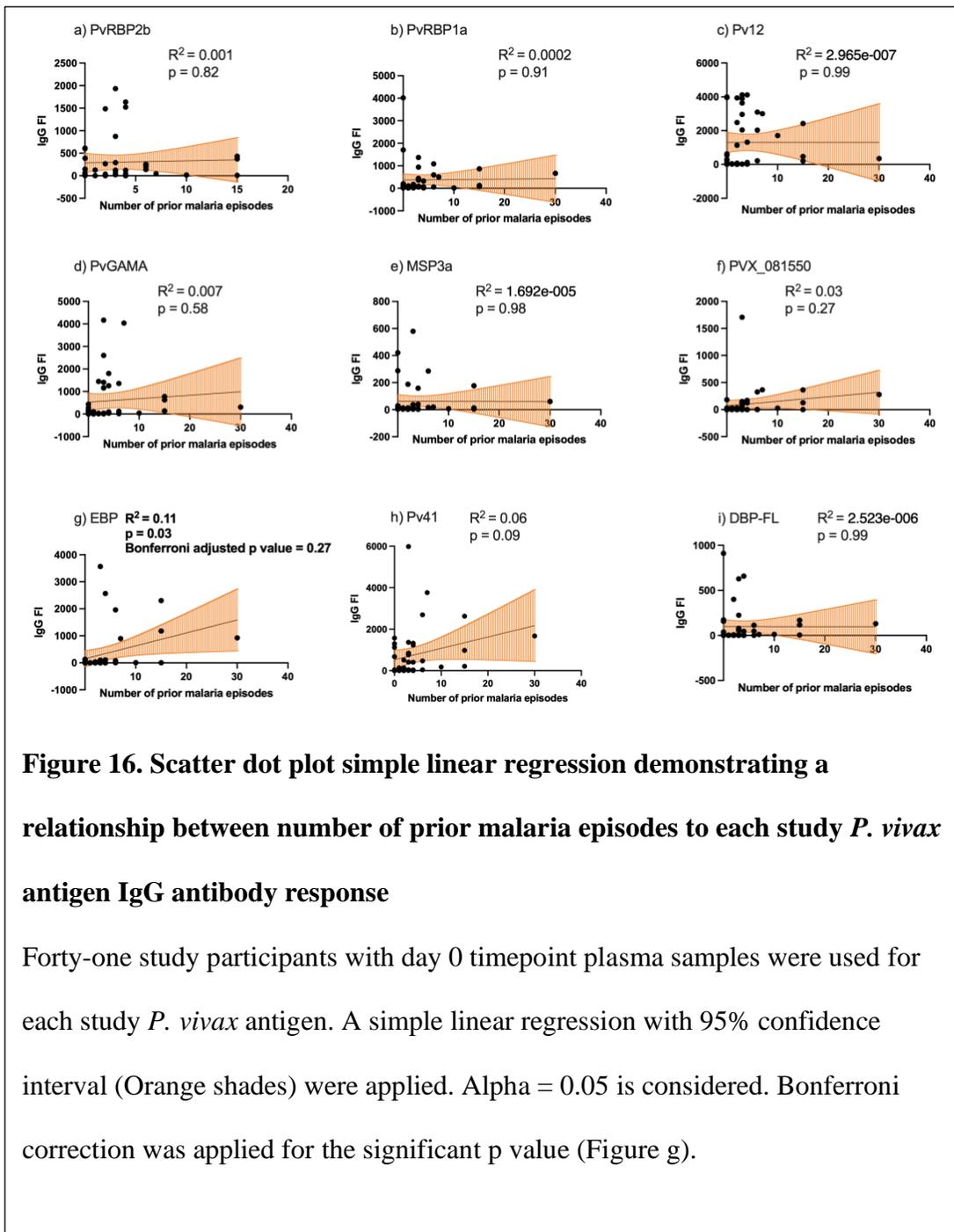


Figure 16. Scatter dot plot simple linear regression demonstrating a relationship between number of prior malaria episodes to each study *P. vivax* antigen IgG antibody response

Forty-one study participants with day 0 timepoint plasma samples were used for each study *P. vivax* antigen. A simple linear regression with 95% confidence interval (Orange shades) were applied. Alpha = 0.05 is considered. Bonferroni correction was applied for the significant p value (Figure g).

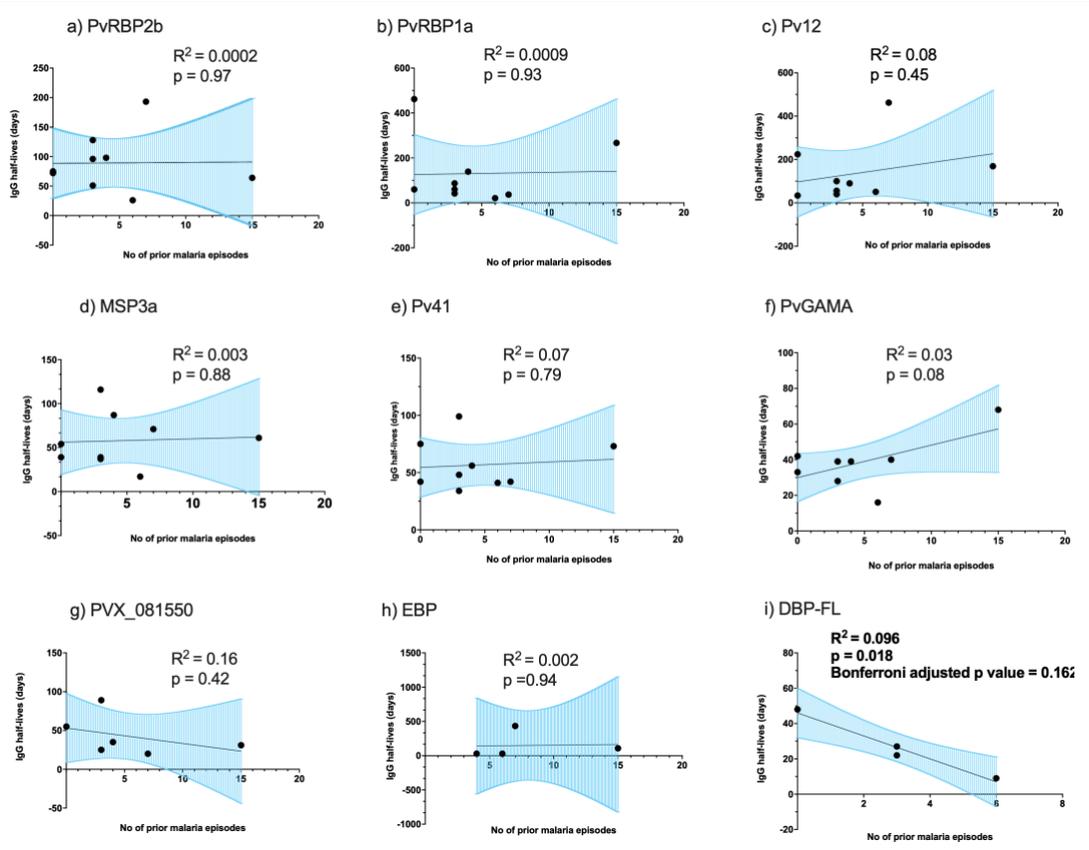
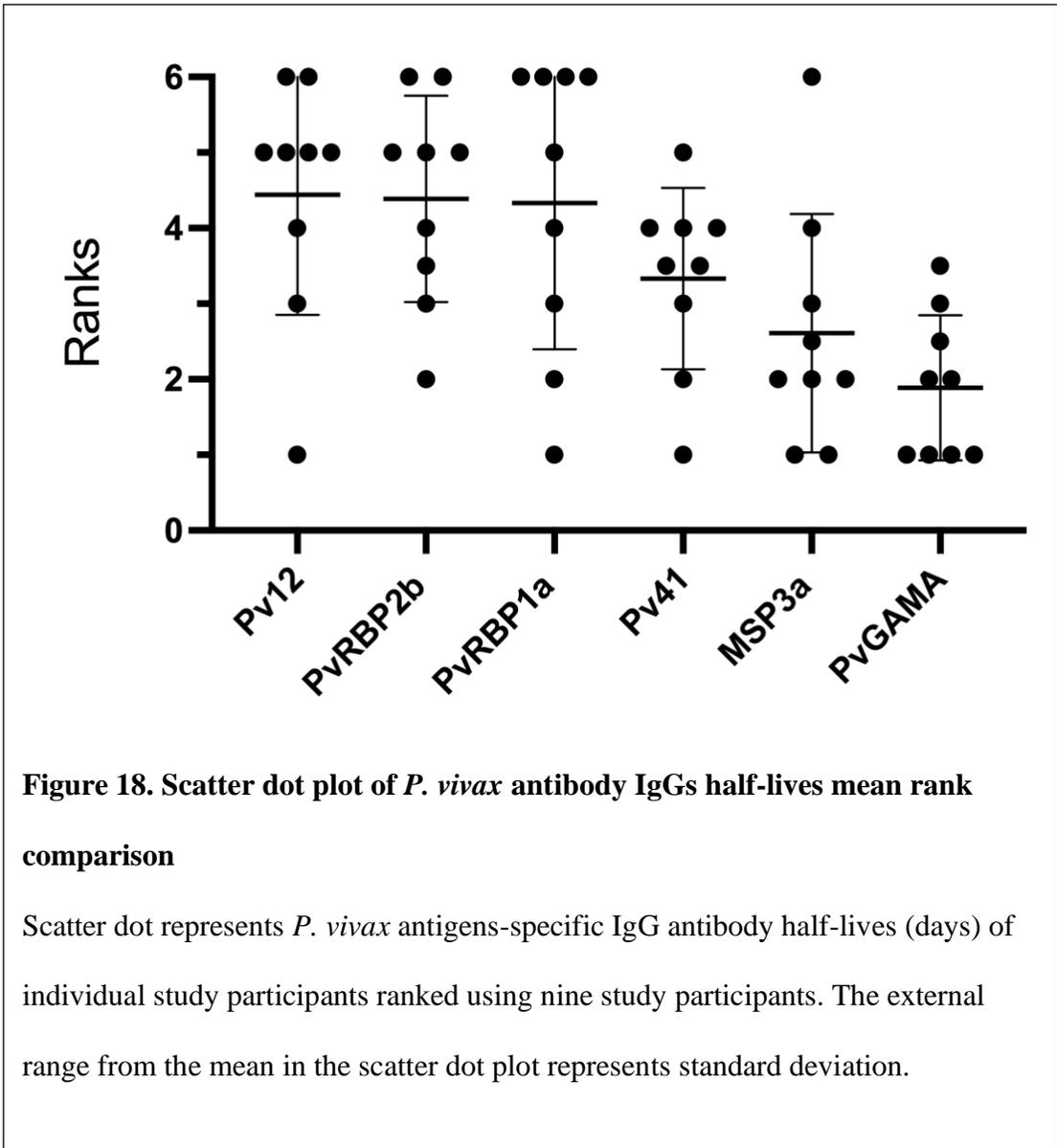


Figure 17. Scatter dot plot linear regression analysis demonstrating comparison between number of prior malaria episodes and *P. vivax* antigen IgG antibody half-lives

Nine study participants were used for most study *P. vivax* proteins except PVX_081550, EBP, and DBP-FL with 6, 4, and 4 study participants respectively. A simple linear regression with 95% confidence interval (Blue shade bands) were applied. Significance in relationship were illustrated in bold. Alpha = 0.05 is considered. Bonferroni correction was applied for significant p values (Figure i).

3.3.4 *P. vivax* IgG Antibody Half-Lives Mean Rank Comparison

To compare *P. vivax* antigen-specific IgG antibody longevity across the cohort, we ranked antigens by their longevity (as measured by antigen-specific IgG half-lives) within each of 9 subjects for whom data was available from all 4 timepoints and then compared the ranks across subjects by Friedman test. The antigens that demonstrated low seroprevalence (Pvx081550, EBP, DBP-FL) were excluded from the analysis. Among six *P. vivax* proteins ranked, Pv12 antibody demonstrated the longest-lived IgG response (global Friedman $p=0.011$), but this result was not significant after adjustment for multiple comparison (post-hoc Dunn's $p>0.05$) (Figure 18a).



Discussion

Given the prior literature supporting a select number of vivax antigens as promising vaccine candidates, further characterization of natural IgG responses to these antigens in other malaria-transmission settings may inform how their natural immunogenicity may influence vaccine responses to these antigens. For example, humoral antibodies generated against antigens from previous infection can affect and interfere with antibody response generated by a vaccine targeting that antigen [58, 59] or, in the case of viral vector vaccines, antigens that displayed by modified viral vector [60]. Additionally, identifying an antigenic target that naturally elicits a robust, durable antibody response can help focus vaccine development on those candidate antigens that would benefit from boosting during natural exposure to the parasite.

In this study, we evaluated naturally acquired IgG antibody responses against nine *P. vivax* vaccine candidate antigens in Brazilian patients with acute vivax malaria and who were longitudinally followed for a period of up to 180 days. We compared the longevity of antibody responses between antigens and examined the associations between patient age and prior malaria exposure and the magnitude and durability of IgG responses to each vivax antigen.

We identified Pv12 as the most long-lived circulating IgG antibodies eliciting *P. vivax* antigen whereas IgG antibodies against DBP-FL displayed the shortest-lived using the Friedman test. We observed peak IgG reactivity against all study *P. vivax* antigens at day 30, which is consistent with the IgG response to *P. vivax* apical membrane protein-1 (PvAMA1) observed in a prior study that used the plasma from the same cohort [61].

Additionally, Pv12 and Pv41 demonstrated high IgG seroreactivity across our study participants. These two 6-cysteine proteins have been shown to be highly immunogenic [77, 78], and antibodies specific to either have been associated with a reduced risk of vivax malaria [56]. Pv12 localizes to the rhoptry of the merozoite and is highly conserved among *Plasmodium* species [79]. Pv41 has been characterized as a merozoite surface protein [78]. Their *P. falciparum* homologs Pf12 and Pf41 form a heterodimer and are thought to be involved in erythrocyte invasion [80]. It has been speculated that Pv12 and Pv41 might also form a heterodimer and have comparable erythrocyte invasion functions due to their ability to interact with each other [63]. Considering above characteristics, and their antibody durability, Pv12 along with its functional counterpart (Pv41) may be considered for incorporation when designing a blood-stage *P. vivax* vaccine.

Plasmodium vivax Duffy binding protein region II (PvDBPII) is a leading vaccine candidate against asexual blood-stage *P. vivax* and has been shown to elicit strain-transcending antibody responses in a Phase 1 clinical trial in India [81]. Studies in Papua New Guinea demonstrated the presence of protective strain-transcending IgG antibodies against DBP that correlated with a reduced risk of malaria [82]. Prior studies showed a high prevalence of DBPII IgG antibodies in a cohort of residents in the Brazilian Amazon [83]. Thus, it was surprising that full length DBP (DBP-FL) demonstrated low seroreactivity and elicited the shortest-lived IgG antibodies among the nine *P. vivax* antigens tested here (Appendix A, Figures 1 and 2). However, low immunogenicity of DBP in acute vivax infection and short-lived IgG response has also been reported in Brazilian studies in which PvDBP_{II-IV} was used as an antigen [84, 85]. There are few

possible explanations for the discrepancies in the natural immunogenicity of DBP individuals naturally exposed to vivax malaria. Region II, the functional binding domain of DBP, has been found highly polymorphic, with evidence of positive selection to favor amino acid variation that helps evade immune responses without negatively impacting its binding function [86, 87]. Thus, the differences in immunogenicity may simply reflect differences in each study population's prior exposure to circulating vivax strains with the similarity to the reference vivax strain (Sal I) used for constructing the recombinant antigens. Alternatively, the low seroprevalence of DBP-FL observed in the current study and studies that used PvDBP_{II-IV} could be due to masking of highly immunogenic conformational DBP_{II} epitopes by other DBP regions or loss of conformational epitopes due to improper folding of DBP-FL. The latter reason is unlikely given that DBP-FL construct used for this study has been demonstrated to be highly seroreactive in a Cambodian acute vivax malaria cohort [63].

As an alternate analysis strategy for determining durability of antibody response, we determined seroprevalence to each of the nine *P. vivax* antigens at each timepoints (day 0, 30, 60, 180). PvGAMA had the highest seroprevalence during acute infection (day 0) (Appendix A: Figure 1a, 2a), while Pv12 demonstrated the highest seroprevalence at day 30 and beyond (Appendix A: Figure 1b, c, d and Figure 2b, c, d). This finding is partially supported by an earlier study that evaluated IgG seroreactivity against a panel of *P. vivax* antigens similar to the ones assessed here [88]. In their findings, PvGAMA demonstrated high seroprevalence using plasma from an adult malaria study cohort in Papua New Guinea [88]. Furthermore, Longley et al. developed a panel of serological exposure markers capable to classify individuals with recent *P. vivax* infections who

could be carrying hypnozoites of *P. vivax* and targeted for anti-hypnozoite therapy [89]. They validated candidate markers using samples from year-long observational cohorts conducted in Thailand, Brazil and the Solomon Islands and antibody responses to eight *P. vivax* proteins classified *P. vivax* infections in the previous nine months with 80% sensitivity and specificity. Similarly, our findings observed biomarkers: PvGAMA and Pv12, can be used as a diagnostic biomarker of *P. vivax* exposure during and after acute vivax infection in general population.

Both age and prior malaria exposure may affect malaria-specific antibody responses [90]. We examined associations between patient age and prior malaria exposure and the magnitude and durability of IgG responses to each *P. vivax* antigen. Based on the earlier findings, anti-PvDBP antibodies increase with exposure to *P. vivax* [83] and reaching peak with long-term exposure in the Amazon Brazil [91]. However, we did not find any association between number of prior malaria episodes and antibody response against DBP or other study *P. vivax* antigen. Although we did not find any association between number of prior malaria episodes and antibody response against DBP or other study *P. vivax* antigen, our study was not sufficiently powered to detect such associations.

Several studies have reported a strong association between age of *P. vivax* malaria patients and the antibody responses against *P. vivax* antigens. For instance, DBP [75, 92, 93], PvRBP1a, MSP3a, PVRBP2b, EBPII [74], MSP3a, Pv12, Pv41 [56], PvGAMA and PVX_081550 [94]. Also, Qiang and team claimed that both PvRBP2b and PvRBP1a in Thailand and Brazil demonstrated positive correlation with age [95]. However, in our study we did not find any correlation between age and antibody response against our all-

study *P. vivax* antigens. One possibility could be due to less study power to detect such associations.

Based on the earlier studies by Costa et al. in Amazon region, Brazil, they reported that number of prior malaria episodes in study participants was positively correlated with the longevity of the IgG antibody against *P. vivax* MSP1-19 [76]. In our studies, we did not find any significant correlation between each *P. vivax* antigen IgG antibody durability and the number of prior malaria episodes (Figure 19). However, we required more study power to confirm this finding because we used only seven study participants to analyze the relationship.

There are several limitations to our study. First, we have a limited number of study participants. Out of 47 study participants, only nine provided all four timepoint plasma samples (Table 1). Thus, we used only nine study participants to determine the *P. vivax* IgG antibody half-lives. For antibody kinetic profiling, we used 37 study participants, but with uneven timepoints (two to four) plasma samples for each study participant. Regardless of sample size for both analyses, we observed similar findings. However, having more study power with all four timepoints plasma samples will strengthen our findings. Second, we have not determined IgG subclass response for this study. Based on previous studies, the longevity of the antibody response against *P. vivax* antigen is partially determined by the isotype- and subclass-specificity of the elicited immunoglobulins, with IgM having shorter half-lives than IgG isotypes, and IgG1 generally being the longest-lived IgG subclass [96]. *Plasmodium vivax*-specific IgG antibody responses are predominantly skewed towards the IgG1 and IgG3 subclasses [68, 83, 96, 97]. According to earlier studies with *P. falciparum*, protein structures and

antigenic regions can impact the nature of IgG subclasses responses [98]. Although IgG1 has the longest half-life among other IgG subclasses, IgG3 subclasses have shown to drive the overall decreasing rate of the total IgG and the subclasses switch can influence the longevity or half-life of IgG antibody as well [99]. IgG subclass switching is believed to be influenced by age and nature of exposure to *P. vivax* antigen [99, 100]. Third, we did not study the B-cell compartment directly. The durability of IgG antibody response is maintained by long-lived antibody secreting cells (ASCs) called plasma cells that is generated after activation of memory cells in response to antigen re-exposure [101, 102]. Thus, determining the magnitude and the longevity of antibody-secreting cells and memory B cells specific to our study *P. vivax* antigens will provide more immunological information to determine the ideal blood-stage vivax vaccine target. Lastly, one of the main challenges in targeting malaria proteins for vaccination is the presence of naturally occurring polymorphisms that may limit the effective use of a vaccine [103-105]. For this study, we used *P. vivax* antigens that were sequenced and optimized from the reference *P. vivax* strain Salvador I, which, as previously mentioned in the context of DBP, may limit the generalizability and interpretability with studies of populations exposed to antigenically diverse vivax parasites.

This study has demonstrated that Pv12 generates a durable IgG antibody response after natural vivax malaria episodes. Thus, a vaccine that targets Pv12 may benefit from natural boosting after breakthrough vivax infections which will likely occur for non-sterilizing blood-stage vaccines that aims to prevent clinical disease and not infection. For instance, Wang et al., observed that COVID vaccines benefit from natural boosting after breakthrough COVID-19 infections. Their findings demonstrated that immunity in

individuals who recovered from COVID-19 infection were long lasting and the convalescent individuals who also received mRNA vaccination generated better humoral antibodies and memory B cells that could protect against circulating SARS-CoV-2 variants [106]. Moreover, this study also provided an insight into the acquisition and longevity of natural humoral immune responses against promising *P. vivax* vaccine candidate antigens. These immunological insights from malaria endemic region Americas will benefit to rationally design an effective blood-stage vaccine that is strain transcending. Future studies will be needed to determine the functional role of naturally acquired IgG antibodies specific for the vivax antigens evaluated here. Identification of antigenic targets, that elicit long-lived, parasite-inhibitory antibodies will inform the development of *P. vivax* blood-stage vaccines.

Appendices

Appendix A: Seroreactivity Comparison Between Nine Study Proteins

Although all our study participants are acute vivax patients, we observed seronegative among our study *P. vivax* antigens. Thus, we performed seroreactivity analysis and ranked the nine study *P. vivax* proteins. First, we used two groups, each having at least two timepoints, to compare the seropositivity ranking. For instance, one group with 36 study participants each having day 0 and 30 plasma samples, and other group with 10 study participants each having day 60 and 180 plasma samples (Figure 1). To further confirm this finding, we used 8 study participants each having all four timepoints plasma samples to compare seropositive among nine study participants. Seropositivity threshold cut-off for each comparison was mean + 3 standard deviation of nine North American malaria naïve controls.

Under both analyses, PvGAMA showed the best seropositivity during acute infection (day 0 timepoint plasma), and Pv12 demonstrated best seropositivity during day 30, 60, and 180 (Figure 1, 2). This suggests that PvGAMA and Pv12 can be used as biomarker for *P. vivax* exposure during and after acute vivax infection respectively.

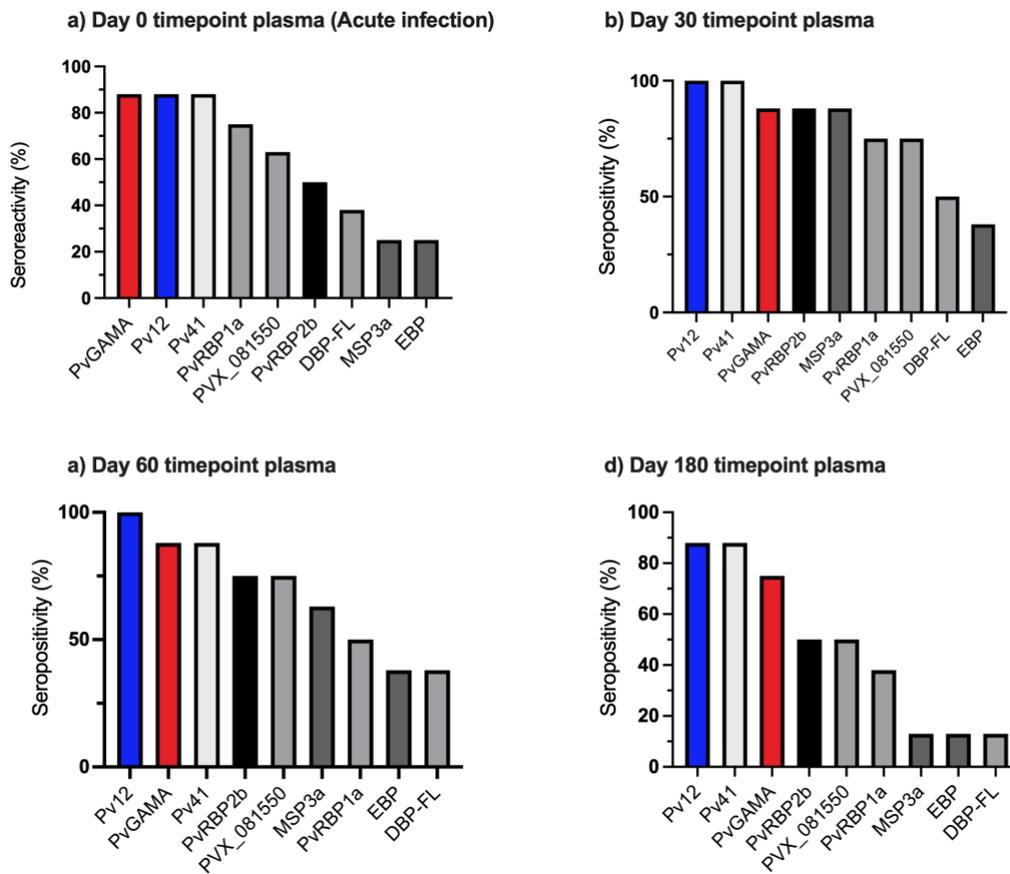
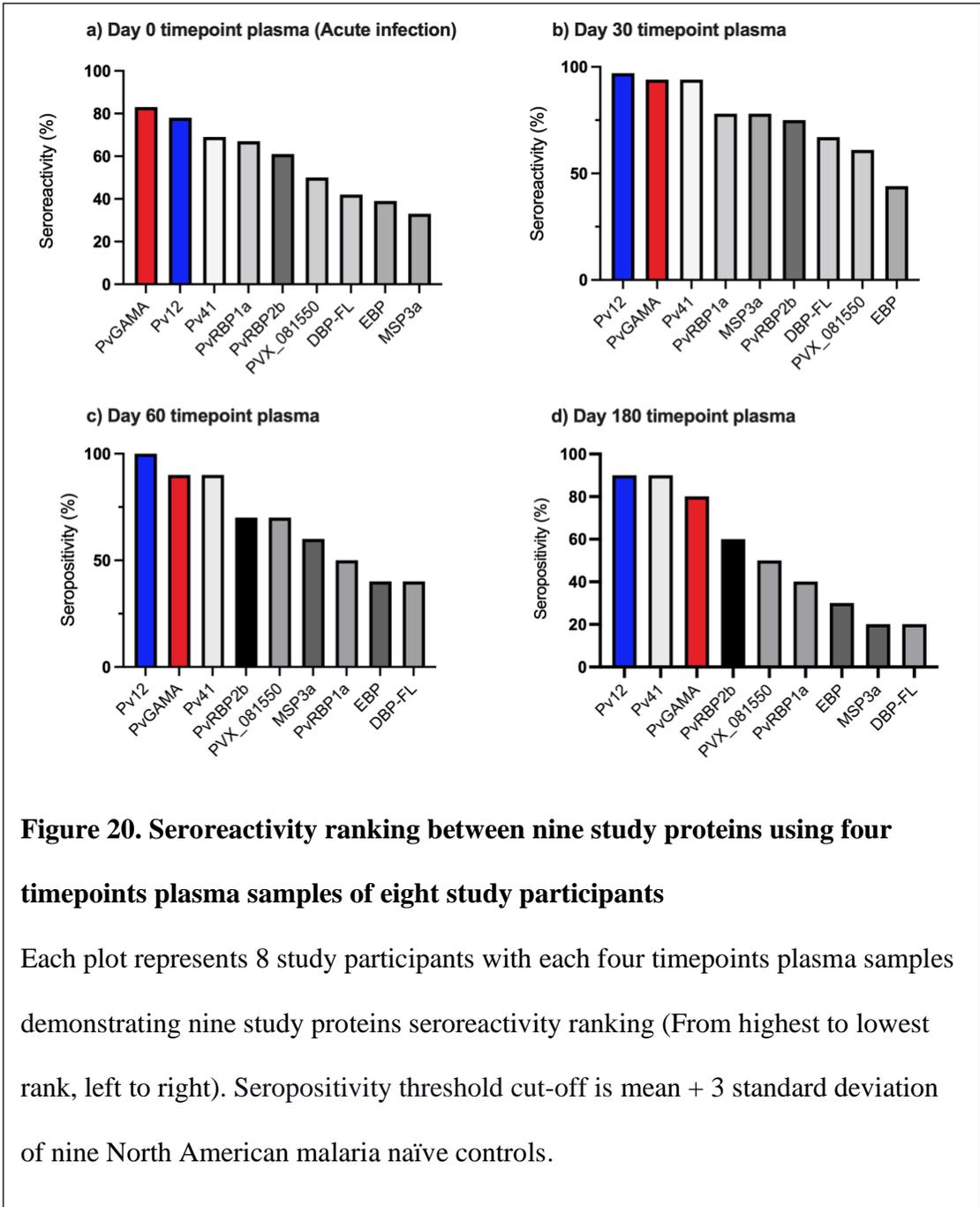


Figure 19. Seroreactivity ranking between nine study proteins using four timepoints plasma samples. Plot a and b represents 36 study participants with two timepoints plasma samples demonstrating nine study proteins seroreactivity ranking (From highest to lowest rank, left to right). a) Day 0 timepoint plasma samples, b) Day 30 timepoint plasma samples. Plot c and d represents 10 study participants with two timepoints plasma samples demonstrating nine study proteins seroreactivity ranking (From highest to lowest rank, left to right). a) Day 60 timepoint plasma samples, b) Day 180 timepoint plasma samples. Seropositivity threshold cut-off is mean + 3 standard deviation of nine North American malaria naïve controls.



Appendix B: Transformation, Expression, and Purification of PvRBP2b

Although we initially aimed to use the PvRBP2b that we produced to run functional and multiplex immunoassays, however, we were able to produce only few amounts of proteins that were used to optimized western immunoblot assay and multiplex immunoassay techniques. For the actual experiment for the study, we used PvRBP2b that was received from WEHI, AU.

We expressed and purified *Plasmodium vivax* reticulocyte binding protein2b (PvRBP2b) in house in collaboration with Dr. Quyen Quoc Hoang laboratory in the Department of Biochemistry & Molecular Biology at IUSM. Briefly, competent Shuffle T7 *E. coli* (K12 strain, Catalog: C3026J, New England Biolabs) was heat-shock transformed with 1 µg of modified pET32a plasmid containing a *pvrpb2b* (Sal I strain) gene insert encoding amino acids 161-1454 (kindly provided by Dr. Wai-Hong Tham) (4). Transformed shuttle T7 *E. coli* cells were plated onto Luria-Bertani (LB) agar containing with ampicillin and incubated overnight at 37° C. Starter cultures were initiated by inoculating single colonies in LB broth containing 100 µg/ml ampicillin (LB-amp), which were incubated at 37° C in a shaker (250 RPM) for ~8 hours. These starter cultures were then diluted 1:1000 500 ml LB-ampicillin for larger scale expression at 37° C in a shaker (250 RPM). Protein expression was induced with 1 mM isopropyl β-D-thiogalactoside (Catalog: 12481C5, GOLDBIO) when the optical density (OD) at 600 nm reached between 0.6 and 0.8. Induced cultures were incubated at 16°C overnight in a shaker (200 RPM). Cells were centrifuged at 6,000 x g in 4°C for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in a lysis buffer (50 mM Tris pH 7.5, 500 mM NaCl, 10% glycerol) with protease inhibitor cocktail (Catalog:

78410, Thermo Scientific, USA). The cells were then lysed by sonication for 20 mins (3 sec on / 3 sec off) and centrifuged at 100,000 G (Ultracentrifuge, 45Ti rotor, Beckman Coulter) at 4° C for 45 minutes. The supernatant was filtered through a 0.22 µm filter (Catalog: SLGVM33RS, Millipore Sigma) prior to incubation with nickel-NTA agarose beads (Catalog: H-350-25, GOLDBIO) for 2 hours at 4° C. The bead slurry was packed in a column and washed with wash buffer (20 mM Tris pH 7.5, 500 mM NaCl, 10 mM imidazole). Proteins were eluted using elution buffer (20 mM Tris pH 7.5, 500 mM NaCl, 300 mM imidazole). Eluted proteins were dialyzed overnight at 4°C using a dialysis membrane with a 10 kDa molecular weight cut-off (Catalog: 68100, SnakeSkin Dialysis Tubing, ThermoFisher Scientific) and HEPES buffer (20 mM HEPES pH 7.4, 100 mM NaCl).

Proteins were further purified via fast protein liquid chromatography using a HiTrap Q HP ion-exchange column (Catalog: 17-5156-01, GE Healthcare) at 4°C. Eluted fractions are collected and assessed for presence of protein of interest by SDS PAGE. Positive fractions were pooled and concentrated by centrifugation (Catalog: UFC905008, Amicon Ultra-15, Merck Millipore). For final purification, pooled concentrated proteins were passed through a size-exclusion column (Catalog: 29-091596, Superose 6 Increase 10/300 GL, GE Healthcare). The protein purity and concentration were confirmed via SDS PAGE, western blot, and Bradford assay. The purified PvRBP2b protein was stored at -80°C until use.

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Curriculum Vitae

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