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TITLE OF THE PROTOCOL

Determination of the protective efficacy of the *Pv*CS/Montanide ISA-51 vaccine formulation against controlled infection with *Plasmodium vivax* sporozoites

Protocol number: 2201-3

Sponsor name:
International Vaccine Center (CIV)

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Version: 1.0 - INVIMA
August 7, 2025

SIGNATURE PAGE

Sponsor Signature

_____ _____

Printed Name: **Date:**
Position: Sponsor

Investigator's Signature

_____ _____

Printed name: **Date:**
Position:

I agree to conduct this clinical trial in accordance with the design described in this protocol and to comply with all provisions of this protocol.

The signatures recorded herein constitute acknowledgment of this protocol and its annexes. Furthermore, they guarantee that this clinical study will be conducted in accordance with the provisions of the protocol, including all confidentiality requirements and in compliance with legal and regulatory requirements, as well as the principles set forth in the GCP guidelines.

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GLOSSARY OF ABBREVIATIONS

ABBREVIATIONS	DEFINITIONS
A.	*Anopheles*
Anti-HBc	Anti-core antibodies Hepatitis B
DNA	Deoxyribonucleic acid
AMA	Artificial membrane feeding technique
AL	Latin America
BUN	Blood Urea Nitrogen (US)
BPC/GCP	Good Clinical Practices
BPL/GLP	Good Laboratory Practices
B-HCG	Human chorionic gonadotropin-beta subunit (US)
C	Terminal carboxyl
CEI-ASOCLINIC	ASOCLINIC Research Ethics Committee
CI	Informed Consent
CIV	International Vaccine Center
CQ	Chloroquine
CS	Circumsporozoite Protein
EA	Adverse Event
SAE	Serious Adverse Event
EKG	Electrocardiogram
ELISA	Enzyme-Linked Immunosorbent Assay (US)
EPS	Health Promotion Company
ETV	Vector-Borne Disease
FDA	Food and Drug Administration
FRC/CRF	Case Report Form
FTA-ABS	Fluorescent-Treponemal Antibody Absorbed (US) - Treponemal Antibody Absorption Test
FWA	Federalwide Assurance
G6PD	Glucose-6-Phosphate Dehydrogenase
GG	Thick Smear

HBsAg	Hepatitis B Surface Antigen
HTLV	Human T-Lymphotropic Virus
IFAT	Immunofluorescence Antibody Test = Indirect Immunofluorescence Technique
INS	National Institute of Health (Colombia)
INSALPA	Pacific Health Institute
IP/PI	Principal Investigator
IPS	Health Services Provider Institution
IRB	Institutional Review Board = Ethics Committee
LDH	Lactate Dehydrogenase
Mabs	Monoclonal Antibodies
MAPs	Multi-Antigenic Peptides
MVDC	Malaria Vaccine and Drug Development Center = International Vaccine Center – CIV)
N	Amino terminal
NIH	National Institutes of Health
NIAID	National Institute of Allergy and Infectious Diseases
NMRC	Naval Medical Research Center
WHO	World Health Organization
P.	*Plasmodium*
SOP	Standard Operating Procedure
PQ	Primaquine
PSL	Long Synthetic Peptides
spp.	Species
RAS	Radiation-attenuated sporozoites
SARS-CoV 2	Severe Acute Respiratory Syndrome Coronavirus 2 (COVID-19)
SP	Sulfadoxine-Pyrimethamine
SGSS	General Social Security System for Health
SSDC	Departmental Health Secretariat of Choco
SSMQ	Municipal Health Secretariat of Quibdó
VES	Erythrocyte Sedimentation Rate
HBV	Hepatitis B virus

1. SUMMARY OF THE PROTOCOL

Title	To determine the protective efficacy of the *Pv*CS/Montanide ISA-51 vaccine formulation against controlled infection with *P. vivax* sporozoites.
Product Name	*Pv*CS.2 / M-51
Study Objectives	**Primary Objective**: To determine the protective efficacy of the *Pv*CS/Montanide ISA-51 vaccine formulation against controlled infection with *P. vivax* sporozoites **Specific Objectives:** 1) Confirm the safety of the vaccine in naïve volunteers immunized with *Pv*CS. 2) To determine the immunogenicity of *Pv*CS in individuals previously exposed to malaria. 3) Determine the protective efficacy of the vaccine against infectious challenge with viable *P. vivax* sporozoites in the above groups.
Study Design	Randomized, double-blind, controlled, Phase IIa/b study comparing two groups of naïve volunteers and volunteers previously exposed to malaria.
Schedule	Three intramuscular injections on days 0, 60, and 180, followed by infectious challenge on day 210.
No. of Volunteers	Step #1: volunteers **(**immunization**)** n=60; plus 4-6 alternates. Step #2 = patients with active *P. vivax* infection, blood donors to artificially infect mosquitoes: minimum 5, maximum 15.

Population	Healthy adults of both genders: 30 volunteers with a history of *Plasmodium vivax* malaria for the semi-immune group based on medical history and serology, and 30 volunteers with no history of malaria for the naïve group. Participants will be residents of Quibdó.

Study procedures	**Step 1. Selection and immunization of volunteers** Sixty subjects who meet the inclusion criteria for each of the groups will be selected: a) naïve (n=30) and b) semi-immune (n=30) from Quibdó and other municipalities in Chocó. The subjects in each main group (A, B) will be randomly distributed into two subgroups: Vaccine (A1 and B1, with n=20 each), Control (A2 and B2, with n=10 each). Subgroups A1 and B1 will be immunized with the vaccine (*Pv*CS protein) and subgroups A2 and B2 with the placebo. The vaccine group will be immunized intramuscularly (IM) on days 0, 60, and 180, with the formulation, composed of peptides derived from the circumsporozoite protein (CS) of *P. vivax (Pv*CS) at doses of 100 µg and 150 µg formulated in Montanide ISA-51 adjuvant (*Pv*CS.2/M51 vaccine). Repeated blood samples will be taken to assess safety through renal, hepatic, and hematological function tests at 0, 1, 3, 5, 7, 8, and 18 months. Adverse events (AEs) induced by immunization will be assessed during the 7 days following immunization. These AEs will be reported immediately to the ASOCLINIC Ltda. research Ethics Committee (IRB). The immunogenicity of the vaccine will be evaluated by testing for specific humoral and cellular responses against the peptides used in immunization and against the parasite. **Step 2. Parasite donor volunteers:** Patients naturally infected with *P. vivax* (*Pv*CS-VK210) will be selected as parasite donors for the infection of *Anopheles* mosquitoes. These donors will be identified at health posts in endemic areas using microscopic testing (thick smear). The group of parasite donor patients (n=5-15) will consist of individuals infected with *P. vivax* who will be asked to donate a 20 mL blood sample that will undergo laboratory testing to rule out co-infections detectable by standard blood banking. Only blood samples infected exclusively with P. *vivax* according to the PCR test will be used for feeding and experimental infection of *Anopheles* mosquitoes.

	3. Infectious challenge of volunteers from step 1: To evaluate the protective efficacy of the immunogen, volunteers will be challenged by bite from 3+1 mosquitoes infected with *P. vivax* sporozoites on one occasion: approximately 1 month after the third immunization. Starting on day 5 post-bite, medical and parasitological follow-up will be performed to determine the onset of infection. All procedures in this study will be performed using Good Laboratory Practices (GLP) and Good Clinical Practices (GCP).

Protection of volunteers and social security	All participants must be affiliated with the General Social Security System for Health (SGSS), under any of its schemes, and provide the corresponding supporting documentation. In addition, as an additional protective measure, the study will provide volunteers with a prepaid medical plan that will cover medical care throughout the process. Likewise, a civil liability policy for clinical trials will be taken out to cover any incident related to their participation.

Procedures to minimize risks associated with the challenge	The potential risks associated with the challenge will be evaluated in accordance with a specific biosafety protocol, which includes the following aspects in more detail and the corresponding contingency plan: ● Detailed analysis of medical history and laboratory tests to evaluate donor inclusion and exclusion criteria. ● Testing of donated blood for infectious diseases to infect mosquitoes, in accordance with Blood Bank standards, to rule out the presence of other pathogens in the donor sample. ● Restriction of personnel access to infected mosquito rooms to minimize the risk of accidental transmission of malaria to researchers and/or the community. ● Close post-challenge follow-up at the time of diagnosis and 28 days after completion of antimalarial treatment (hematological, parasitological, and blood chemistry); in addition, physical examination (personalized assessment). ● Antimalarial treatment will be administered immediately upon documentation of parasitemia ≥ 100 p/μL evaluated by thick smear. Parasitemias will be confirmed retrospectively by qPCR. ● Immediate treatment for any individual exposed to accidental infection with malaria. ● Analysis of volunteers' blood samples to detect antibodies against HIV, hepatitis B, and hepatitis C will contribute to the protection of health and laboratory personnel. ● Standard biosafety procedures will be followed for the handling of blood and body fluid samples.

Data processing	Study data will be entered into an online database using REDCap version 5.10.1 (http://project redcap.org/). Final data processing will be performed using STATA®, SPSS®, or R® statistical software. Data will be collected using REDCap v5.10.1 and managed on a secure server. Data entry will be performed using electronic forms verified by the clinical monitor and corrected according to the SOPs. After quality control, the data will be analyzed using Stata, SPSS v16.0, and GraphPad Prism v6.0. The data will be available in the article and supplement; raw data may be requested from the corresponding author, respecting the confidentiality of the participants.
Study Duration	30 months
Study sites	**Asoclinic IPS Research Center** - Clinical Laboratory Calle 21a - Carrera 23. Zona Minera -La Virginia. Quibdó, Chocó

2. INTRODUCTION:

Malaria continues to be a global public health problem due to high morbidity and mortality, mainly in countries located in tropical and subtropical areas of the planet (WHO 2021). Despite major efforts to reduce it, in 2020 there were 241 million clinical cases and nearly 1 million deaths reported in 85 endemic countries. In the same year in Latin America, Central American countries achieved reductions in incidence of more than 90%. However, in countries such as Brazil, Colombia, and Venezuela, which account for ~77% of the ~1.5 million cases reported in the region, an average reduction of 58% (to ~650,000 cases) was observed (PAHO 2022).

These large differences in the results of the fight against malaria are due, among other causes, to the failure of classic control measures, such as the use of insecticides and antimalarial drugs, and to numerous socioeconomic and political problems, which together demand their strengthening and the development of new control tools and strategies.

2.1. Epidemiology of malaria.

Malaria is a disease that currently affects ~250 million people and is responsible for ~600,000 deaths per year, representing an enormous economic impact globally, mainly for populations living in developing regions in sub-Saharan African countries (Breman, 2001; Sachs and Malaney, 2002), but also in some regions of Asia and Latin America (LA). Epidemiological indicators report that infections caused by P. *vivax* remain widely distributed worldwide, even more so than infections caused by *P. falciparum,* representing a significant cause of morbidity and mortality among the 2.85 billion people living at risk of infection (Guerra, 2010). Most cases of P. *vivax* originate in Southeast Asia and the Western Pacific, accounting for ~70% of cases in LA and a smaller proportion (5-20%) in some African countries (Guerra, 2010; Mendis, 2001, WHO 2023).

Most endemic areas where both *P. falciparum* and *P. vivax* are transmitted share the same mosquito vectors, and therefore *Anopheles* resistance to insecticides affects the transmission and control of both *Plasmodium* species (Rodríguez, 2009). On the other hand, although the disease caused by P. *vivax* is less lethal than that caused by P. *falciparum*, it is more complex due to the development of liver parasitic forms (hypnozoites) that remain silent (asymptomatic) and create a reservoir of parasites with periodic clinical reactivation of the infection (Sattabongkot 2004). Additionally, the hemolytic effect of Primaquine (PQ) in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency is known, with the consequent risk to these patients when treated with conventional regimens (Galappaththy, 2013). Furthermore, in recent years, the limited effect of PQ in patients with certain polymorphisms of the cytochrome P450 (2D6) enzyme has been demonstrated, a fact that contributes to the development of relapses after conventional treatment (Manjurano, 2016).

During the period 2000-2015, efforts to control malaria were intensified with a view to moving towards its global elimination. The Millennium Development Goals established by the United Nations set a target of reducing the incidence of malaria by 75% by 2015, and

contributions to global control efforts were increased by international agencies such as the Global Fund to Fight AIDS, Tuberculosis and Malaria, the US President's Malaria Initiative (PMI), and the Bill and Melinda Gates Foundation, among others (WHO, 2015).

This led to a notable reduction in malaria worldwide, from nearly 500 million cases in 2000 to approximately 250 million in 2015, which, although representing a significant decrease in incidence, still continues to have an enormous impact on global public health. In this context, between 2000 and 2015, a total of 17 countries achieved the elimination of transmission in their territories. In addition, regions such as Central America reduced their incidence by about 90%, and in Colombia the reduction was around 75%. At the same time, progress was made in the search for vaccines that could reinforce the elimination strategy, particularly for P. *falciparum*, the species responsible for about 90% of all cases globally and 99% of cases reported in Africa. Progress was also made in the study of molecules with potential for the development of vaccines against P. *vivax*, a parasite with a much lower incidence but with a wider global distribution; this species is predominant in Asia, Oceania, and the Americas. (WHO, 2021)

Unfortunately, due to reduced international funding and the inability of endemic countries to maintain control measures, primarily global access to early diagnosis and timely treatment, there was a resurgence in malaria transmission in the following years, and in some countries, such as Colombia, malaria has doubled between 2016 (~50,000 cases/year) and 2024 (~100,000 cases/year), highlighting the importance and urgency of developing vaccines against P. *falciparum* and *P. vivax*, the two most prevalent species of *Plasmodium*.

Fortunately, significant progress has been made over the last 10 years in the development of two vaccines aimed at preventing *P. falciparum* infection (WHO, 2023). Although vaccines will not be able to eliminate malaria on their own, their contribution to control and elimination strategies will be enormous; vaccines are considered the most cost-effective mechanism for combating communicable diseases, and the contribution of clinical immunity to reducing morbidity and mortality under natural conditions is incalculable (Bloland, 2002, Doolan, 2009) (see state of the art).

2.2. Development of natural immunity against malaria caused by P. *vivax*

In general, exposure to malaria can be fatal. As mentioned, approximately 250 million clinical cases are reported worldwide each year, with nearly 600,000 deaths, mainly in children under 5 and pregnant women (WHO 2023). However, if patients survive the first infections, their repeated exposure to *Plasmodium* produces a progressive reduction in the intensity of the disease and causes individuals in endemic areas to eventually develop clinical immunity, in which people only have mild symptoms or no clinical manifestations (asymptomatic), despite harboring long-term infections. These communities also develop immunity that blocks mosquito infection, because when the mosquito bites the patient, it ingests not only the parasite but also antibodies that block its transmission (Arévalo-Herrera 2011c).

In this context, in highly endemic regions, children and adolescents between the ages of 10

and 15 achieve a moderate degree of immunity, which is associated with a decrease in the intensity of clinical manifestations, the frequency of infection (Cattani, 1986b), and the transmission of the infection itself. Unfortunately, this immunity disappears when individuals leave the endemic area (Doolan, 2009) and become susceptible again. Furthermore, this immunity is species-specific (Collins and Jeffery, 1999), and although there is cross-reactivity between parasites, this reactivity does not necessarily induce sufficiently effective natural cross-protection.

Because *P. vivax* has different biological properties than *P. falciparum*, it is unlikely that one species of parasite can be effectively controlled by a heterologous vaccine, although under certain conditions there is cross-reactivity between components (antigens) of one species and another that could contribute partially to protection. Therefore, the identification of specific P. *vivax* components is required for the development of vaccines for this species and, with further research, the identification of antigens that would ideally allow for the development of multispecies vaccines.

2.3. Vaccines as tools for malaria control and elimination

Due to limitations in classic malaria control strategies, such as early diagnosis and timely treatment, vector control measures, and others, vaccines have been considered a complementary strategy. Over the last three decades, the scientific community has worked intensively to identify molecules that can be used for vaccine development as a tool to strengthen control and accelerate the elimination of malaria (Dhiman, 2019).

The feasibility of malaria vaccines is based on multiple lines of evidence: **1**) individuals in endemic areas become clinically immune through repeated exposure to malaria and can remain asymptomatic carriers for indefinite periods, leading an almost normal life; 2**)** It has been demonstrated that the passive transfer of specific antibodies or cells from semi-immune patients in endemic areas confers protection to non-immune individuals in areas without malaria transmission (Druilhe, 1994). **3)** Human volunteers vaccinated through the bite of mosquitoes infected with *P. falciparum and P. vivax* sporozoites *and* irradiated develop strong immunity in more than 90% of those vaccinated (Clyde, 1975). 4**)** Similarly, inoculation with attenuated sporozoites, extracted from the mosquito by microscopic dissection and cryopreserved, induces similar protection and is being studied for industrialization (Hoffman, 2002). **5)** In the case of P. *vivax,* the International Vaccine Center (CIV) recently achieved remarkable protection in primates of the genus *Aotus and* in healthy adult volunteers exposed to vaccination with experimentally infected and irradiated mosquitoes. These mosquitoes were carriers of sporozoites in their salivary glands (Arévalo-Herrera, 2016). 6**)** After more than three decades of research, it has been confirmed in recent years that the Pf-RTS,S and Pf-R21 vaccines developed in Europe by large international alliances achieve efficacy greater than 50% and 70%, respectively, in endemic areas of Africa. This achievement recently led the WHO to approve their implementation in some African countries with high P. *falciparum* transmission (Penny, 2016). 6**)** More recently, in Colombia, the CIV found that the PvCS/Montanide ISA-51 formulation, which had been in development for nearly three

decades, achieved for the first time nearly 40% total protection (sterile) in naïve volunteers from the city of Cali and semi-immune volunteers from the municipality of Buenaventura (endemic area), and total protection greater than 50% and 60%, respectively, taking into account a notable reduction in parasitemia in those who did not produce sterile immunity (Arévalo-Herrera, 2022).

Over the last three decades, ~30 *P. falciparum* antigens have been identified worldwide, and their immunogenicity and protective efficacy have been evaluated in animal models and humans (Richie and Saul, 2002). Among these antigens are those expressed in sporozoites (CSP, SSP2/TRAP) (Rogers, 1992), in hepatic stages (LSA1, LSA3, EXP1), in the erythrocytic phase (MSP-1, MSP-2, AMA-1), and in sexual stages (PfS25, PfS45/48), among others. Colombia, through the CIV, has contributed to the development of many of these (Arévalo-Herrera 1998; Arévalo-Herrera 2001; Herrera 2005; Arévalo-Herrera 2010; Herrera 2011a; Arévalo-Herrera 2001; Arévalo-Herrera 2011a; Arévalo-Herrera 2001b; Arévalo-Herrera 2016; Arévalo-Herrera 2022).

Methodological strategies for vaccine production include, among others, synthetic peptides and recombinant proteins formulated in different adjuvants, as well as live recombinant viruses and DNA vaccines. Most of these antigens have been identified by screening genomic libraries with sera against whole parasites or fractions thereof, as well as with monoclonal antibodies (Mabs). The immunogenicity and protective efficacy of several of these antigens have been experimentally tested in animals and humans (Kumar, 2002), showing a wide range of immunogenicity and/or protection in these models (Genton and Corradin, 2002).

However, the Pf-RTS-S/ASO2A) and Pf-R21-Matrix M vaccines, which are recombinant hybrid products containing a fragment of the *P. falciparum* CS protein and hepatitis B AgS, are the only vaccines that, after extensive phase II and III studies on the African continent, have been approved by the WHO for use in selected countries on that continent (Hill, 2011, Penny, 2016). The *Pf*-RTS,S/AS02A vaccine initially showed modest protection of approximately 30% against clinical malaria in young children, while a new formulation, Pf-RTS,S/AS01E, showed an increase in protective efficacy, reaching around 53% in children aged 5 to 17 months (Bejon 2008). Meanwhile, the more recently reported candidate PfR21-Matrix-M achieved superior protection of 77%, making it, together with Pf-RTS,S, one of the most advanced vaccines currently available (Samuels, 2022; Datoo, 2024). However, there are numerous other vaccines in preclinical and clinical trials against P. *falciparum* (Hoffman, 2015; Webster, 2021; Kublin, 2017), including other platforms, which more recently include mRNA vaccines (Tsoumani, 2023; Tang, 2025) and the use of recombinant nanoparticles (Karsk, 2017; Zhuo, 2024, Herrera, SM 2024).

2.4. Limitations to the development of vaccines against *P. vivax*

In contrast to the significant advances in the development of vaccines against *P. falciparum*, explained by its high importance and significant global investment, only a limited

number of antigens have been identified and partially characterized in *P. vivax*, such as the MSP1 protein (del Portillo, 1991), AMA1 (Thomas, 1994), MSP3, MSP4, MSP5, RBP, and DBP from the asexual blood stages (Barnwell and Galinski, 1995; Barnwell, 1999; Chitnis, 2001; Galinski, 1999; Galinski., 2001; Miller, 1977), Pvs25 and Pvs28 from oocysts/oocysts, and PvCS and PvSSP2/TRAP antigens from the pre-erythrocytic phase (Templeton and Kaslow, 1997). Of this last phase of the cycle, only PvCS has been extensively analyzed in preclinical and clinical studies (Arévalo-Herrera, 2010). However, advances in "omic" technologies, mainly genomics and proteomics, are allowing for a more rapid analysis of the parasite. In our case, this applies to the pre-erythrocytic phase of *P. vivax* (Mo 2018, Herrera 2024). On the other hand, progress has been made in other phases, such as the sexual blood phase of the parasite, where the proteins Pvs48/45, Pvs230, and Pvs47 expressed in gametocytes and in the sporogonic phase are being studied in the discovery and preclinical analysis phases (Malkin, 2005; Arévalo-Herrera, 2015, Molina-Cruz, 2025). Similarly, in the asexual erythrocytic phase, promising candidate antigens such as *Pv*DBP, *Pv*RBP, *Pv*AMA-1, SPECT1, FALSTATIN, and CELTOS have been identified and are being analyzed in recent experimental studies (López-Pérez Soares 2023, and coiled-coil (Cespedes 2014).

The limited progress in the study of *P. vivax* antigens identified as candidates for vaccine development can generally be explained by several factors, such as: **1**) the impossibility of obtaining continuous cultures (*in vitro*) of blood forms of the parasite that in turn allow mosquitoes to be infected regularly in the laboratory; 2**)** difficulty in maintaining a constant and adequate production of sporozoites for infectious challenge studies; 3**)** the consequent difficulty in the experimental use of vaccination with irradiated *P. vivax* sporozoites, which would allow the generation of reagents (serum and cells) of great value for the identification of antigens potentially associated with protection and, therefore, candidates for the development of vaccines against this species (Barnwell, 1985; Arévalo-Herrera, 2010); 4**)** the existence of genetic factors that limit the development of clinical studies, such as: a) Duffy blood group polymorphism (Fy+/-) essential for the invasion of red blood cells by the parasite, b) glucose-6-phosphate dehydrogenase (G6PD) deficiency, which can affect human volunteers' tolerance to primaquine (PQ) treatment of the parasite, and c) the prevalence of hemoglobinopathies in some endemic populations.

Despite these difficulties, the CIV in Cali has been making progress in developing infrastructure and experimental models for conducting studies on vaccines against *P. vivax* in Colombia. This process includes a comprehensive approach to the parasite, ranging from discovery, preclinical studies, and clinical trials of proteins from the three phases of the parasite's cycle: 1) hepatic or pre-erythrocytic phase; 2) asexual blood phase; and 3) sexual and sporogonic blood phase, with a valuable component of national and international scientific cooperation.

In this context, with financial support from national and foreign institutions and scientific cooperation from Latin America, Europe, and the United States, genomic and proteomic screening studies of the parasite have been carried out, using a collection of biological reagents such as sera from animal models (mice and primates) and individuals from

endemic areas on different continents, such as Latin America (Brazil, Peru, Panama, Guatemala, and Colombia), Africa (Mali, Burkina Faso, Senegal, Nigeria). In Colombia, the CIV has had access to very valuable reagents (serums and cells) from various endemic areas such as Nariño, Cauca, Valle, Chocó, and Córdoba, which have enabled comparative studies on the natural immune response to the parasite and its relationship with naturally induced clinical protection. On the other hand, the establishment of insectaries and the development of mosquito infection models and their use for controlled experimental human infection models has allowed access to sera and cells from volunteers experimentally vaccinated with irradiated sporozoites (Arévalo-Herrera, 2016a; 2016b) or naturally exposed (Molina, 2012) in the search for proteins critical to the parasite during the hepatic phase.

Additionally, the study of the blood phase proteome in the search for protein domains, characterized by the expression of alpha-helix structures (*coiled-coil motifs*) and by the induction of antibodies associated with protection (Céspedes, 2014; 2017). Furthermore, the group has focused significant efforts on characterizing the Pvs48/45 protein expressed in *P. vivax* gametocytes/gametes, whose neutralization blocks the development of the sporogonic cycle in the *Anopheles* mosquito (Arévalo-Herrera, 2022a; 2021; 2015; 2011c; 2005).

This infrastructure gives Colombia an important position as a global reference point for the study and development of vaccines for the different stages of the parasite, mainly but not exclusively against *P. vivax* malaria. Given the significant protective efficacy of the *Pf*CS component of Pf-RTS-S and Pf-R2, which, as mentioned previously, were the first vaccines to be approved by the WHO for implementation in a large number of endemic countries and as part of regular vaccination programs after more than 30 years of study (Stoute, 1997; Hill, 2011).

The present clinical study focuses on PvCS, an orthologous protein of *Pf*CS, specifically on confirming the safety and reproducibility of this vaccine candidate (Herrera, 2011b; Herrera, 2009c; Arévalo-Herrera, 2022a). The process of immunological characterization and analysis of *Pv*CS in preclinical and clinical phases is described in detail below.

2.4.1. Identification and characterization of *Pv*CS protein

The CS proteins of *P. falciparum* and *P. vivax* were identified using sera from individuals immunized with irradiated sporozoites (Druihle., 1998; Hoffman and Doolan, 2000). *P. vivax* CS *(Pv*CS) was identified and its gene cloned in 1985 (Arnot, 1985). *Chemical characterization* of the protein indicated that its structure is like that of the homologous protein in other *Plasmodium* species *(*Sinnis and Nussenzweig, 1996). *Pv*CS is composed of 373 amino acids and is characterized by a central domain (90-261 aa) consisting of short repetitive units flanked by non-repetitive protein fragments at its amino (N) and carboxyl (C) termini. The flanking regions contain small, highly conserved sequences called Region I (85-89 aa) and Region II-plus (338-355 aa), which have been identified as parasite binding domains for the invasion of hepatocytes by sporozoites (Cerami, 1992; Frevert, 1993). The central domain of

the protein is composed of 19 blocks of 9 amino acids each, of which two allelic forms can be found in nature, the VK210 or common type (GDRADGQPA) (Arnot, 1985) and VK247 or variant type (ANGAGNQPG) (Tsuji and Zavala, 2001) (Figure 1 and Figure 3). Probably due to its repetitive nature, this region has been shown to be immunodominant and is believed to mask the response against the N and C flanks and their invasion domains.

In addition to this dimorphism, limited polymorphism has been observed in the regions encoding the amino (N) and carboxyl (C) flanks of the protein. (Arnot, 1990; Gonzalez, 2001; Kain, 1992; Machado and Povoa, 2000; Maheswary, 1992; Mann, 1994; Qar, 1992; Rosenberg, 1989; Wirtz, 1987). This polymorphism does not appear to have a major influence on the immunogenic regions (epitopes) of the protein.

Over the last two decades, different research groups, including the CIV, have carried out extensive *immunological characterization* of the protein, using sera from individuals vaccinated with irradiated sporozoites (RAS) and from semi-immune individuals from endemic areas. These individuals recognize the CS protein and induce a precipitation reaction on the surface of live sporozoites (CSP reaction) (Cochrane, 1976), which neutralizes the invasion of sporozoites into hepatocytes (Nussenzweig, 1969).

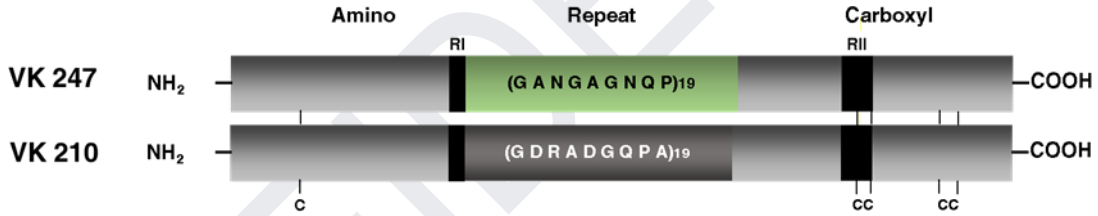


Figure 1. Structure of the *Pv*CS, VK247, and VK210 variants.

Using these sera, different B epitopes have been identified throughout the entire protein sequence (Arévalo-Herrera, 1998; Franke, 1992a), and the VK210 and VK247 sequences were found to be significantly recognized by sera from immune individuals from different endemic areas, indicating their wide distribution (Arévalo Herrera, 1998; Burkot, 1992; Cochrane, 1990; Franke, 1992b; Ramasamy, 1994; Wirtz, 1990). The VK210 variant contains the AGDR sequence, which is highly recognized by individuals from malaria-endemic communities, as well as by monoclonal antibodies capable of protecting *Saimiri* monkeys against challenge with infectious *P. vivax* sporozoites *(*Charoenvit, 1991).

Multiple helper T epitopes have also been recognized in the context of class II Major Histocompatibility Complex (MHC) molecule haplotypes (Herrera, 1994; Nardin, 1991). Furthermore, using nona- or decapeptides containing binding motifs for class I MHC antigens, our group identified five peptide sequences in the CS protein of *P. vivax* capable of stimulating human CD8+ lymphocytes from HLA-A*0201 individuals (Figure 2). These peptides induced

the production of IFN-γ, a cytokine involved in protection against malaria, by mononuclear cells from individuals previously naturally infected with *P. vivax* malaria *(*Burkot, 1992; Franke, 1992b).

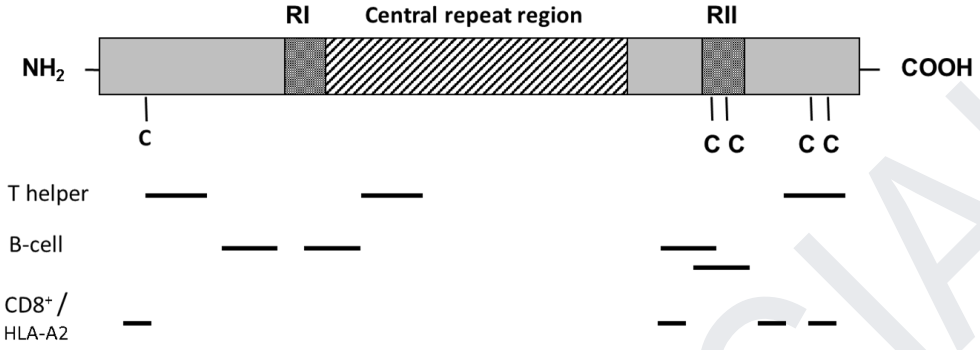


Figure 2: Mapping and identification of T helper, B cell, and cytotoxic (CD8+ HLA-A2) epitopes in the CS protein of *P. vivax*

2.4.2. Development of the *P. vivax* CS protein as a candidate for an antimalarial vaccine.

In 1987, the *Pv*CS protein was initially proposed as a vaccine candidate by Dr. Ruth Nussenzweig's group at New York University when it was tested as a recombinant protein (rPVCS-1) in mice, in which it induced a strong neutralizing antibody response (Cattani, 1986b). Later, two clinical trials were conducted in the USA using recombinant proteins, which failed to induce significant immune responses that would justify their development as a vaccine (Gunewardena, 1994; Williams, 1996); and during the following decade, no further clinical trials with the same protein were reported.

2.4.2.1. Preliminary preclinical studies in Colombia and other countries.

CIV has focused significant efforts on the immunological characterization of *Pv*CS, with the aim of obtaining a rational vaccine design (Arévalo-Herrera, 2001). Initially, using epitopes identified in the protein, a series of Multi-Antigenic Peptides (MAPs) were constructed containing various combinations of the B and Th epitopes. Two of the seven MAPs designed produced strong specific antibody responses against CS and IFN-γ production in preclinical trials conducted in primates. However, these MAPs could not be purified in the quantities required for subsequent clinical trials (Herrera, 1997). Therefore, within the framework of cooperative studies between the CIV and the group of Dr. G. Corradin of the University of Lausanne (Switzerland), they decided to use the strategy of Long Synthetic Peptides (LSPs) with sufficient length (>70-mer) to contain multiple B, Th, and CTL epitopes. LSPs derived from the CS proteins of P. *falciparum* and *P. vivax* were synthesized separately and tested in preclinical trials in *Aotus* monkeys *(*Arévalo-Herrera, 1998).

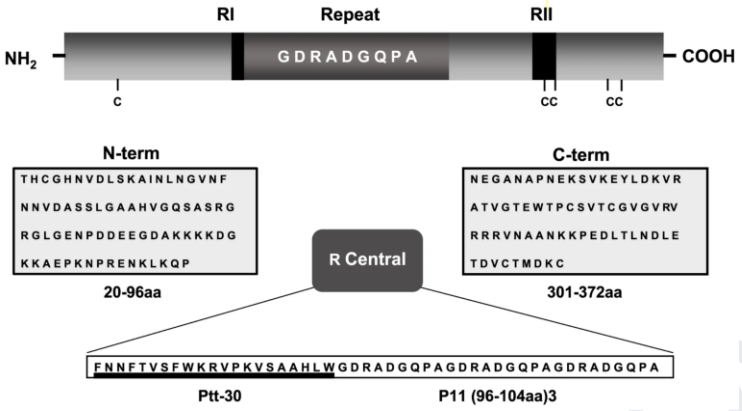
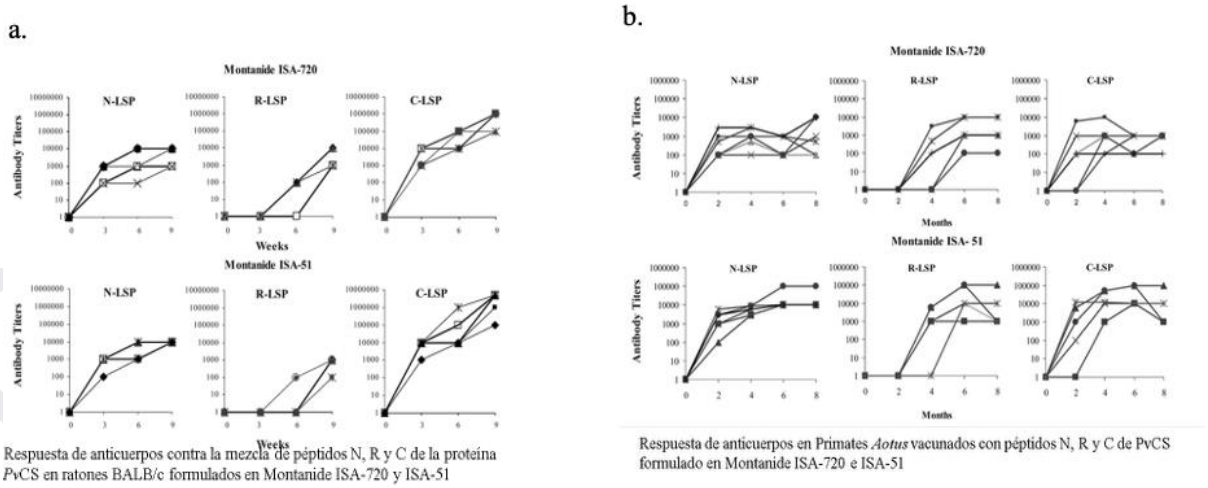


Figure 3: Structure of the *Pv*CS protein, amino (N-terminal) region, central region, and carboxyl (C-terminal) region with their respective amino acids.

These studies indicated high immunogenicity and the ability of *P. vivax* sporozoites to *boost* this immune response (Herrera, 1997). In both trials, the animals produced high titers of specific antibodies capable of recognizing the native protein in antigenic preparations of the parasite, by immunofluorescence (IFAT), in addition to stimulating the release of IFN-γ *in vitro*, determined by the ELISpot technique. Simultaneously with these studies in Colombia, the Walter Reed Institute (WRAIR) group in Silver Spring (MD) in the USA developed a recombinant chimeric protein with sequences from different variants of *Pv*CS. The recombinant vaccine formulated in Montanide ISA was highly immunogenic in mice, and the CS protein was recognized by sera from individuals infected with P. *vivax (*Yadava, 2007).



Respuesta de anticuerpos contra la mezcla de péptidos N, R y C de la proteína *Pv*CS en ratones BALB/c formulados en Montanide ISA-720 y ISA-51

Respuesta de anticuerpos en Primates *Aotus* vacunados con péptidos N, R y C de PvCS formulado en Montanide ISA-720 e ISA-51

Figure 4: Antibody response determined by ELISA, induced by the *Pv*CS protein formulated in Montanide ISA-720 and ISA-51 adjuvants (a) in BALB/c mice and (b) in *Aotus* primates immunized with the same formulation. Each line represents an individual animal.

In Colombia, the CIV developed a new preclinical study of the vaccine to test its immunogenicity in BALB/c mice and *Aotus* monkeys (Céspedes, 2013; Arévalo-Herrera, 2011a). For these studies, combinations of the three synthetic peptides corresponding to the amino (N), central repetitive (R), and carboxyl (C) regions of the CS protein were used, formulated in the adjuvants Montanide ISA-720 and Montanide ISA-51. Both formulations were highly immunogenic in both animal species. Mice developed a better antibody response against the C and R polypeptides, while the N polypeptide was more immunogenic in primates.

Because *Aotus lemurinus griseimembra* primates develop variable infections in their prepatent period after infectious challenge with *P. vivax* sporozoites, it was not possible at that time to reliably test the protective efficacy of vaccination in primates. Furthermore, experimental mice are also not susceptible to infection with human parasites. *In vitro* tests were used as an alternative to determine the protective capacity of antibodies induced by immunization, to determine whether specific IgG antibodies against the *Pv*CS protein had the ability to block the invasion of viable sporozoites into cultured liver cells (HepG2 cell line) (Arévalo-Herrera, 2011b).

Although during the period in which preclinical studies were conducted in mice and primates, it was not possible to measure protective efficacy in animal models, given the safety, tolerability, and immunogenicity of the *Pv*CS + Montanide ISA 51 (*Pv*CS.2 / M-51) formulation, we decided to move forward with its development in the clinical phase.

However, in recent years, a murine model has been developed in which it is possible to determine the protective efficacy of vaccines against human malaria in mice, using murine (mouse) parasites such as *Plasmodium berghei* transfected with human parasite genes (Raghunandan, 2020) from the *PbPf* model. Subsequently, a transgenic parasite containing a fragment of *Pv*CS was developed (Espinosa 2013).

More recently, taking advantage of the advances made by CIV in the clinical phase and these developments at Johns Hopkins University in Baltimore (MD) (USA), we decided to work together on adapting this model to study several antigens from the pre-erythrocytic phase of *P. vivax*. This cooperation began with a modification of the transgenic parasite, and two versions were produced containing the *Pv*CS gene in its two most prevalent variants in nature (*Pv*CS VK210 and *Pv*CS VK247). Using the formulation used in the phase II clinical trial in Cali (*Pv*CS + Montanide ISA-51 (Arévalo-Herrera, 2022) and using the same strategy used in humans, in which mice are initially vaccinated with the N+C mixture and subsequently with N+R+C, C57Bl/6 mice were immunized. Figure 5 presents a summary of the study: **panel a** shows the general design of the trial, with an immunization phase on days 0, 30, and 60, followed by the infectious challenge, and finally the analysis of liver infection using bioluminescence. **Panel b** shows the antibody immune response against the central region determined by ELISA; panel c shows a histogram indicating the level of protection achieved. Immunization induced a vigorous immune response against the protein with ELISA titers > 2 x 10^5 and recognition of the native *Pv*CS protein in IFA studies. Most notably, protective

efficacy against challenge with the *P. berghei/P. vivax*-CS parasite was demonstrated to be slightly higher (68%) than that observed in humans (64%) (Herrera 2025).

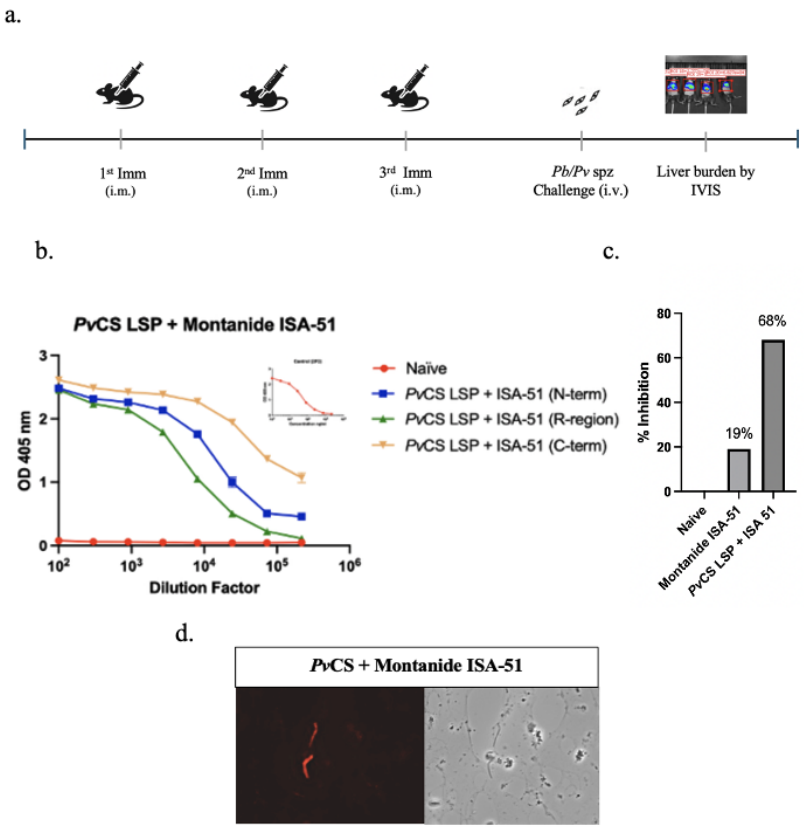


Figure 5: Evaluation of immunogenicity and protective efficacy of the *Pv*CS-LSP vaccine formulated in Montanide ISA-51 evaluated in mice. (a) Schematic of immunization and challenge protocol. (b) Specific antibody response against *Pv*CS N, R, and C peptides determined by ELISA. (c) Inhibition of liver invasion compared to naïve controls and Montanide ISA-51. (d) Recognition of sporozoites by antibodies from animals immunized with *Pv*CS by IFA.

This study has therefore validated not only the reproducibility of the immunogenicity and protective efficacy of *Pv*CS, but also the potential usefulness of the murine model for predicting the future performance of vaccines for human use (see non-clinical studies in the Investigator's Manual).

Simultaneously, in this context, studies have been conducted using the same animal model with nanoparticles based on self-assembled recombinant proteins (*Self-Assembled Protein Nanoparticles* - SAPN), containing the two most common natural variants, *P. vivax* CS VK210 and VK247, as well as two immunostimulants incorporated into the CpG nanoparticle and flagellin, meaning that the formulation does not require external adjuvants. These studies showed great similarity to those developed with the synthetic *Pv*CS formulation (Figure 6) (Herrera, SM, 2025b submitted), indicating the consistent protective capacity of the *Pv*CS protein with both synthetic and recombinant proteins, and the reproducibility of the murine

model. These latest studies have made it possible to advance more rapidly, at significantly lower cost and without the use of humans or experimental primates, with a new *Pv*CS formulation which, although not corresponding to the studies proposed here, represents a promising path for this antigen and for other pre-erythrocytic candidates in the difficult field of vaccines against *P. vivax.*

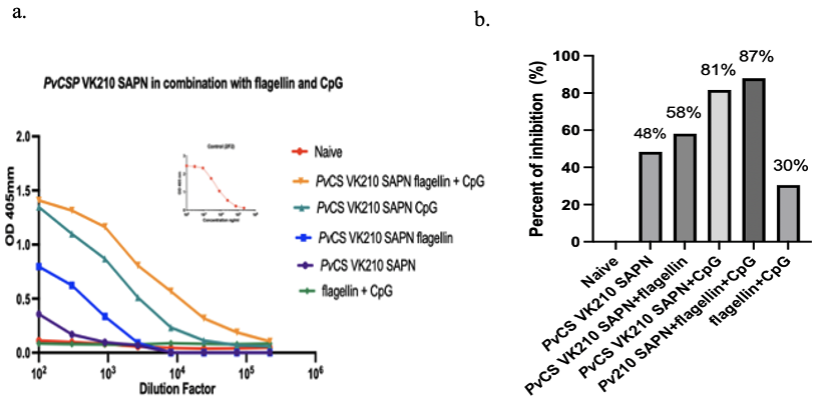


Figure 6: Evaluation of the immunogenicity and protective efficacy of the *Pv*CS VK210-SAPN vaccine evaluated in mice. (a) Anti-*Pv*CS antibody titers evaluated by ELISA in mice immunized with different combinations of immunostimulants (flagellin and CpG). (b) Immune response and activity induced in combination with immunostimulants and *Pv*CS VK210-SAPN.

2.4.3. Phase I clinical trials with *P. vivax* CS protein

First Phase I clinical trial

Based on the results of the preclinical phase, in 2000 the CIV decided to initiate Phase I clinical studies to determine the safety, tolerability, and immunogenicity of three (3) long synthetic peptides (LSPs) derived from *Pv*CS in human volunteers residing in Cali, an area without malaria transmission, and subsequently conduct clinical studies aimed at standardizing a method for infecting healthy volunteers with viable *P. vivax* sporozoites, in preparation for the development of protective efficacy trials in subsequent Phase II trials of the vaccine.

The initial studies were conducted in Cali, jointly by the CIV and the Fundación Clínica Valle de Lili, and were financially supported by Colciencias, the Ministry of Social Protection, and the US National Institutes of Health (NIH/NIAID). They were monitored by the WHO and are briefly described below.

This Phase I study evaluated the safety, tolerability, and immunogenicity of *Pv*CS LSPs in young (18-45 years old), healthy volunteers with no history of malaria. This trial was a randomized, double-blind study conducted in 69 male and female volunteers with no previous

exposure to malaria, as demonstrated by medical history and serological tests, who met all the inclusion criteria. The volunteers were immunized with the three LSPs corresponding to different regions (Amino= N; Central e Repetitive= R, Carboxyl= C) of *Pv*CS, formulated in the Montanide ISA-720 adjuvant (Seppic, Inc.). The three peptides were administered in escalating doses of 10 µg, 30 µg, and 100 µg, which were safe and proved to be well tolerated and highly immunogenic. The incremental doses of the vaccine were also administered in a staggered manner over time; only after the first two doses of 10 µg had been administered was the 30 µg dose tested, and the same strategy was used for the 100 µg dose.

Although the volunteers presented minor signs and symptoms at the injection site, none developed serious or severe adverse events (AEs). Table 1 describes the occurrence of AEs recorded after the administration of the different doses (see Annex IV *Safety and Efficacy* in the Investigator's Manual).

Number (%) of adverse events

Symptom	Dose group 10 µg	Dose group 30 µg	Dose group 100 µg	Control group
Most probably related adverse events				
Local pain at injection site	21 (100)	21 (100)	21 (100)	6 (100)
Erythema	1 (0.05)	2 (0.1)	0 (0)	0 (0)
Local edema	6 (29)	5 (24)	7 (33)	1 (17)
Other common adverse events*				
Axilar adenopathy	5 (24)	6 (29)	3 (14)	1 (17)
Cervical adenopathy	6 (29)	5 (24)	8 (38)	2 (33)
Cefalea	12 (57)	14 (67)	15 (71)	4 (67)
Common cold	12 (57)	17 (81)	21 (100)	3 (50)
Hypoglycemia	7 (33)	8 (38)	8 (38)	1 (17)

* Includes adverse events classified as not related to immunization.

Table 1: Adverse events observed after immunization with different doses of the *Pv*CS vaccine (10, 30, and 100 µg) compared to the control group.

After immunization, all individuals seroconverted as demonstrated by the ELISA technique, although there were differences in antibody titers against the different peptides. These antibodies recognized the native protein in *P. vivax* sporozoites in IFAT tests and demonstrated the ability to block sporozoite invasion of liver cell lines *in vitro*, as assessed by the sporozoite invasion inhibition (ISI) test (Arévalo-Herrera, 2011b).

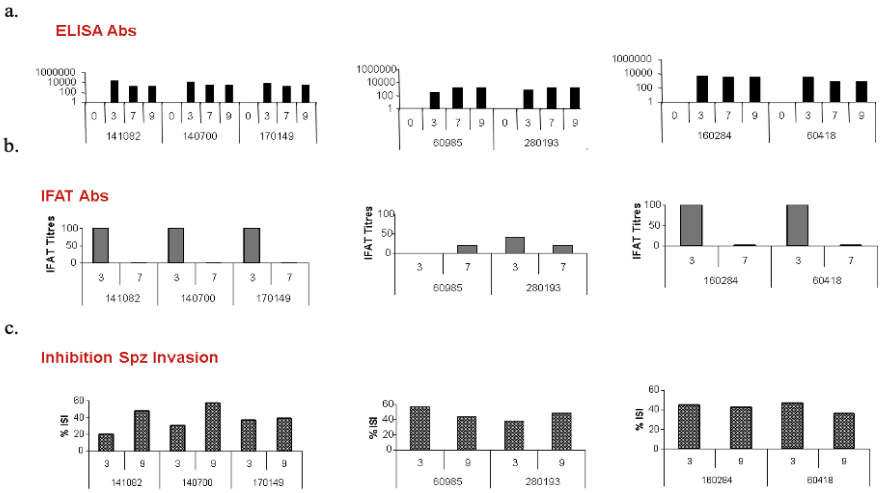


Figure 7: Specific antibodies against *Pv*CS fragments, measured by (a) ELISA (b) IFAT (c) Inhibition of sporozoite invasion of human hepatoma cells (HepG2 *in vitro*).

The CIV subsequently conducted a study of the cellular and antibody immune response in 21 of the 69 patients in this clinical trial (Arévalo-Herrera, 2011b). The antibodies were predominantly of the IgG1 and IgG3 isotypes and again showed recognition of parasitic proteins (IFAT) and partial blocking of invasion in the ISI test. The high antibody response, its activity in blocking liver invasion, and the stimulation of *in vitro* IFN-γ production by peripheral blood mononuclear cells in most volunteers provided evidence and encouraged further studies. The first Phase I clinical trial was therefore successful and led to a new trial to optimize the vaccine formulation (Herrera, 2011a).

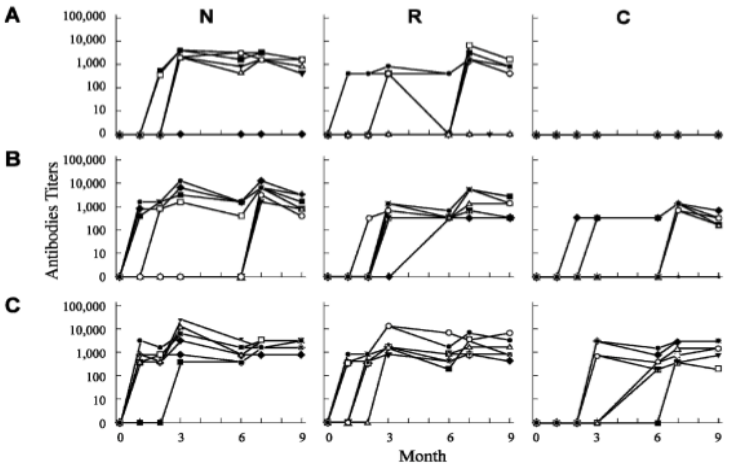


Figure 8: Specific antibody response against *Pv*CS N, R, and C peptides in volunteers immunized during Phase I clinical trial.

Second Phase I clinical trial with combined peptides.

A study was designed to determine the safety, tolerability, and immunogenicity of a mixture of LSPs formulated in Montanide ISA-720 and Montanide ISA-51 (SEPPIC Inc.), two of the most potent adjuvants for use in humans (Herrera, 2011a). This second Phase I clinical trial was proposed to identify which of these two adjuvants generated better safety, tolerability, and the optimal dose for use in subsequent Phase II trials.

The trial consisted of a randomized, double-blind, dose-escalation controlled clinical study. Forty healthy volunteers from the city of Cali who had not previously been exposed to malaria were randomized to receive three injections of the mixture of synthetic peptides N, R, and C at doses of 50 µg or 100 µg/dose/peptide, formulated in the Montanide adjuvants described, and the corresponding control group received the corresponding adjuvant as a placebo. The first immunization consisted of a mixture of the N+C peptides, while the second and third immunizations were mixtures of the three peptides N+C+R, a strategy aimed at reducing the immunodominance of the R region, described previously, which could affect the protective capacity induced by the other two fragments N and C. Once again, the vaccines were well tolerated and there was no association with serious or severe AEs. Table 2 describes the occurrence of AEs recorded after the application of the different formulations (adjuvant and dose) (see Annex IV *Safety and Efficacy* in the Investigator's Manual).

Number of volunteers reporting vaccine-related adverse events per dose and adjuvant group

		Montanide*								
		ISA 51			ISA 720			Placebo		
Adverse event	No. doses	1	2	3	1	2	3	1	2	3
	n =	(16)	(15)	(15)	(16)	(15)	(14)		(N = 8)	
Local										
Injection site pain		14	12	9	14	13	12†	4	8	3
Swelling		5	2	2	11	6	8	4	1	2
Erythema		1	0	1	1	0	2	1	0	1
Systemic										
Fever (> 37.5°C)	0	1	0	1	1	0	2	1	1	
Headache	3	2	1	5	4	0	2	2	0	
Dizziness	1	1	0	1	0	0	3	0	0	
Nausea/Emesis	1	2	0	2	0	0	2	1	0	
Abdominal pain	1	0	0	2	0	0	1	0	0	
Adenopathy (Axilar/cervical)	0	1	0	1	0	0	0	0	0	

*Montanide at 150 µg or 300 µg dose.
†Two-tailed *P* values < 0.05 by Fisher exact test, for comparison among adverse events (AE) in adjuvant and placebo groups.

Table 2: Adverse events related to immunization reported in healthy volunteers after administration of different formulations of *Pv*CS LSP in Montanide ISA-51, Montanide ISA-720, or placebo adjuvants.

The antibody response determined by ELISA again showed seroconversion, but as in the previous trial, the N peptide induced earlier antibodies and higher titers. The response against the C and R peptides appeared after the second immunization, with 97% of volunteers responding against these peptides.

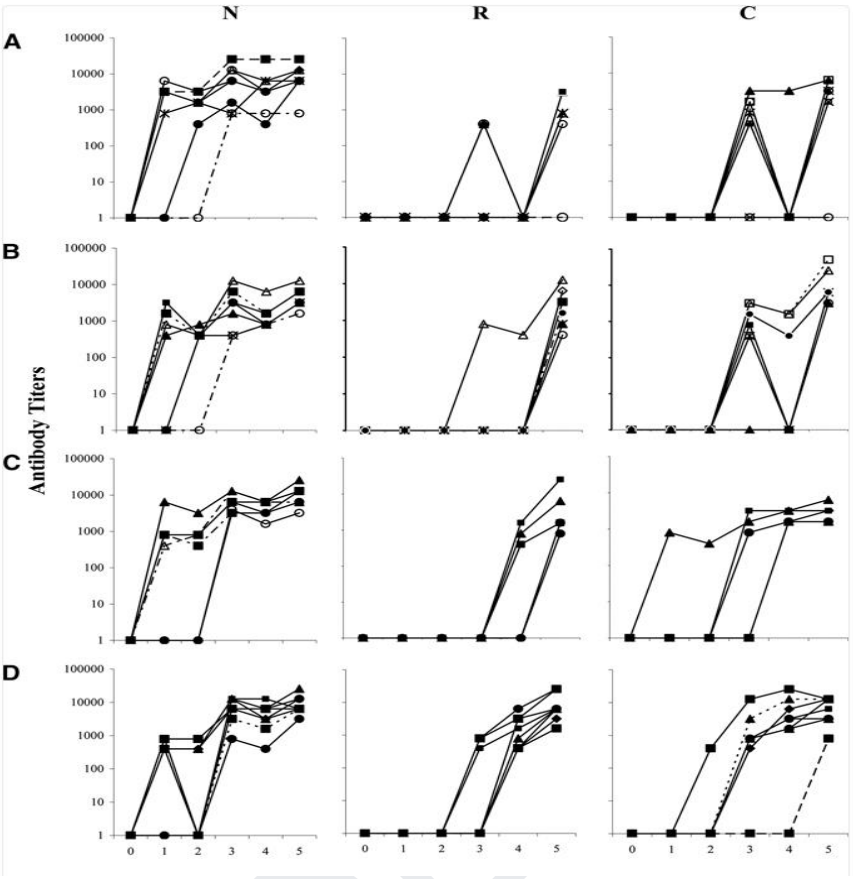


Figure 9: Antibody response against *Pv*CS N, R, and C peptides in volunteers from the phase IIa/b clinical trial in Chocó, Colombia.

Confirmation of the safety, tolerability, and immunogenicity of these formulations prompted the initiation of studies aimed at establishing an infectious challenge model with sporozoites for use in vaccine protective efficacy trials (see 2.4.5 Infectious Challenge Models).

First Phase IIa/b clinical trial

Based on the success of the two Phase I trials and the establishment of the infectious challenge model (described below), the CIV planned and carried out, in partnership with the Imbanaco Medical Center (CMI) in Cali, the development of a randomized, double-blind, controlled clinical trial to evaluate, as a primary outcome, the safety and protective efficacy of the synthetic *P. vivax* circumsporozoite (CS) protein formulated in the Montanide ISA-51 adjuvant in healthy young people (aged 18-45), both men and women, including a group that had not suffered from malaria (naïve) (phase IIa) and volunteers with previous clinical malaria, considered here as semi-immune (phase IIb). Participants (n=35) were randomly selected from a larger group (n=121) of volunteers from Cali city without local malaria transmission (naïve group) and from Buenaventura, an area with indigenous malaria transmission (semi-immune group). The 35 participants were divided into naïve (n=17) and semi-immune (n=18) groups and were immunized at months 0, 2, and 6 with the *Pv*CS protein formulated in Montanide ISA-51 adjuvant, or with Montanide ISA-51 adjuvant alone, as a placebo.

Adverse events[a]	Group	Phase IIa						Phase IIb					
		Naive ($n = 11$)			Control ($n = 5$)			Semi-immune ($n = 11$)			Control ($n = 5$)		
	Doses	1	2	3	1	2	3	1	2	3	1	2	3
Local													
Injection site pain		5	4	5	3	2	3	1	3	3	1	2	
Swelling		1	1										
Systemic													
Headache		2		1	3	1	1	1	1	1	1		
Malaise		3	1		2		1					2	1
Fever		2			2			1				1	
Nausea/Emesis		2			1				2				
Chills										2			
Diarrhea		3			1			1					
Abdominal pain		1			1								

Table 3: Adverse events observed in participants in phase IIa and phase IIb clinical trials after immunization with the *Pv*CS protein formulated in Montanide ISA-51, compared to the control groups.

After immunization, specific antibodies and the *in vitro* IFN-γ release response to stimulation of participants' mononuclear blood cells with *Pv*CS LSPs were evaluated; all experimental volunteers developed specific IgG and IFN-γ production.

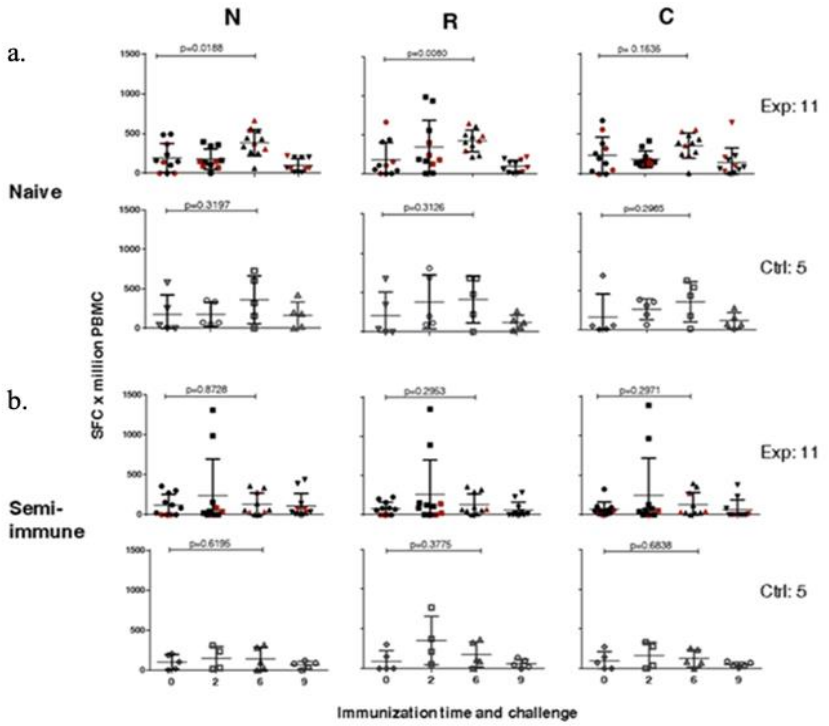


Figure 10: IFN-γ response against *Pv*CS N, R, and C peptides in naïve (a) and semi-immune (b) volunteers immunized with *Pv*CS formulated in Montanide ISA-51.

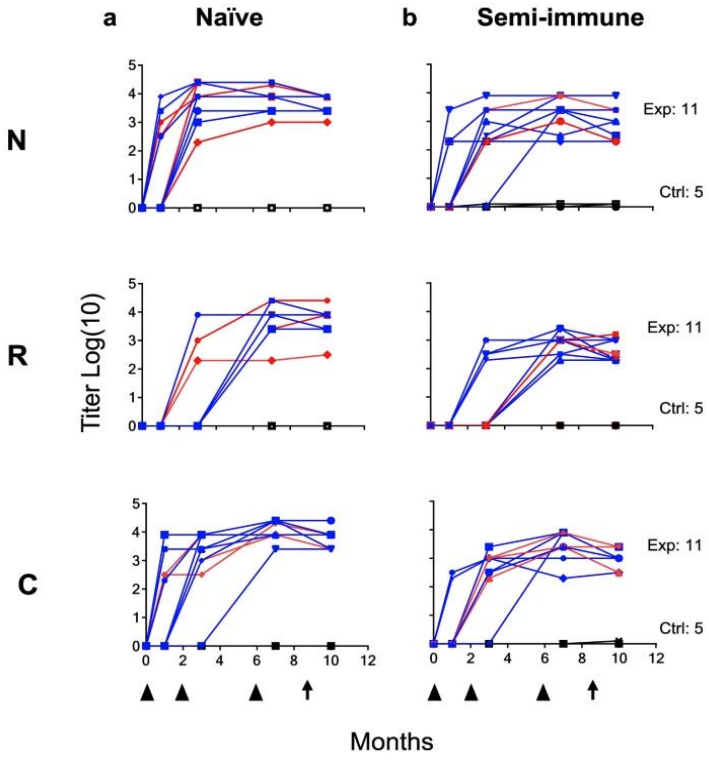


Figure 11: Antibody response against *Pv*CS N, R, and C peptides in naïve (a) and semi-immune (b) volunteers immunized with *Pv*CS formulated in Montanide ISA-51.

Three months after the last immunization, all participants underwent a challenge infection (*CHMI*) with *P. vivax* parasites (sporozoites), inoculated through mosquito bites, using the method developed by the CIV, described below (see 2.4.5 Challenge infection models). The parasites were inoculated in a controlled manner by the bite of 2-4 *Anopheles* mosquitoes previously infected by artificial feeding through a membrane.

Within the experimental groups, there was a dramatic reduction in parasitemia, including sterile protection (no infection), in several volunteers in the experimental group, both in phase IIa (6/11) (54%, 95% CI 0.25-0.84) and phase IIb (7/11) (64%, 95% CI 0.35-0.92), however, no differences in parasitemia were observed between the two phase IIb subgroups. All placebo-vaccinated controls (except one control from the semi-immune group) became infected, as expected.

During the study, mild AEs occurred, like those induced by vaccination in expanded immunization programs (classical vaccines), as well as mild reactions in response to mosquito bites during the infectious challenge. Table 4 describes the occurrence of AEs recorded after the application of the different formulations (adjuvant and dose) (see Annex IV Safety and Efficacy in the Investigator's Manual).

In conclusion, this study indicated significant protection in both naïve and semi-immune volunteers (Arévalo-Herrera, 2022).

Phase	Group	Code	Doses	Mosquitoes		Pre-patent period (Days)	Parasite /uL (Microscopy)
				Bites**	Spz***		
IIa	NAIVE	CS1001	3	4	32	14	120
		CS1006	3	2	32	16	100
		CS1013	3	2	32	17	100
		CS1015	3	2	32	15	100
		CS1023	3	3	22	16	80
		CS1025	3	2	25	16	60
		CS1028	3	3	22	16	280
		CS1030	3	3	46	P	0
		CS1031	3	2	10	P	0
		CS1036	3	2	100	P	0
		CS1038	3	2	100	P	0
	CONTROL	CS1003	3	3	215	16	400
		CS1005	3	2	32	16	100
		CS1012	3	3	100	15	220
		CS1018	3	3	32	16	240
		CS1037	3	3	22	16	100
		CS1016	2*	NA	NA	NA	NA
IIb	SEMI-IMMUNE	CS1506	3	2	100	12	60
		CS1511	3	2	32	P	0
		CS1535	3	2	100	P	0
		CS1537	3	2	316	19	20
		CS1538	3	2	1000	15	60
		CS1547	3	2	32	P	0
		CS1553	3	3	22	19	400
		CS1565	3	3	100	15	160
		CS1569	3	2	100	16	340
		CS1575	3	2	100	14	128
		CS1581	3	2	10000	17	60
		CS1584	1*	2	NA	NA	NA
	CONTROL	CS1549	3	4	56	17	50
		CS1554	3	2	100	16	400
		CS1570	3	3	22	17	70
		CS1572	3	2	1000	P	0
		CS1574	3	2	32	17	220
		CS1579	1*	2	NA	NA	NA

P: Protected; NA: Not Apply; *Withdraws; **No. Infected mosquitoes; ***Spz density

Table 4: Results of the infectious challenge in naïve (phase IIa) and semi-immune (phase IIb) volunteers immunized with *Pv*CS formulated in Montanide ISA-51.

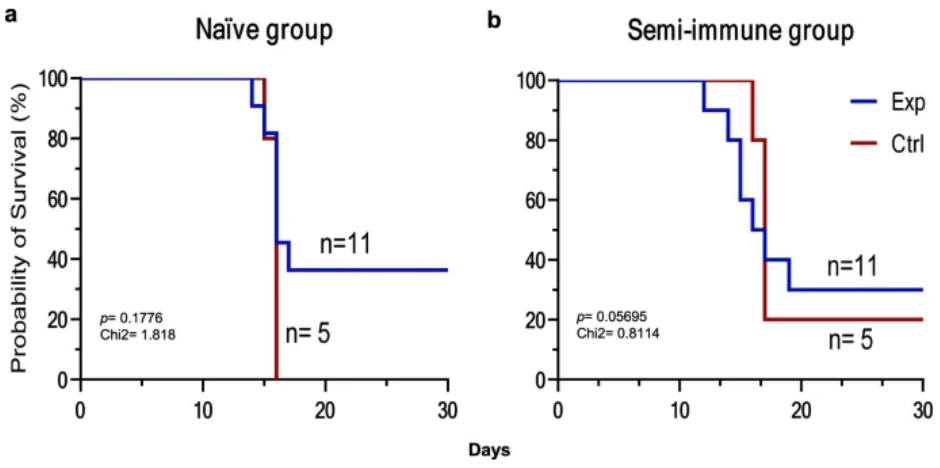


Figure 12: Kaplan-Meier curves of survival without parasitemia in naïve (a) and semi-immune (b) volunteers after infectious challenge.

To the best of our knowledge and after a systematic literature search, this study is the first report of a clinical trial of protective efficacy in *P. vivax* indicating these levels of protection. A previous study conducted by the Walter Reed Army Institute of Research (WRAIR) and reported in news reports and clinical records indicated that the vaccine evaluated did not achieve significant protection against parasitemia; it did show a small but significant delay in the time to parasitemia in a percentage of vaccinees (≈59%), and led to the conclusion that it was necessary to optimize candidates and models before advancing to field trials (Bennet, 2016).

These results prompted a clinical trial of the *Pv*CS vaccine in a larger number of volunteers and in other endemic areas of the country with a higher prevalence of malaria, such as the Department of Chocó. For logistical reasons, the study will be conducted in Quibdó, a municipality with better infrastructure and health conditions than the rest of the department.

2.4.4. Establishment of infectious challenge models for *P. vivax*.

Although chronologically the establishment of the controlled human malaria infection model (CHMI) was developed prior to the phase II clinical trial, it is included here so as not to alter the sequence of the safety studies (phase I) and the protective efficacy study (phase II) reported above. Therefore, we describe below the background of experimental human infection with malaria.

The possibility of experimentally infecting human volunteers with *Plasmodium* is a practice that has been carried out for nearly 100 years (Grassi, 1899) (Fairley, 1947), and during the 1950s and 1960s, experimental malaria infection was routinely used as a treatment for neurosyphilis ("malaria therapy") (Glynn, 1995). Later, a challenge model with *P. falciparum* sporozoites was developed and used to test the protective efficacy of a vaccine against *P. falciparum* based on radiation-attenuated sporozoites (Clyde, 1975; Clyde, 1973; Egan, 1993; Herrington, 1991; Rieckmann, 1979). More recently, the *P. falciparum* challenge model was improved and used extensively in major research centers around the world, primarily at the Naval Medical Research Center (NMRC) in the United States (Rockville, MD) (Hoffman, 2002), The Center for Clinical Vaccinology and Tropical Medicine, Oxford University (Walther, 2005) and the Department of Medical Microbiology at the University Medical Center in Nijmegen, Netherlands, and more recently at SANARIA Inc. under the direction of Dr. S. Hoffman (Hoffman, 2015), to determine the protective efficacy of various vaccines based mainly on the use of whole attenuated parasites. However, the challenge with *P. vivax* sporozoites was only used in a few patients and about 40 years ago.

Given the need to develop vaccines against the hepatic forms of *P. vivax* (pre-erythrocytic stages) and, in particular, the need to test the protective efficacy of the *Pv*CS protein, the CIV invested significant efforts in developing a Controlled Human Malaria Infection (CHMI) challenge system (*Controlled Human Malaria Infection - CHMI)* under controlled conditions of biosafety, volunteer protection, statistical significance, and reproducibility, taking into account the enormous logistical difficulties involved in working with this species.

2.4.5. Standardization of the challenge model with *P. vivax* sporozoites for the evaluation of protective efficacy

The lack of continuous *in vitro* cultures of *P. vivax* creates the need to use parasites from infected patients, which is why work on this species must be carried out in endemic areas with greater access to patients with parasites circulating in peripheral blood. For obvious reasons, this work is extremely difficult in non-endemic areas and in developed countries without malaria transmission.

Faced with the need for a method to test the protective efficacy of the described *Pv*CS candidate and other parasite antigens, the CIV, together with partners such as the Pacific Health Institute, ASOCLINIC Ltda., and others, has worked systematically on the standardization, optimization, and use of infection protocols using parasites from patients infected with P. *vivax* from the endemic area of the Pacific coast of Colombia, specifically Buenaventura (Valle).

Using the facilities of the Pacific Health Institute, particularly its malaria diagnostic service, and a colony of *An. albimanus* laboratory mosquitoes, preliminary studies were conducted to determine the susceptibility of mosquitoes to experimental infection under captive conditions, and optimal conditions were established to achieve reproducible infections (Hurtado, 1997). Subsequently, another experimental colony of *Anopheles* was established at the CIV headquarters in Cali, with the required biosafety measures. The biosecurity of these colonies includes, but is not limited to, the use of enclosed spaces, restriction of access by personnel not belonging to the unit, use of safety suits for operators, use of aspirators and light traps, and the use of security locks.

Under these conditions, a **first clinical challenge trial** in humans was conducted, in which 17 out of a total of 18 healthy young volunteers (aged 15-45), both men and women, were successfully infected. They had been screened to determine the presence of the Duffy group in their red blood cells (Fy+), which is essential for the parasite to invade the blood. Individuals (Fy-) are refractory to *P. vivax* infection. In addition, the normality of the glucose-6-phosphate dehydrogenase (G6PD) protein was examined, an enzyme that protects the integrity of cell membranes and whose deficiency can affect patients' tolerance to primaquine (PQ) treatment. The volunteers were exposed to *P. vivax* sporozoites administered through the bites of batches of 2 to 10 mosquitoes. All volunteers exposed to the bites developed malaria, with pre-patent periods of 9-11 days, determined by thick smear (TS) and PCR, and all were treated immediately upon confirmation of the presence of parasites in the blood, when parasitemia was minimal. The volunteers were closely monitored clinically and parasitologically to ensure early detection of potential adverse events (AEs), which were mild and most frequently related to mosquito bites; these events resolved within 24 to 72 hours after curative treatment (Herrera, 2009a; Herrera, 2009b). The study showed that there was no difference in the development of patent parasitemia in volunteers exposed to the different doses used, which is why it was decided that in future studies the lowest dose consisting of 3+1 bites from infected mosquitoes, confirmed microscopically, will be used. The minimum effective dose used here (2 infected mosquitoes) contrasts with the minimum dose used in *P. falciparum*

models in the United States and Europe, where at least 5 infected mosquitoes have been required to ensure infection in *Controlled Human Malaria Infection* (CHMI) studies (Roestenberg, 2013). Table 5 describes the occurrence of AEs recorded after volunteers were exposed to mosquito bites (see Annex IV Safety and Efficacy in the Investigator's Manual).

Consistent Safety and Infectivity in Sporozoite Challenge Model of *Plasmodium vivax* in Malaria-Naive Human Volunteers

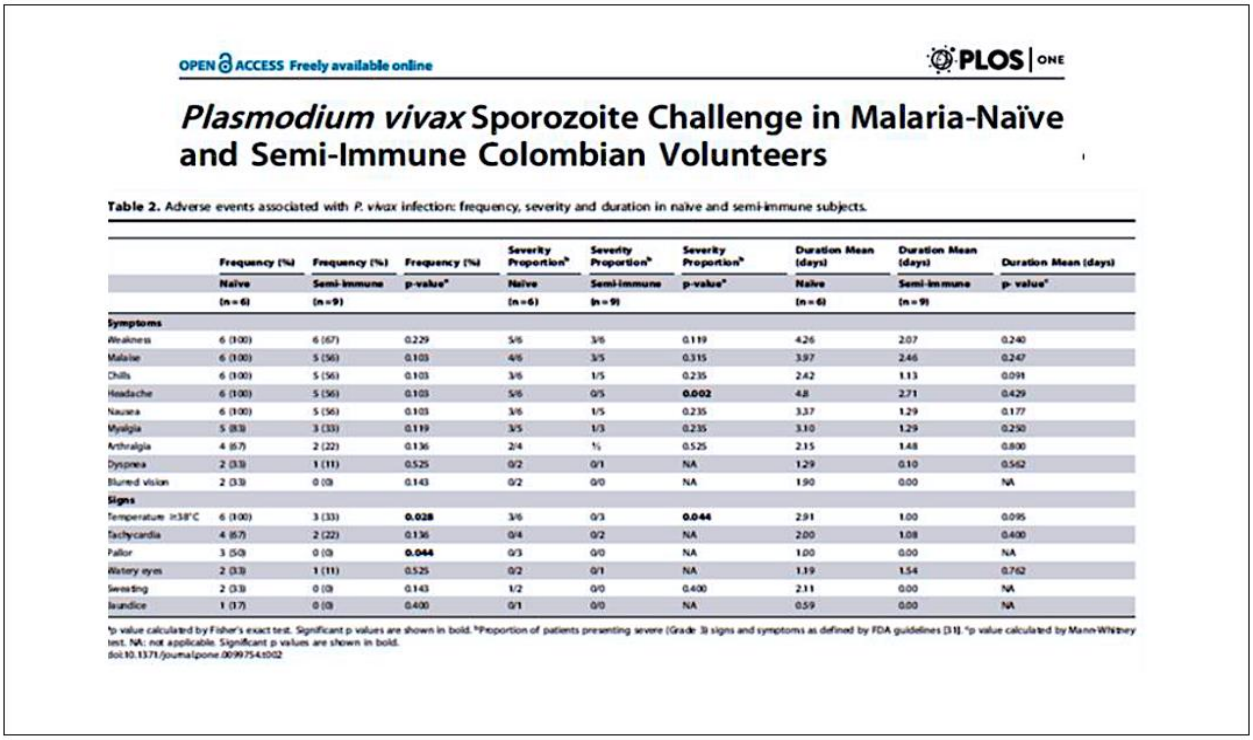
Local adverse events (AEs) related to mosquito bite

	Group			Total
Local AEs	A ($N = 8$) n*	B ($N = 8$) n	C ($N = 6$) n	($N = 22$) n
Pruritus	6	2	4	12
Swelling	0	2	0	2
Erythema	4	5	3	12

* Number of events.

Table 5: Local adverse events associated with *P. vivax*-infected mosquito bites in naïve volunteers included in the challenge model. The most frequent events were pruritus and erythema, both reported in 12 cases in total. All events were mild and transient.

To ensure the reproducibility of infection with potentially different *P. vivax* strains, a **second clinical challenge trial** was conducted in healthy volunteers selected using the same parameters as in the previous study. In addition, a control group of five volunteers selected for being Fy- and consequently refractory to P. *vivax* infection (n=20) was included. They were randomly assigned to groups 1, 2, and 3, which were exposed to doses of 2-4 bites from mosquitoes infected with parasites from three different donors, with the aim of determining their possible variability when exposed to genetically potentially different parasites. This time, infection occurred in all volunteers, with pre-patent periods like those in the first study, confirming the reproducibility of the challenge system (Solarte, 2011), except in Fy- volunteers. The AEs were again mild and like those in the previous study, and the volunteers were treated as soon as the infection in peripheral blood was diagnosed by thick smear examination. Table 6 describes the occurrence of AEs recorded after the volunteers' exposure to the bite of the mosquitoes (see Annex IV Safety and Efficacy in the Investigator's Manual).



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Plasmodium vivax Sporozoite Challenge in Malaria-Naïve and Semi-Immune Colombian Volunteers

Table 2. Adverse events associated with *P. vivax* infection: frequency, severity and duration in naïve and semi-immune subjects.

	Frequency (%) Naïve (n = 6)	Frequency (%) Semi-immune (n = 9)	Frequency (%) p-value[a]	Severity Proportion[b] Naïve (n = 6)	Severity Proportion[b] Semi-immune (n = 9)	Severity Proportion[b] p-value[a]	Duration Mean (days) Naïve (n = 6)	Duration Mean (days) Semi-immune (n = 9)	Duration Mean (days) p- value[a]
Symptoms									
Weakness	6 (100)	6 (67)	0.229	5/6	3/6	0.119	4.26	2.07	0.240
Malaise	6 (100)	5 (56)	0.103	4/6	3/5	0.315	3.97	2.46	0.247
Chills	6 (100)	5 (56)	0.103	3/6	1/5	0.235	2.42	1.13	0.091
Headache	6 (100)	5 (56)	0.103	5/6	0/5	**0.002**	4.8	2.71	0.429
Nausea	6 (100)	5 (56)	0.103	3/6	1/5	0.235	3.37	1.29	0.177
Myalgia	5 (83)	3 (33)	0.119	3/5	1/3	0.235	3.10	1.29	0.250
Arthralgia	4 (67)	2 (22)	0.136	2/4	½	0.525	2.15	1.48	0.800
Dyspnea	2 (33)	1 (11)	0.525	0/2	0/1	NA	1.29	0.10	0.562
Blurred vision	2 (33)	0 (0)	0.143	0/2	0/0	NA	1.90	0.00	NA
Signs									
Temperature ≥38°C	6 (100)	3 (33)	**0.028**	3/6	0/3	**0.044**	2.91	1.00	0.095
Tachycardia	4 (67)	2 (22)	0.136	0/4	0/2	NA	2.00	1.08	0.400
Pallor	3 (50)	0 (0)	**0.044**	0/3	0/0	NA	1.00	0.00	NA
Watery eyes	2 (33)	1 (11)	0.525	0/2	0/1	NA	1.19	1.54	0.762
Sweating	2 (33)	0 (0)	0.143	1/2	0/0	0.400	2.11	0.00	NA
Jaundice	1 (17)	0 (0)	0.400	0/1	0/0	NA	0.59	0.00	NA

[a]p value calculated by Fisher's exact test. Significant p values are shown in bold. [b]Proportion of patients presenting severe (Grade 3) signs and symptoms as defined by FDA guidelines [31]. [c]p value calculated by Mann-Whitney test. NA: not applicable. Significant p values are shown in bold.

doi:10.1371/journal.pone.0099754.t002

Table 6: Adverse events associated with the challenge in naïve and semi-immune volunteers. The frequency, severity, and duration of symptoms recorded after infection are shown, including fever, chills, headache, myalgia, arthralgia, asthenia, among others. In general, the events were mild to moderate, self-limiting, and comparable between groups, with no serious complications.

Finally, with the aim of accelerating the clinical development of the vaccine against *P. vivax* and taking into account that, following studies in malaria-free areas such as Cali, vaccines should be evaluated in endemic communities with a semi-immune population due to their previous contact with the parasite, a **third clinical challenge trial** was conducted with experimentally induced infections in naïve and semi-immune volunteers, which aimed to compare the evolution of malaria infection in individuals with and without malaria experience. In this trial, seven healthy adults with no malaria experience and nine semi-immune adults (total n=16) were subjected to bites from 2–4 *Anopheles* mosquitoes infected with parasitized blood from a single donor; all volunteers developed infections confirmed by microscopy and RT-qPCR. No significant differences were observed in the pre-patent period (mean 12.5 and 12.8 days for naïve and semi-immune patients, respectively). The volunteers developed classic signs and symptoms of malaria, while the semi-immune volunteers showed minor symptoms or no symptoms on the day of diagnosis. The infection induced an increase in specific antibody levels in both groups; this trial was safe and reproducible with mild AEs similar to the two previous studies (Arévalo-Herrera, 2022). Table 7 describes the occurrence of AEs recorded after exposure of volunteers to bites from mosquitoes (see Annex IV Safety and Efficacy in the Investigator's Manual).

Case Report: Successful Sporozoite Challenge Model in Human Volunteers with *Plasmodium vivax* Strain Derived from Human Donors

Adverse events associated with mosquito bites

	A				B				C			
			Severity grade†				Severity grade†				Severity grade†	
Adverse events	Frequency	Duration (days)*	1	2	Frequency	Duration (days)*	1	2	Frequency	Duration (days)*	1	2
Local Pruritus	1/6	1	X	-	4/6	1.25	X	-	1/6	5	X	-
Edema	1/6	1	X	-	1/6	1.25	X	-	0/6	-	-	-
Erythema	1/6	1	X	-	2/6	1.50	X	X	0/6	-	-	-
Arm pain	1/6	1	X	-	0/6	-	-	-	1/6	5	X	-

*Geometric mean.

†Grade 1 = low, Grade 2 = moderate.

Table 7: Local adverse events associated with the bite of infected mosquitoes in naïve and semi-immune volunteers. The main events reported were pruritus, erythema, and pain at the bite site, which were mild to moderate in intensity and of short duration. No serious events were observed.

Once the remarkable safety and reproducibility of this infectious challenge model (CHMI) had been established, it was proposed to determine the efficacy of immunization of naïve and semi-immune volunteers with the synthetic N+R+C peptides of *Pv*CS (Herrera 2009, Herrera 2011) described above (section 2.4.6), and that of naïve, healthy volunteers with irradiated sporozoites described below (Arévalo-Herrera 2016a).

As mentioned previously (section 2.3), in 1967, at the beginning of the search for a malaria vaccine, Clyde and colleagues conducted a study in which healthy volunteers were exposed to vaccination with irradiated sporozoites inoculated through mosquito bites. This study, conducted in the United States (Maryland, Walter Reed Army Institute of Research), demonstrated for the first time the extraordinary efficacy of this vaccination, which achieved protection greater than 90–95% for a duration of approximately 12 months (Nussenzweig, 1967, Clyde, 1973).

Subsequently, multiple studies with similar protocols were conducted and, as described in the last decade, Stephen Hoffman and his company SANARIA Inc. have worked to industrialize a vaccine that uses irradiated, dried, and cryopreserved sporozoites for intravenous administration (Hoffman et al., 2010; Epstein et al., 2011).

With regard to *P. vivax*, the model of immunization with radiation-attenuated sporozoites was only tested in 1970 by Collins et al. in two volunteers, and then not again until 2014, when the CIV (Cali, Colombia) conducted the first human study with *P. vivax*, which evaluated the safety of experimental exposure to infection by mosquitoes infected with irradiated and non-irradiated sporozoites, thus establishing the *Controlled Human Malaria Infection (CHMI)* platform for *P. vivax* (Arévalo-Herrera et al., 2014, 2016).

S4 Table. Adverse events during immunizations and after CHMI

Local	Grade	Radiation attenuated spz, n=14														Control, n=7											
		After immunizations													After CHMI	After immunizations											After CHMI
		1st		2nd		3rd		4th		5th		6th		7th		1st		2nd		3rd		4th		5th		6th		7th	
		Related		Related		Related		Related		Related		Related		Related	Related	Related		Related		Related		Related		Related		Related		Related	Related
		Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not	Yes	Not
Erythema in immunization site	Mild	28		24		17		5		1		4		1		12		10		4		1				1			
	Moderate			2				2														1							
	Severe																												
	Life threatening																												
Hyperesthesia in immunization site	Mild	26		22		12										2		10											
	Moderate																												
	Severe																												
	Life threatening																												
Wheals	Mild							9		7		4		4						1		2		1		3		4	
	Moderate					1		4		1				1								2		2					
	Severe																												
	Life threatening																												
Induration in immunization site	Mild			11		2																							
	Moderate																												
	Severe																												
	Life threatening																												
Local edema in immunization site	Mild											6		1															
	Moderate									3		1		1															
	Severe																												
	Life threatening																												
Pain in immunization site	Mild			4																									
	Moderate																												
	Severe																												
	Life threatening																												
Hyperpigmentation in immunization site	Mild									1																			
	Moderate																												
	Severe																												
	Life threatening																												

Table 8: Systemic adverse events observed during immunizations and after CHMI in volunteers. In the group vaccinated with radiation-attenuated sporozoites (n=14), the most frequent symptoms were headache, fever, myalgia, chills, and nausea, mostly mild or moderate and without serious outcomes. In the control group (n=7), fewer events were reported, mainly limited to mild headache and fever after immunization or CHMI.

2.5. State of the art

The recent approval by the WHO to proceed with an implementation phase for the RTS,S and R21 vaccines against *P. falciparum* represents an important achievement for the international scientific community in general and, in particular, for their developers, Glaxo Smith Kline, and the PATH *Malaria Vaccine Initiative* consortium, which for nearly four decades have pursued the goal of developing a vaccine that is sufficiently effective, cost-effective, and accessible to communities affected by malaria globally. On the other hand, it is also a significant achievement for the University of Oxford, which has worked intensively to improve the protective capacity of a vaccine related to the former; the two represent the most advanced vaccines in development worldwide.

Pf-RTS,S is a recombinant chimeric protein composed of fragments from the central and carboxyl-terminal regions of the *P. falciparum* circumsporozoite protein (*Pf*-CS), linked to the hepatitis B virus surface antigen (HBsAg), initially formulated in AS02A adjuvants and subsequently in AS01E. The proportions of the protein correspond to approximately 25% Pf-CS fragments and 75% HBsAg. This vaccine was a pioneer in the development of malaria vaccines and paved the way for others in research. In phase II studies formulated with AS02A, it induced protection of close to 34%, but when replaced with the AS01E adjuvant, efficacy increased to 50–55% in children in endemic areas.

Pf-R21, which is very similar to Pf-RTS,S, optimized its structure by increasing the proportion of Pf-CS relative to HBsAg and reducing the number of redundant epitopes, as well as being formulated with the Matrix-M (MM) adjuvant, which has greater immunostimulatory potential.

Both vaccines have been tested in endemic communities in several African countries (including Ghana, Malawi, and Kenya for Pf-RTS,S; and Burkina Faso and Mali for R21). They have now been approved by the WHO for pilot implementation programs in several African countries.

On the other hand, several vaccines based on the use of live parasites attenuated by radiation (Radiated Attenuated Sporozoites-RAS) or genetic manipulation (*Genetically Attenuated Sporozoites-GAP*) have been progressing in studies in recent years. In contrast to *Pf*- or *Pv*CS-based vaccines, these attenuated parasites, being complete, trigger responses against many of the more than 5,500 *Plasmodium* proteins. These formulations induce more vigorous and comprehensive responses in terms of the number of recognized antigens, but they represent a challenge from an industrial standpoint.

As mentioned previously, the development of vaccines against *P. vivax* represents a phenomenal challenge due to the lack of continuous *in vitro* cultures, the scarcity of animal models, the limitations imposed by the lack of insectaries with access to parasite samples, and, consequently, limited financial support. Nevertheless, the CIV's efforts to overcome these obstacles have led to the development of a very comprehensive infrastructure for the study of P. *vivax* and the development of vaccines to the level discussed above, which can be summarized as follows: **a)** exhaustive immunological analysis of the *Pv*CS protein (Arévalo-Herrera, 1998, 2001, 2002, 2010, 2011a, 2011b); b) antigenicity studies of the protein in humans naturally exposed to the parasite in endemic areas (Herrera, 2009c, 2011b, Solarte 2011); c) preclinical trials (Herrera 1994, 1997, Arévalo-Herrera 2010, 2011a,) and d) clinical phase trials (Herrera 2005, 2011a, Arévalo-Herrera 2001, 2010, 2016a, 2022). Interaction with international colleagues and bibliographic analysis allow us to affirm that, although two decades ago the Walter Reed Army Institute of Research (WRAIR) in Silver Spring (MD, USA) produced different formulations of the *P. vivax* circumsporozoite protein (*Pv*CS) that reached phase IIa clinical trials, these studies (Benett 2016, Lata De 2022), and probably the program in general, were suspended around 2003.

More recently, a group at the University of Oxford (UK) published a preclinical study in a mouse model on the immunogenicity and protective efficacy of the *Pv*CSP-21 protein (known as *Pv*21), formulated in the Matrix-M™ adjuvant (Novavax AB, Sweden), against challenge with a transgenic *P. berghei* parasite expressing *Pv*CS. This work demonstrated high immunogenicity and significant levels of protection (Salman 2017). However, to date, there has been no concrete progress with this candidate toward clinical development phases in humans.

Given this background, the progress made by the CIV in Colombia probably represents the most advanced *P. vivax* vaccine candidate in development worldwide. This progress is due to the comparative advantages of the CIV and the country described above, in terms of: **1)** access

to endemic communities in different latitudes (continents); 2**)** availability of insectaries in endemic and non-endemic areas; 3**)** access to animal facilities with rodents and non-human primates; 4**)** laboratory infrastructure with capacity for immunological, proteomic, chemical, and clinical studies; 5**)** cooperation with top-level national clinical research centers on an international scale; 6**)** national and international scientific partnerships and funding.

2.6. Scientific Justification

Except for Africa, where 99% of malaria cases are caused by *P. falciparum* infections, most endemic areas of the world, including Colombia, experience simultaneous transmission of *P. falciparum* and *P. vivax.* For this reason, despite valuable advances in the development of vaccines against *P. falciparum,* the identification and production of *P. vivax* antigenic components is required both for monovalent vaccines specific to this species and for the possible production of multi-species vaccines that simultaneously protect against *P. vivax and P. falciparum.*

The *Pv*CS protein was recognized four decades ago, and like *Pf*CS and several CSPs from other *Plasmodium* species, it was shown to have the same structure, immunogenic and protective capabilities. This protein is highly immunogenic and contains functional regions (epitopes) that are essential for the parasite's invasion of host cells in humans and mosquitoes. Antibodies against these epitopes or domains (RI, R2, Repeat) have consistently demonstrated the ability to block invasion of the liver cell in *in vitro* and *in vivo* studies, as well as to generate protection through both active immunization and passive transfer of specific antibodies (Atcheson 2020), anti-mosquito infection (RII) (Sinnis 1994) and *anti-repeat* (Arévalo-Herrera 1998, 2011b, 2016, Herrera 2005, 2011a).
Additionally, numerous pieces of evidence indicate the feasibility of developing vaccines against malaria and represent a solid scientific justification for the development of the proposed clinical study.

1) Immunization with radiation-attenuated sporozoites has consistently demonstrated the induction of sterile immunity (>90%) to several species of parasites, including *P. falciparum* and *P. vivax* in animals and humans (Nussenzweig 1969, Druille 1998, Hoffman 2002, Arévalo-Herrera 2016a).
2) Several groups are advancing in the development of vaccines based on the use of *P. falciparum* attenuated by different methods, including genetic manipulation of the parasites, with promising results (Richie TL, 2023, Van der Boor SC, 2023; Vaughan AM, 2017).
   A significant number of trials conducted over the last decade have demonstrated the protective capacity of the RTS-S and R-21 vaccines containing *P. falciparum* CS in both individuals with no previous exposure to malaria and in endemic communities (Guinovart, 2009; Macete, 2007) (Hill, 2011; WHO, 2023), currently representing the most advanced vaccines approved by the WHO for implementation in Africa.
3) Although, for the reasons outlined above, vaccine research in the case of *P. vivax* has not progressed as far, the CIV has successfully developed several Phase Ia clinical trials with *Pv*CS, which demonstrated the safety and tolerability of the *Pv*CS + Montanide ISA 720 and Montanide ISA - 51 formulations. (Herrera, 2011a; Herrera, 2005).

4) The CIV has developed a safe and reproducible infectious challenge system essential for studies of the protective efficacy of antimalarial vaccines. This model has been successful in three (3) clinical studies using parasite isolates (strains) from different donor patients, even with doses as low as two (2) infected mosquitoes. (Herrera, 2011b; Herrera, 2009c, Arévalo-Herrera 2014).
5) The immunization of healthy, naïve volunteers in Colombia with irradiation-attenuated parasites (*Pv*RAS) demonstrated significant protective efficacy in Phase II clinical trials, comparable to that obtained in the United States with *P. falciparum*, and the sera of protected individuals preferentially recognized *Pv*CS, establishing a correlation between anti-*Pv*CS antibodies and protection (Arévalo-Herrera M, 2016a, Lopez-Perez 2024). These reagents have enabled the initiation of a program to discover new antigens with potential for vaccine development (NIAID Contract 1U01AI155363), (Herrera SM 2025).
6) More importantly, as evidence for the present study, the formulation proposed here, *Pv*CS + Montanide ISA 51, recently demonstrated high safety, tolerability, and protective efficacy greater than 60% (Arévalo-Herrera M, 2022).
7) Additionally, CIV, in association with Johns Hopkins University in Baltimore (MD) (USA), confirmed the protective capacity of this formulation in a murine (mouse) model that induced a vigorous immune response against the same formulation previously used in humans and equivalent protective efficacy against infection with a murine parasite (P. *berghei)* transfected with the *Pv*CS protein (Herrera SM 2025) (see non-clinical studies).

All these studies represent solid scientific evidence supporting the feasibility and potential success of the study proposed in this protocol.

3. HYPOTHESIS

- The application of the N, R, and C peptides of *P. vivax* CS formulated in Montanide ISA-51 adjuvant offers protection against malaria infection in naïve individuals and those previously exposed to malaria in malarial areas.

- Immunization with the *Pv*CS protein is safe in volunteers previously exposed to malaria in a natural way (semi-immune).

- Immunization of volunteers naturally exposed to malaria with the *Pv*CS protein produces a booster effect on the previous immune response present in these volunteers.

- Vaccination with *Pv*CS in individuals previously exposed to malaria induces a response that may offer sterile immunity.

4. OBJECTIVES

4.1.General objective

To determine the protective efficacy induced by the *Pv*CS vaccine formulated with the Montanide ISA-51 adjuvant in naïve and previously exposed volunteers in the department of Chocó.

4.2.Specific objectives

1. Confirm the safety of the vaccine in naïve volunteers immunized with *Pv*CS.
2. To determine the immunogenicity of *Pv*CS in individuals previously exposed to malaria.
3. Determine the protective efficacy of the vaccine against infectious challenge with viable *P. vivax* sporozoites in the above groups.

5. STUDY POPULATION

The study will have a total population of approximately 80 young volunteers aged 18-45, both men and women, residing in the urban area of Quibdó (Chocó), of whom 65 will be volunteers selected from a larger population of individuals who agree to participate in the immunization process and subsequently in an infectious challenge with *P. vivax* parasites administered through mosquito bites. Of these 65 volunteers, 60 will be regular participants in the process and the remaining 5 will be potential substitutes (or alternates) who would participate only if any of the regular participants are suspended or decide to withdraw early from the study (before the second vaccine dose). These participants must be semi-immune (n=30) and naïve (n=30) volunteers who may come from malaria-endemic areas of Chocó but reside in Quibdó and must meet all inclusion criteria and not present any exclusion criteria to be included in the study. The other participants will be a group of between 5 and 15 volunteer parasite donors in whom acute infection with P. *vivax* is confirmed.

The study has been structured in three main steps. In step #1, the 65 participants in the immunization and infectious challenge and their potential substitutes or alternates will be selected. In step #2, volunteer parasite donors will be selected for feeding and infecting *Anopheles* mosquitoes, and in step #3, the infectious challenge will be performed on the 60 final immunized volunteers.

5.1 Steps 1 and 3. Immunization and infectious challenge volunteers.

A total of 60 volunteers, semi-immune and naïve volunteers from malaria-endemic areas of Chocó, who are residents of the urban area of Quibdó, who meet the inclusion criteria and do not present any exclusion criteria, will be included in the study.

5.1.1.1 Group of healthy volunteers with no history of malaria (naïve).

Subjects must meet all the following inclusion criteria to be selected as participants in

this study:

- Healthy men and women between the ages of 18 and 45.
- Freely and voluntarily sign an informed consent form (IC), accompanied by two witnesses who will also sign.
- Be a resident of the urban area of the municipality of Quibdó.
- No history of malaria as assessed by medical history and serology.
- For women, use an appropriate contraceptive method from the start of the study until the contraceptive restriction is lifted by a study physician.
- Agree not to travel to areas considered to have higher malaria transmission in the department of Chocó or Colombia during the infectious challenge period (1 month).
- Be reachable by telephone throughout the study period.
- Hemoglobin (Hb) levels >11 g/dl.
- Availability to participate during the study period.
- Not participating in another clinical study.

To be selected as participants in this study, subjects must **NOT** meet any of the following exclusion criteria.

- Confirmed pregnancy by laboratory test, breastfeeding, plans to become pregnant from the time of recruitment.
- Duffy negative phenotype (Fy-).
- G-6-PD deficiency (d-G-6-PD).
- Any hemoglobinopathy.
- Personal history of allergies to medications or insect bites.
- Having received vaccination against malaria.
- Positive IFAT antibodies > 1:20 or ELISA greater than 1:200 for *P. vivax* in screening tests.
- Living in a malaria-endemic area during the 6 months prior to the study (municipalities with recognized endemic areas in Chocó, the Pacific Coast, and others).
- Clinical or laboratory evidence of systemic disease, including kidney, liver, cardiovascular, lung, psychiatric, or other diseases that could have a significant negative impact and alter the study results.
- Evidence of active hepatitis B and C or HIV infection.
- History of transfusion of any blood product in the six (6) months prior to the study.
- Plans to undergo surgery from the recruitment period until the end of post-challenge follow-ups.
- Presence or history of autoimmune disease (lupus, rheumatoid arthritis, thyroiditis, or other).
- Splenectomized volunteers.
- Volunteers undergoing treatment with drugs that affect the immune system (steroids, immunosuppressive agents, or immunomodulators).
- History of alcoholism or drug abuse is defined as a habit that interferes with the individual's normal social functioning.
- Any condition that may interfere with the ability to provide free and voluntary Informed

Consent (IC).

5.1.1.2 Healthy volunteer group with a history of malaria (semi-immune)

Subjects must meet all of the following inclusion criteria to be eligible to participate in this study:

- Non-pregnant, healthy men and women between the ages of 18 and 45.
- Freely and voluntarily sign an IC, accompanied by two witnesses who will also sign.
- Have a history of malaria infection(s) and positive serological tests for *P. vivax*.
- Be a resident of the urban area of the municipality of Quibdó
- For women, use an appropriate contraceptive method from the start until the contraceptive restriction is lifted by a study physician.
- Agree not to travel to areas considered endemic for malaria during the infectious challenge period (1 month) (municipalities with recognized endemic areas in Chocó, the Pacific Coast, and others).
- Be reachable by phone throughout the study period.
- Availability to participate during the period in which the study will be conducted.

To be selected as participants in this study, subjects must NOT meet any of the following criteria:

- Be negative for antibodies against *P. vivax* by the IFAT method <1:20 or ELISA less than 1:200 in screening tests.
- All other criteria used in the case of naïve volunteers, except for a history of living in the endemic area during the last 6 months.

To be selected as participants in this study, subjects must **NOT** meet any of the following exclusion criteria described above, except for a history of malaria determined by clinical history and serological tests:

5.2 Step 2. Volunteers donating blood infected with *P. vivax*

A total of 5-15 people infected with *P. vivax* who attend diagnostic centers in Quibdó or other malaria-endemic areas near Quibdó, with detected parasitemias ≥ 0.1% and who meet the inclusion criteria for parasite donors and do not have any exclusion criteria, will be included in the study.

To be selected as volunteer blood donors, subjects must meet all the following inclusion criteria:

- Non-pregnant men and women between 15 and 60 years of age
- Have a positive diagnosis of *P. vivax* malaria determined by thick smear examination.
- The patient must not have other detectable *Plasmodium* species in their bloodstream, such as *P. falciparum* or *P. malariae*.
- Have a *P. vivax* parasitemia ≥ 0.1% by thick smear.

- Have Hb ≥9 g/dL at the time of malaria diagnosis.
- Be able to provide informed consent freely and voluntarily. If illiterate, be able to affirm their decision to participate by placing their fingerprint on the informed consent form. Minors between the ages of 15 and 17 who wish to participate must sign the informed consent form, and one of their parents must sign the informed consent form, accompanied by two witnesses who will also sign.

To be selected as volunteer blood donors, subjects may **NOT** meet any of the following criteria:

- Have chronic or acute diseases other than *P. vivax* malaria.
- Have a history of diseases or clinical conditions that, according to medical criteria, could significantly increase the risk associated with participation in this study.
- Hb levels <9 g/dL at the time of recruitment.
- Have received antimalarial treatment prior to diagnosis.
- Have a history of diseases or clinical conditions that, according to medical criteria, could significantly increase the risk associated with participation in this study.

6. STUDY DESIGN

Diagram #3 provides an overview of the main activities of the study, which are described in detail below. These activities include: 1) the immunization process, 2) the infectious challenge, 3) clinical and laboratory analysis to determine the immunogenicity of the formulation administered and the laboratory parameters used to determine safety. Additionally, it indicates 4) the scheduled times for evaluation by the DSMB.

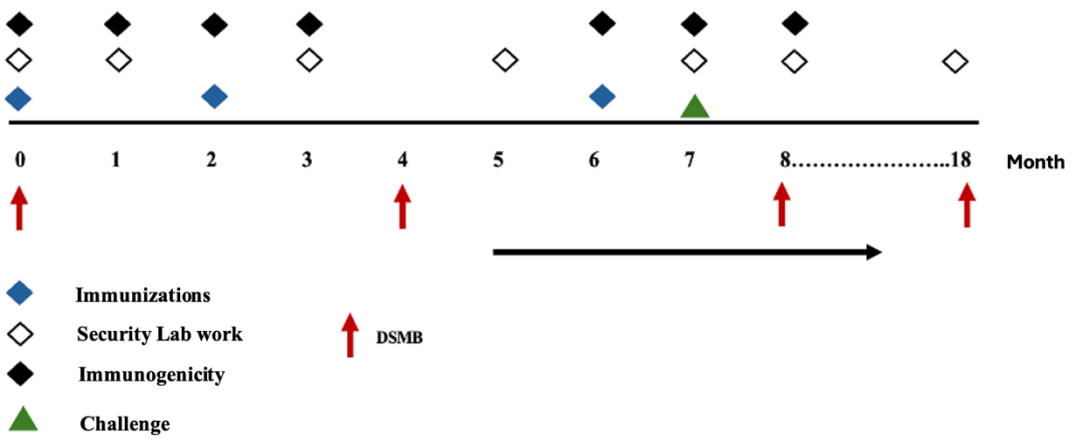

Figure 13: Summary diagram of the main procedures of the study.

6.1 Recruitment process

Candidates will be recruited through radio messages targeting the general population and through graphic material (posters, flyers) targeting those who attend health facilities, with an emphasis on the diagnostic centers of the Vector-Borne Diseases (ETV) network of the SDS

of Chocó in Quibdó. Candidates must be residents of the urban area of Quibdó, although they may come from municipalities such as Tadó, Lloró, Alto Baudó, and others. All candidates for this study must meet the inclusion criteria and will be excluded if any of the exclusion criteria are present. **Naïve** volunteers will be those who, based on their medical history and serological tests (ELISA, IFAT)**,** are determined to be negative for antibodies against *P. vivax* and *P. falciparum, and* individuals who have antibodies against *P. vivax* will be considered **semi-immune**. Some volunteers may react serologically with *P. falciparum,* but this reactivity will not be considered for admission.

During the call for volunteers, only individuals who are affiliated with the general social security health system (SGSS), in any of its regimes, can prove it with an official document will be considered. Eligible volunteers must sign up for an IC after being instructed by a team member on the purpose of the study. The person(s) providing this information will try to simplify the language to make it understandable to all candidates, avoiding the use of technical terms as much as possible. In addition, volunteers will be given a verbal test of their understanding of the study, particularly regarding the implications of informed consent (IC), the concept of blinding, confidentiality, and the risks and benefits of participating in the study, among others, to ensure their freedom and acceptance of the IC.

If it is found that the potential volunteer donor does not understand the study, up to three attempts will be made to explain it to them, and if the lack of understanding persists, the subject will not be included. Understanding of their participation in the study will be ensured by asking them to repeat the explanation given. Any elements that are not fully understood through this test will be explained again by the evaluator. Additionally, they will be encouraged to ask questions if they have any doubts. All IC procedures and volunteer questionnaires will be documented in the participants' table. In addition to the IC for participation in the vaccine trial, an IC form will be signed for HIV testing. If the individual is HIV-positive, they will be informed and referred for counseling and treatment. All volunteers will receive a copy of the IC.

6.1. Identification process

Volunteers will be assigned a five-character identification code: characters #1, 2, and 3 correspond to the project initials: Vaccine CS (VCS), character #4 is the description of the process (1: Immunization; 2: Infectious challenge; and 3: Selection of *P. vivax-infected* donors for mosquito feeding). Characters #5-6 correspond to the Quibdó site (12). The next two characters,#7-8, will be the number randomly assigned to each volunteer according to the randomization table for each group designated by the person in charge. Example: VCS11201 would correspond to being the first volunteer immunized in the randomly and blindly assigned group.

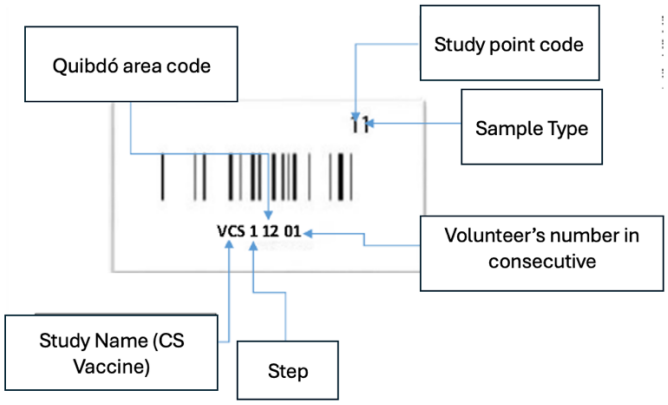

Figure 14: Structure of the identification code for volunteers in the CS vaccine study.

6.2. Selection process

The selection procedures (medical history, physical examination, and blood sampling) will be carried out after the volunteer signs the IC. If a volunteer has been selected and immunization has not begun within 12 weeks, the selection tests will be repeated. At the screening visit, medical history and concomitant therapy will be documented by one of the clinical investigators. A complete physical examination will be performed, including assessment of the sensory organs, cardiovascular (CV), pulmonary, neurological, gastrointestinal (GI), musculoskeletal, and dermatological systems. A spontaneous urine sample and 35 mL of blood will be collected for laboratory screening tests. When a woman is considered eligible for the study, she will be asked to use a hormonal method of contraception during the clinical trial period until the follow-up period is completed, at which time a final pregnancy test will be performed.

A physician at the study site (Quibdó) will oversee the clinical and paraclinical evaluation of volunteers during recruitment and selection for the study.

The following volunteer screening tests will be performed within 12 weeks prior to the first immunization:

Hematological tests:
Complete blood count, G-6-PD determination, Fy phenotype, hemoglobin electrophoresis, blood classification, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP).

Renal function tests: Urinalysis, creatinine, and BUN.

Infectious diseases other than *P. vivax*:
HIV (two rapid tests), Hepatitis B surface antigen (HBsAg), Hepatitis C virus (HCV), Human T-cell Lymphotropic Virus (HTLV) 1 and 2, RPR test for syphilis, and rapid test for Chagas disease.

Confirmatory tests:

If the result of either (or both) rapid HIV tests is positive, a fourth-level confirmatory ELISA test will be performed.

If the RPR is positive for syphilis (at any dilution), an FTA-ABS test will be performed.

If HBsAg is positive, a Hepatitis B anti-Core antibody test (anti-HBc) will be performed.

Liver function tests:

ALT, AST, total bilirubin, conjugated bilirubin, PT, and PTT.

Pregnancy test: determination of β-HCG in urine and serum.

OTHERS:

Blood glucose, electrocardiogram.

Immunological tests:

IFAT: for antimalarial antibodies and ANAs (antinuclear antibodies)

6.3. Group formation

A total of 60 people between the ages of 18 and 45 who voluntarily agree to participate in the study by signing an informed consent form and who meet the inclusion criteria will be included in the study and assigned to one of two main groups: **naïve** (groups A, n=30) and **semi-immune (**groups B, n=30) and will be randomly distributed into four subgroups corresponding to two experimental subgroups **(**A1 and B1) of 20 individuals per subgroup and two control subgroups **(**A2 and B2) of 10 individuals each. In the event of withdrawal of any volunteer, only those who withdraw from the study before the second immunization (month two) will be replaced; after this stage, it will not be possible to replace volunteers.
The vaccination schedule for new volunteers (replacements) will be adjusted so that their last vaccine dose (3rd) matches that of the other volunteers. Volunteers will be stratified into subgroups as shown in Table 1:

	Experimental	**Control**
Naïve	A1 (n=20)	A2 (n= 10)
Semi-immune	B1 (n= 20)	B2 (n= 10)
Total	40	20

Table 9: Group formation (Step 1)

6.4. Immunization process

6.4.1. Group formation

Groups A1 and B1 (n = 20 volunteers/group) will be immunized with the vaccine, and groups A2 and B2 (n = 10 each) will be immunized with placebo [See immunization schedule (Figure 2)]. The preparation of the assigned vaccine will be pre-coded to reduce potential selection biases and balance potential confounding factors, and its code translation must remain blind to the vaccinating investigator and clinical study staff (Herrera, 2005). To ensure double blinding of the study, the statistical staff will be responsible for randomizing the volunteers' codes and assigning them to the experimental and placebo groups. Software (OxMaR) will be used for randomization.

Once the scientific staff has formulated the vaccine, a member of the data management group will assign the codes of the subjects in each group to a sealed envelope, which will be delivered to the clinical staff who will immunize the volunteers with knowledge of the composition of the groups. Neither the principal investigator nor the laboratory staff nor the statistician in charge of data analysis will be allowed to know this composition. Before each immunization, each volunteer will be evaluated by one of the research physicians. If the volunteer reports the presence of any AE, it will be recorded on a Case Report Form (CRF). To determine the safety and tolerability of the vaccine, groups A1 and B1 will receive three doses of the vaccine. The first dose will consist of a mixture of peptides N and C only (50 µg/peptide; total dose 100 µg/dose) formulated in Montanide ISA 51, immunizations 2 and 3 will consist of peptides N, R, and C (50 µg/peptide/dose; total 150 µg/protein/dose). This peptide escalation strategy is aimed at reducing the volunteer's exposure to the central R fragment, considered immunodominant under natural conditions, which apparently competes with protective responses from the N and C fragments. Volunteers in groups A2 and B2 will be immunized with a placebo consisting of a saline emulsion in the same adjuvant. The immunization phase will last 6 months.

6.5.2 Vaccine formulation

The vaccine will be prepared directly in Quibdó by a pharmaceutical chemist (QF), as described in **Annex 1** (Biosafety)**;** this QF will have access to the composition of the groups. Using a 1 mL syringe with a 25G needle, a total of 500 µL of solution will be taken. The vaccine will be injected intramuscularly into the deltoid muscle opposite to the one used for blood sampling. A physician will oversee administering the vaccine to volunteers during this phase.

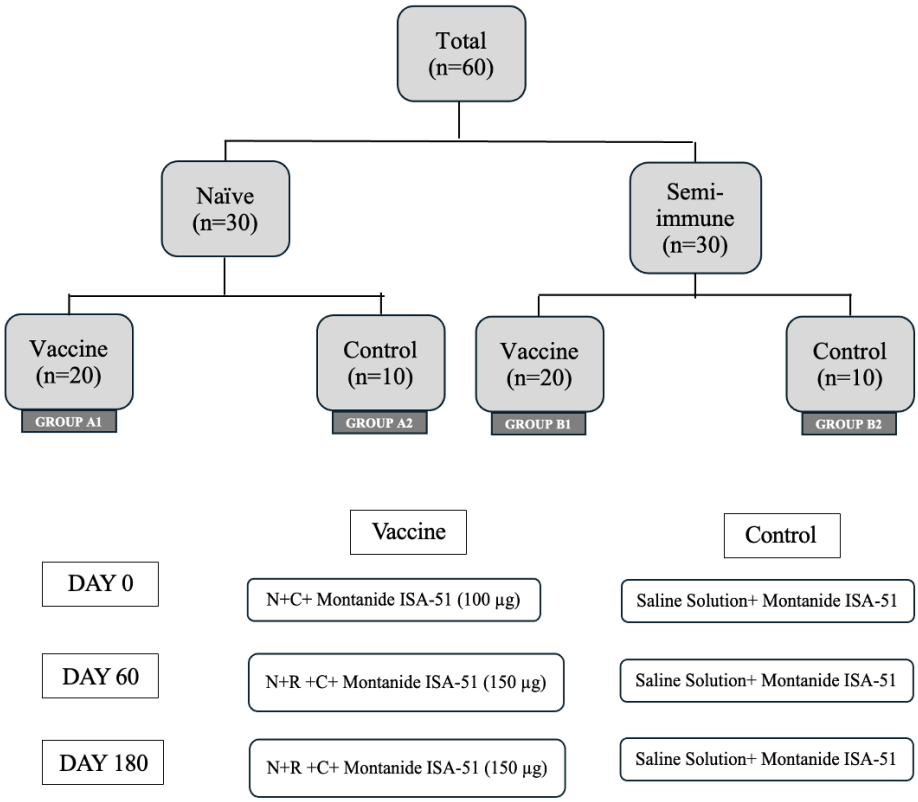

Figure 15: Immunization schedule.

6.5. Post-immunization follow-up

6.5.1. Immediate follow-up

Volunteers will be under direct medical observation for one hour after immunization to detect any adverse reactions to the vaccine injection. After a one-hour observation period, a physical examination will be performed to confirm the volunteer's condition. If the volunteer is physically well, they will be discharged and may leave the vaccination site. Eight hours after immunization, each volunteer will receive a phone call to check on their physical condition. Any manifestation of AE will be reported as detailed in the AE section.

In the event of any serious acute AE that compromises the patient's physical stability, e.g., severe allergic reaction (anaphylaxis), it will be treated immediately by the treating physician using specific equipment and medications (Epinephrine 1:1000, diphenhydramine, cimetidine, and methylprednisolone). If hospital care is required, the volunteer will be transported by available ambulance, accompanied by the study physician, to San Francisco de Asís Hospital, where an ICU is available if necessary.

6.5.2. Late follow-up

Additionally, personal follow-ups will be conducted the day after each immunization and then two weeks before the next immunization. These follow-ups will include a new clinical evaluation and AE report. Volunteers will be provided with all the information required to contact members of the research team at any time (including cell phone numbers); in addition, they will be encouraged to ask questions if they have any concerns. In this phase of the study, the clinical and paraclinical follow-up of the immunized volunteers will be carried out by the study physician.

6.6. Step 2: Donation of infected blood to produce sporozoites

In this step, patients infected with *P. vivax* who consult the laboratories of IPS ASOCLINIC Ltda. or others in the public malaria diagnosis network in Quibdó and referred to ASOCLINIC Ltda. will be identified for parasite donation. Each selected donor will be asked to sign an informed consent form freely and voluntarily prior to donating parasitized blood.

From each donor, 35 mL blood will be obtained by venipuncture of the arm, which will be distributed as follows: 5 mL will be used for infectious disease screening like that used in blood banks and that performed on naïve and semi-immune volunteers. The remaining 30 mL will be used to feed batches of adult *An. albimanus* mosquitoes. Infectious disease screening tests will be performed in the laboratory and mosquito infection will be carried out in the ASOCLINIC Ltda. insectarium, located in the same building (Pacific Health Institute - INSALPA) in Quibdó, in accordance with standard protocols.

6.7.1 Donor recruitment

Patients will be seen by a certified bacteriologist or microscopist, who will perform the thick smear test and read the results. If the result is positive for P. *vivax* without other concomitant species and if the parasitemia is ≥ 0.1%, a physician assigned to the study will explain to the donor the methodology and objectives of the study and invite them to participate in the clinical trial as a parasite donor. If the patient agrees to participate in the study, they will be asked to sign two informed consent forms: the first for their participation as a parasite donor, and the second for HIV testing.

6.7.2 Patient identification and collection of the parasitized blood sample

Volunteer donors will be assigned an identification code with two characters preceded by the study code, as explained previously in section 6.2: Example: VCS11201: 01 would correspond to the first immunized volunteer in the randomly assigned and blinded group. Blood samples will have a code following the patient identification according to the time of collection and type of sample taken (see Table 9) as follows: Example: VCS1120156: the time was collection 1 at the time of challenge and 6 corresponds to a sample taken on filter paper.

Study Point Identification Code	Procedure	Sample Type Code	Sample Type Name
1	Selection	1	Whole Blood
2	Immunization (1st blood test)	2	Plasma
3	Immunization (2nd blood test)	3	Cells
4	Immunization (3rd blood test)	4	Thick Blood Smear
5	Challenge	5	Urine
6	Post treatment follow-up (day 0-15)	6	Filter Paper
7	Post treatment follow-up (day 16-28)	7	Serum
8	Post treatment follow-up (after day 28)	8	ADN
		9	RNA
		0	Resport

Table 9: Structure of the identification code for blood samples.

6.7.3. Blood donation and donor treatment

Once the volunteer has signed the IC and been assigned an identification code, one of the doctors from the research group will take a medical history and perform the corresponding physical examination. This step will result in one of two possible situations:

- The subject does not meet the inclusion criteria: they will be given medication for the treatment of malaria, in accordance with the national guidelines of the Colombian Ministry of Social Protection (MPS). In addition, the reasons why they will not continue in the study and therefore will not donate blood will be explained to them. Even if the subject is not included in the study, they will be asked to return two weeks later (Day 15 after starting treatment) for a GG to ensure that the malaria has been cured. If the GG is positive on Day 15, the treatment regimen will be repeated. If CQ resistance is present, it will be managed as described in the treatment and follow-up section (see below).

- The subject meets the inclusion criteria: 35 mL of blood will then be taken by brachial venipuncture, of which 5 mL will be used for infectious disease screening tests similar to those used by blood banks, a process that will take between 3 and 5 days, and the other 30 mL will be used immediately to feed mosquitoes through an artificial membrane (AMA); The blood must be kept at 37°C between collection and mosquito feeding. Immediately after blood donation, volunteers will receive medication according to current national guidelines for the treatment of malaria recommended by the MSP, and the response to treatment will be confirmed as mentioned in the previous point. In addition, volunteers will be asked to return approximately one week later to receive the results of the blood bank screening tests. In the event of a positive result for any of the tests performed, including HIV, the volunteer will be referred to the appropriate health care provider according to the Social Security Health regime to which they are affiliated, with a copy of their results for counseling and appropriate medical assistance. If the volunteer has a private doctor, they will be referred to their doctor with a copy of all the results, and if they are not affiliated with any social security

system, they will be referred to one of the hospitals in the public health network affiliated with the SSDC. The case will be handled by the corresponding EPS or the SSDC, but the study team will follow up to ensure that the EPS provides the required care.

Treatment and follow-up of volunteer donors according to MSP parameters:

- CQ: 600 mg (4 tablets of 150 mg) on the first day, 450 mg (three tablets) on the second day, and 450 mg (three tablets) on the third day.
- PQ: 30 mg (2 tablets of 15 mg) per day for 14 days.
- Follow-up: they will be asked to return two weeks after starting treatment (day 15 after the first dose) for a GG to ensure that the malaria has been cured. If the GG is positive on day 15, the treatment will be repeated.

In case of resistance to CQ (*), the infection will be treated with the combination sulfadoxine/pyrimethamine (Falcidar® 25 mg pyrimethamine/500 mg sulfadoxine) 3 tablets single dose as an alternative treatment.

If the patient has a contraindication to Falcidar® (e.g., allergy to sulfa drugs), they will receive amodiaquine in doses of 3 tablets (each tablet contains 200 mg, dose of 600 mg/day) for 3 days (Bosman, 2001) and will be asked to return a week later for a GG to confirm cure.

(*) If the donor's parasite resistance to CQ is confirmed in a timely manner (7-12 days), the batch of mosquitoes infected with that sample will be discarded. In the exceptional case that the donor only reports when the batch of mosquitoes has already been used for the challenge, the treatment of immunized and challenged volunteers (step 3) will be modified as described below.

6.7.4 Laboratory tests on donated blood

***Plasmodium* PCR:** 500 µL of the donor sample will be used to perform molecular PCR testing for *P. vivax*, *P. falciparum*, and *P. malariae*, to rule out the presence of mixed malaria in a timely manner (before the mosquitoes are used, approximately 7-12 days).

The PCR technique has higher specificity and sensitivity values than any other diagnostic method currently available (WHO, 2000); it is therefore considered the *gold* standard for malaria diagnosis in the research setting. The test achieves sensitivities and specificities of up to 100% compared to other available diagnostic methods (Pöschl B., 2009). In the clinical setting, however, PCR is not the diagnostic method of choice, as it is a test that requires expensive equipment and reagents, which means it is not readily available in many clinical centers. Furthermore, as it is a time-consuming test to process and report, having to wait for the result would delay the start of treatment for the patient. This difficulty will not arise in this study, as thick smears will be used as the diagnostic method, and appropriate treatment will begin immediately if the result is positive, as confirmed by the two microscopists.

PCR testing to rule out infection by other *Plasmodium* species will be performed on the sample collected from the donor after it has been used to infect mosquitoes but before the infected batch is used to challenge healthy volunteers (7-12 days), so that it can be ensured that the sample only contains *P. vivax*. If PCR is positive for a *Plasmodium* species other than *P. vivax*, the fed mosquito batch will be discarded in accordance with biosafety regulations and will not be used for challenge under any circumstances.

Although the possibility of false negatives for *P. falciparum* by PCR is very low, if a donor tests positive for mixed malaria not initially detected by PCR, this may be evidenced during post-treatment follow-up of the donor, as their symptoms would not improve with the antimalarial regimen initiated against *P. vivax*, given that this regimen (based on the use of CQ) is not effective against P. *falciparum.* At that point, a thick smear and PCR would be performed again, and appropriate treatment against *P. falciparum* would be initiated. Additionally, mosquitoes fed with such blood would be discarded.

- **Blood bank analysis:** 5 mL of donor blood will be used to perform the following tests : Two rapid HIV tests from different manufacturers, antibodies against HTLV 1 and 2, hepatitis B surface antigen (HBsAg), rapid test for hepatitis C, rapid test for Chagas disease, and RPR for syphilis.

- **Confirmatory tests:** If any of the rapid HIV tests are positive, fourth-generation ELISA tests will be performed as a confirmatory test. If HBsAg is positive, hepatitis B core antibodies will be tested to confirm the diagnosis. Similarly, if the RPR for syphilis is reported positive at any dilution, an FTA-ABS will be performed. Although it is theoretically possible for mosquitoes to transmit the hepatitis B virus within 72 hours after feeding on blood (Blow, 2002), no evidence has currently been found that any of these diseases are transmitted by *Anopheles* mosquitoes. In addition, mosquitoes have an incubation period of about 15 days, during which transmission of any of these diseases is unlikely.

- **Additional analysis:** In addition to routine blood bank testing, other pathogens that could be inadvertently transmitted by *Anopheles* mosquitoes during a challenge are considered. Below are the diseases that have been found in Colombia that could be transmitted by *Anopheles* mosquitoes.

Discussions held prior to challenge test #1 (Herrera, 2009) with experts in vector-borne diseases indicated that: there is no evidence of current transmission of any species of filaria in Colombia and that testing for these parasites is not necessary.

Although *Leishmania spp.* are endemic in the country, they are not transmitted by *Anopheles* mosquitoes. Similarly, there is no evidence of the need to carry out evaluations of viruses other than those studied in the Blood Bank tests. In addition, more than a decade of follow-up of volunteers participating in our group's previous trials (Herrera, 2009; Herrera, 2011) does not report any type of pathology associated with experimental infection. Furthermore, although

the SARS-CoV-2 virus is not known to be transmitted by arthropod vectors, if the parasite donor is diagnosed with this virus during the mosquito infection stage (14 days), the corresponding batch of mosquitoes will be discarded as explained above.

6.7. Step 3: Obtaining sporozoites and infectious challenge

6.7.1. Infection of mosquitoes

The mosquitoes to be used are kept in the Center's Insectarium under standardized conditions previously established in Cali and Quibdó, as set out in the maintenance and biosafety SOPs. (EN-04-mca-001 Entomology Unit Biosafety Manual). Female *An. albimanus* mosquitoes will be prepared under GLP conditions in the Quibdó insectarium. The reason for selecting only females is that male mosquitoes do not feed on blood and do not transmit the infection. The blood samples selected in step 2, with a parasitemia greater than 0.1% of P. *vivax* confirmed by PCR, correspond to P. *vivax* and correspond to the *Pv*CS VK210 variants. These will be used to infect an equal number of batches of mosquitoes containing 10,000 mosquitoes per batch. The blood samples will be centrifuged at 3000 rpm for 5 min at room temperature, and the autologous plasma will be removed. The blood will be washed with RPMI-1640 medium and reconstituted to 50% hematocrit with equal volumes of a pool of non-immune AB human serum obtained from a blood bank. The AB serum complement will be inactivated by heating at 56°C for 30 min. After washing the blood, 3-4-day-old female mosquitoes that have been fasted overnight will be fed using an artificial membrane feeder at 37°C, as previously described (Hurtado, 1997). The mosquito boxes will be labeled with a feeding code and the date of infection. The day after feeding, females without blood will be removed from the box, and the fed mosquitoes will be kept under strict biosafety standards in the temperature and humidity conditions described in the respective Standard Operating Procedures (SOPs) for optimal operation of the mosquito colony.

As previously mentioned, batches of mosquitoes fed with blood samples confirmed to be co-infected with *P. falciparum* will be discarded under biosafety conditions, while batches fed with samples containing only *P. vivax* will be retained.

Mosquito samples acceptable for challenge will be examined microscopically by abdominal dissection on days 7-8 after feeding to determine the presence of oocysts in their midgut and on days 14-15 to assess the sporozoite load in their salivary glands. For oocyst analysis, 40 mosquitoes will be dissected and their midguts stained with 2% mercurochrome and examined microscopically, as described by Eyles (Eyles, 1950). Oocyst infection will be calculated according to the equation Nx79 /N + 79, where N is the number of live mosquitoes on the day of mosquito dissection. The results will be expressed as the percentage of infected mosquitoes and the arithmetic means of the number of oocysts per dissected mosquito intestine. Positive mosquito batches will be kept inside the insectary's biosafety room, where they will remain for another seven (7) days until they are examined for the presence of sporozoites.

Sporozoites will be examined in a sample of 38 dissected mosquitoes/batches and examined microscopically on day 14. To do this, the salivary glands will be dissected (6 lobes) on a non-slip slide in a drop of PBS (phosphate-buffered saline), crushed by applying pressure, and examined microscopically at 400x magnification to assess the density of sporozoites (spz) per pair of glands; each preparation will be classified as 1+ (1-10 spz), 2+ (11-100 spz), 3+ (101-1000 spz), or 4+ (>1001 spz) (Chulay, 1986).

Exclusion criteria for challenging mosquito batches

- Mosquito batches are infected with blood from volunteers exposed to mixed malaria (*Pv* + *Pf*), HIV, HTVL-1-2, hepatitis B, C, Chagas, syphilis, and any other criteria determined by the investigator, such as the presence of therapeutic failure in the donor, which raises suspicion of resistance to the *P. vivax* strain to CQ.
- Batches with a percentage of infected mosquitoes (with sporozoites) <50%.

6.7.2. Preparation for the infectious challenge

Volunteers participating in the study will be advised to contact one of the study physicians immediately if any of them become pregnant in the period between the selection visit and the challenge. One day before the challenge, the women will be asked to come to the CIV facilities for a blood test to detect pregnancy. If any of them test positive, they will be immediately excluded from the study. If this test reveals any cases of pregnancy, the volunteer will be replaced by one of the alternate volunteers.

Two (2) days before the challenge, volunteers will be invited to visit the Entomology Unit at the INSALPA facilities in Quibdó. This visit will allow them to familiarize themselves with the place where the infection procedure will take place, which will reduce anxiety on the day of the challenge. At this time, 35 mL of blood will be drawn from all volunteers to establish a baseline for their immune system.

On the day of the challenge, all participating volunteers will be evaluated by one of the study physicians, who will take a medical history and perform a brief evaluation, including vital signs. If any of the volunteers are found to have an acute illness that, in the opinion of the evaluator, requires the volunteer's exclusion, they will be replaced by one of the alternate volunteers.

The mosquitoes from the batches selected for the challenge will be distributed in small 7x7x7 cm "feeding cages." Prior to the challenge, the necessary cages will be prepared using mosquitoes from the same batch, with four (4) mosquitoes in each cage.

6.7.3. Infectious challenge with mosquitoes infected with *P. vivax*

The 60 volunteers immunized in step 1 will be challenged on day ~210 of the study, 1 month after the third immunization, by exposure to the bite of 2-4 infected mosquitoes according to the previously established procedure. The procedure will be carried out in a safety room prepared for the study within the Entomology Unit.

The "feeding cages" will be placed on the volunteer's forearm for 10 minutes, allowing the feeding window covered by a mesh to rest against the surface of the volunteer's skin. The box and forearm will be covered with dark cloth to create dim light, as this favors mosquito biting activity.

After feeding, the volunteers will remain in the insectarium while technicians from the Entomology Unit determine the number of mosquitoes that are fed and, through dissection of the salivary glands and microscopic examination, determine the number of infected mosquitoes per cage and the sporozoite load in the salivary glands. If the bite rate (determined by the presence of blood in the mosquito's abdomen) and the infectivity rate (determined by the sporozoite load in the salivary glands) are below the minimum number of infective mosquitoes (n=2) (infective bites) expected for each volunteer, the volunteer will be required to be exposed to additional mosquitoes for feeding, until a total of 2-4 fed mosquitoes (positive for sporozoites in their salivary glands) are reached.

Volunteers will be directly observed by one of the study physicians for one hour after the challenge to immediately detect any adverse reactions induced by the mosquito bites. Approximately eight (8) hours after the challenge, volunteers will be contacted by telephone to document their progress. Subsequently, a personal check-up will be carried out 24 hours later, and daily telephone follow-ups will continue until day 7 post-challenge. Volunteers will have all the necessary information to contact the researchers 24 hours a day (including a cell phone number) and will be encouraged to consult them if they have any questions or require guidance. This direct contact will continue for three weeks. The clinical and paraclinical follow-up of the volunteers in this phase will be carried out by the doctor in charge at each study site.

6.7.4. Post-challenge evaluation of volunteers

Monitoring of the pre-patent parasitemia period: Volunteers will receive instructions about malaria symptoms such as fever, headache, chills, myalgia, and general malaise, which may occur between days 7 and 23 after the challenge. However, from day one (1) to day six (6) post-challenge, volunteers will be monitored by telephone by study staff. According to previous studies, it is unlikely that parasitemia will appear before the ninth day, however, the study team will be available to attend to any volunteer who presents early symptoms of malaria. From day 7 post-challenge until day 28, volunteers will be evaluated daily by a study physician, monitored daily with GG, and a blood sample will be taken for subsequent PCR-TR for comparative purposes at the end of the study. According to our previous studies, most exposed

volunteers develop overt infection around day 10 and the rest within the following week, so it would be exceptional for late infections to appear after day 20. Once all volunteers test negative in response to treatment, the daily collection and examination of thick smears will be suspended.

If the daily GG is negative but the volunteer has a fever (axillary temperature >38°C) and/or other signs/symptoms of malaria, GG and peripheral blood smears will be taken twice a day. If a volunteer presents symptoms compatible with malaria, but their thick smear is negative on three successive occasions, a PCR-TR malaria diagnostic test (Rougemont, 2004) will be performed immediately to clarify the diagnosis. However, the gold standard for treatment should be the thick smear. If any of the volunteers develop the infection and need to be hospitalized, they will be treated at the Ismael Roldán or San Francisco de Asís Hospital in Quibdó by the clinical team physician.

If 28 days after the challenge the volunteers are negative for malaria, they will continue with their parasitological follow-up (GG) once or twice a week, depending on the logistical conditions of access to the volunteer, until 60 days after the challenge. During this period, volunteers will be monitored daily by telephone. Based on the previous clinical trial, it is expected that at least some of the volunteers will develop sterile immunity; however, some may be only partially protected and develop longer pre-patent periods or lower parasitemia. Individuals who become infected and have pre-patent periods or parasitemia densities like those of the control group, i.e., whose pre-patent periods do not differ statistically significantly from those of the unvaccinated controls, will be considered unprotected. If any of the volunteers develop malaria during this phase, the study physician will be responsible for administering and monitoring treatment.

6.7.5. Treatment of volunteers infected with malaria

Once malaria infection is confirmed, volunteers will be treated with the antimalarial regimen recommended by the MSP for the treatment of *P. vivax*, consisting of: CQ (a total of 1,500 mg of CQ orally in divided doses: 600 mg initially, followed by 600 mg 24 hours later and 300 mg 48 hours after the first dose) and PQ for fourteen days (15 mg/day) administered with food. All antimalarial drugs will be administered with food, as they can cause stomach pain, nausea, and vomiting (gastritis) if taken on an empty stomach. Volunteers without parasitemia on day 60 will be treated from that day onwards with the same antimalarial regimen. PQ will be administered directly at ASOCLINIC Ltda., under daily medical supervision for fourteen (14) days. Because PQ can cause hemolytic anemia in individuals with G6PD deficiency, all volunteers accepted for the study must have a normal screening test.

6.7.6. Follow-up after initiation of antimalarial treatment

Each day after the initiation of CQ treatment, a thick smear will be taken until 3 consecutive negative GG results are obtained. In addition, GG follow-up will be performed on days 7, 14,

and 21 after the initiation of treatment to ensure cure. If any volunteer develops a fever or any symptoms compatible with malaria during this period, a GG will be performed again on the day of the symptoms and, if positive, a new treatment will be started using the alternative regimen, if necessary. In two previous trials, this therapeutic regimen has been shown to be effective in completely controlling the infection within 1-2 days. On day 45 after starting antimalarial treatment, volunteers will be evaluated at ASOCLINIC Ltda. by a study physician; in addition, 10 mL of blood will be drawn to measure hematological, renal, and hepatic function and to assess for pregnancy.

6.7.7. Follow-up for relapses or recurrences

In Colombia, there are no documented cases of relapse with supervised high doses of PQ (30 mg/day/14 days). In our previous studies, no relapses were observed during a 2-year follow-up, although on two occasions, infections were diagnosed in volunteers who visited the endemic area after the study, which were interpreted as reinfections. All volunteers will be contacted by telephone at 3-month intervals after completion of PQ treatment and confirmation of negative GG (see **Table 10)**. *P. vivax* resistance *to* CQ has been documented only rarely in Colombia (Comer, 1968; Soto, 2001) and has not been observed with mixed treatment (CQ + PQ) (Soto, 2001). However, in the unexpected event that a GG-positive sample is found at any time during post-treatment follow-up (days 7, 14, 28 after initiation of treatment), the volunteer will receive alternative treatment with Fansidar® (SP) with 3 tablets in a single dose (25 mg of Pyrimethamine plus 500 mg of Sulfadoxine per tablet). If the patient has a contraindication to Fansidar® (e.g., allergy to sulfa drugs), he or she will receive Amodiaquine as described above and will be followed up with additional GG to confirm cure.

Any relapse of *P. vivax* will be treated with CQ and repeat treatment with PQ (at doses identical to the first treatment regimen). Follow-ups will be performed on the same days as the first cycle, as explained above. Long-term follow-up will be performed to detect possible relapses due to *P. vivax* hypnozoites. Although it is not possible to determine unequivocally whether a relapse is due to the reactivation of hypnozoites or natural reinfection, the treatment for the event is similar regardless of its origin. It is also not possible to distinguish whether relapses are due to new hypnozoites (originated by challenge) or old hypnozoites generated by natural infection. Once supervised treatment with PQ (2 weeks) has been completed and negative GG results are obtained in post-treatment controls, all volunteers will be contacted at least by telephone at intervals defined in **Table 10.** In addition, the study physician will be the clinical consultant for the rest of the team in all the phases and in the event of a relapse.

All volunteers who have been exposed to the infectious challenge will be asked to contact the study physician or the people providing medical assistance to report any cases related to the diagnosis and treatment of malaria infection, and in case of fever. If fever, accompanied by chills, convulsions, or other malaria-related symptoms, occurs at any time within 1 year (12 months) after the challenge, the physician examining the volunteer should be informed that they have been exposed to experimental malaria infection, and the volunteer should undergo a

GG test, peripheral blood smear, and PCR. The study procedure schedule, including volunteer follow-up, is outlined below.

		Accepted Window
Within the first month	Weeks 1, 2, 3, and 4	± 3 days
For two months	Weeks 5 and 8	± 5 days
For three months	Weeks 10 and 12	± 7 days
For six months	Week 16, 20, 24	± 10 days
During the first year	Weeks 30, 38, 46, and 52	± 10 days
For a year and a half	Weeks 52, 60, 68, and 76	± 14 days

Table 10: Clinical and parasitological follow-up schedule for detecting relapses or recurrences and accepted windows.

6.8.8 Risk and management of natural infections during the study

Although the urban area of Quibdó is largely free of malaria transmission, given that the study will be conducted in proximity to areas of malaria transmission, and despite the preventive measures implemented to minimize the risk of natural infections, there is a possibility that some volunteers may acquire *P. vivax* or *P. falciparum* infection during the immunization period (days 0 to 180), diagnosed by thick smear.

In the event of a natural infection, the volunteer will be treated immediately in accordance with the therapeutic protocol established for each species. The AE will be reported to the IRB and documented in detail in the CRF and in the study database. The volunteer will not be excluded from the primary efficacy analysis, as the study will adopt an *"Intention to Treat*" approach. This principle implies that all participants will be analyzed according to the group to which they were originally assigned at the time of randomization, regardless of whether they completed the immunization schedule, had intercurrent events (such as natural infections), or deviated from the protocol. This approach preserves the statistical and clinical validity of the results, as it more accurately reflects the efficacy of the schedule under natural conditions in endemic areas.

Additionally, a complementary "*Per-Protocol*" analysis will be performed, in which participants who have experienced natural infections during the immunization phase will be

excluded from the efficacy analysis, with the aim of estimating biological efficacy under ideal compliance conditions.

In all cases, volunteers who develop natural infections will remain enrolled in the study and will be followed according to the procedures and schedule established in the protocol.

7. LABORATORY TESTS

7.1. Malaria diagnosis:

The IPS ASOCLINIC Ltda. laboratory, as an accredited laboratory, can perform the diagnosis to identify malaria infection. Each sample will be read by two independent microscopists. In GG tests, a total of 200 microscopic fields will be examined with immersion oil (x 1000) before reporting it as negative, as no parasites were found.

- **Thick smear**. This microscopic test will be used as the standard test for making decisions related to infection. It will be performed in step #2 for donor group selection and in step #3 during post-challenge and post-treatment follow-up. In addition, it will be used for the diagnosis of any volunteer who presents symptoms of malaria during the immunization phase and in extended follow-ups after definitive treatment. For testing, ~2 drops of blood will be collected by finger prick, GG and thin smears will be performed, which will be stained using the Field staining method employing POE CD-POE-001-03 recommended by the Colombian Ministry of Health.

- **PCR-TR for malaria** (steps #2 and #3): This will be performed retrospectively but will not be used for decision-making regarding infection. For this purpose, DNA will be extracted from whole blood to perform PCR-RT diagnosis for *Plasmodium* (*P. vivax, P. malariae*, and *P. falciparum*) (Rougemont, 2004), with a sample of 500 µl of donor blood, using POE ML-03-POE 007. Procedure for molecular diagnosis of malaria. This technique is characterized by having an analytical level sensitivity that detects up to 1 parasite/µL. Plasmo 1 and Plasmo 2 *primers* and TaqMan species-specific probes for *P. vivax* and *P. falciparum* will be used for this analysis. For each species and assay, positive and negative controls will be used, and the standard curve will be constructed from plasmids for both species to establish and quantify the number of copies per sample.

7.2 Methodology for objective #1.

Confirm the safety of the vaccine in naïve volunteers and those previously exposed to malaria, immunized with PvCS.

1. To determine the safety of the vaccine, the following tests will be performed: complete blood count, PT, PTT, ALT, AST, bilirubin, alkaline phosphatase, BUN, creatinine, partial urine test, pregnancy test (for women), monthly and when deemed appropriate, in order to determine

changes in any of the parameters of these tests. Any changes that occur will be reported, their severity will be established, and their relationship to the vaccine will be determined. If there are any changes that require additional clinical or paraclinical studies, these will be performed, as well as additional controls, beyond those already established. Follow-up will be performed until these parameters return to normal.

7.3 Methodology for objective #2.

Determine the immunogenicity of PvCS in individuals previously exposed to malaria

7.2. Specific immune response assessment tests

The analyses to determine the immunogenicity of the formulation in *Pv*CS2-Montanide ISA 51 studies will include analysis of the B immune response (IFAT and ELISA) and analysis of the T cell-mediated response (IFN-γ by ELISPOT). All serum samples will be stored at -20°C and cells will be stored in liquid nitrogen until use. Results will be archived in the laboratory for further analysis.

7.2.1. Evaluation of B cell response

Specific antibody titers will be measured using IFAT and ELISA techniques on day 0 (zero), and subsequently in months 1, 2, 3, 6, 7, and 8 using as antigens, sporozoites produced by artificial feeding of *An. albimanus* (Hurtado, 1997) and synthetic peptides derived from *Pv*CS.

- IFAT tests will be performed according to the protocol previously described (Herrera, 2005). Briefly, the slides will be incubated in a humid chamber in darkness for 1 hour at 37°C, with 25 µl of serum diluted in 2% PBS-BSA, starting with a 1:20 dilution. After 3 washes in PBS, FITC-labeled human anti-IgG (1:100 in PBS-Evans Blue 0.05%) will be added. It will be washed again and mounted for reading. Antibody titers will be expressed as the last dilution that showed fluorescence.
- Enzyme-linked immunosorbent **assay/ELISA**. Antibodies against the peptides included in the vaccine (N, R, and C) will be measured by ELISA (Herrera, 2005). Briefly, 96-well plates (Nunc-Immuno Plate, Maxisorp, Roskilde-Denmark) will be sensitized with 100 µl of synthetic peptides N, R, and C at 1 µg/mL overnight at 4°C ( ). They will then be blocked with 200 µl of PBS1X/Tween 0.05%, 5% milk for 2 hours at room temperature. Next, 100 µl of each of the serum samples diluted in PBS1X/Tween 0.05%, 2.5% milk will be added and incubated for 1 hour at room temperature. They will then be washed 5 times with PBS 1X/Tween 0.05%. Add 100 µl of goat anti-human IgG conjugate with alkaline phosphatase diluted 1:1000 in PBS1x/Tween 0.05%, milk 2.5%, incubate for 1 hour at room temperature, and wash 4 times with PBS 1X/Tween 0.05%. The reaction will be revealed after incubating for 30 minutes with 100 µl per well of the para-nitrophenol phosphate substrate. The optical density of the reaction will be determined at 405 nm in an ELISA reader (Dynex Technologies, INC MRX Chantilly VA). A sample is defined as positive when the optical density (OD) of the sample is 3 times

greater than the average OD of the negative control.

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7.2.2. Evaluation of T-cell response

The cellular immune response will be evaluated by measuring the *in vitro* production of the cytokine IFN-γ by peripheral mononuclear cells (PBMC) using ELIspot techniques. Cells will be separated by Ficoll-Hypaque gradients from whole blood (Herrera, 2005).

- **ELI spot**. The *in vitro* production of IFN-γ by total PBMCs stimulated with different antigens and/or synthetic peptides will be determined by ELIspot using commercial kits (MABTECH, Stockholm, Sweden). For IFN-γ, microplates are sensitized with 5 µg/mL of human anti-IFN-γ monoclonal antibody (1-D1K MABTECH AB, Sweden) overnight at 4°C. Subsequently, $^{2x10^5}$PBMC/well stimulated with peptides (*Pv*CS=N, R, C) at 1 µg/mL are added, using PHA as a positive control and RPMI medium alone as a negative control. The plates were incubated at 37°C for 40 hours in a 5% CO2 atmosphere and washed with PBS/Tween-20 0.05%. Biotinylated anti-IFN-γ monoclonal antibody (7-B6-1, MABTECH AB, Sweden) was added and incubated at room temperature for 2 hours. Alkaline phosphatase with streptavidin will then be added, and the reaction will be revealed by adding BCIP/NBT (5-bromo-2-chloro-3-indolyl Phosphatase/Nitroblue Tetrazolium) (Sigma, St Louis, MO). Spot-forming cells (SFCs) will be counted using a counting system (AID ELISpot Reader System ELR07, AID GmbH, Germany).

7.4 Methodology for objective #3:

Determine the protective efficacy of the vaccine against infectious challenge with viable P. vivax sporozoites.

To determine protective efficacy, infection will be monitored by medical examination, GG, peripheral blood smear, and diagnostic PCR starting on day 7 post-infection. Once the volunteer is diagnosed as positive for malaria, they will be treated according to the MPS protocol described above. The frequency (number) of individuals who become infected, the pre-patent period of the disease, and the severity and frequency of symptoms will be compared in the study groups.

- Thick smear: The slides will be stained with Field's stain and read independently by two experienced microscopists. Parasitemia will be quantified by observing the microscopic fields corresponding to 300 leukocytes and the estimated leukocyte count per µL of blood.

- Diagnostic PCR: This will be performed using genomic DNA obtained from the parasite from 500 µL blood samples from study volunteers. Species-specific detection of *Plasmodium* will be performed using the previously described and validated PCR-RT technique (Rougemont, 2004).

7.5 Interpretation of results

B-cell response: Due to the low antibody titers induced by *P. vivax* under natural conditions (compared to *P. falciparum*), responses in this study will be quantified as low, medium, and high, bearing in mind that any positive reaction will indicate previous contact with the parasite and an anti-malarial immune response in volunteers (**Table 11**).

Technique	Low	Medium	High
IFAT	<1:40	>1:40 -1:320	>1:320
ELISA	1:100 - 1:500	>1:500-1:5000	>1:5000

Table 11: Antibody titers against *P. vivax*

T-cell response. IFN-γ production will be determined using the ELIspot technique. In this technique, *spot-forming cells (SFCs)* will be quantified and expressed as: i**)** the average number of SFCs per 10^6PMBCs, which will be considered significant if the average number of cells in each well with the experimental antigen is greater than the well with the control antigen ($P < 0.05$, Student's t-test), ii) the net number of SFCs per well (average number of SFCs in the well with the experimental antigen minus the number of SFCs in the well with the control antigen), and iii) the number of SFCs per well (average number of SFCs in the well with the experimental antigen minus the number of SFCs in the well with the control antigen*).* 0.05, Student's t-test), **ii)** the net number of SFCs per well (average SFCs in the well with the experimental antigen minus the number of SFCs in the well with the control antigen) is greater than 5 SFCs per well, and **iii)** The average ratio between the SFCs in the well with the experimental antigen and the average SFCs in the well with the control antigen is greater than 2.0.

8. DATA MANAGEMENT AND ANALYSIS

The data obtained from the study and the medical history will be entered into a database designed with the REDcap program (Vanderbilt University, 2012) and stored on a server with password-restricted access. The data will be entered by study staff into an Electronic Case Report Form (ECRF), verified by the clinical monitor in accordance with standard operating procedures, and corrected, if necessary, by the principal investigator. The data entered in the ECRF will be verified by referring to the source documents and comparing them with the printed data from the database. The Clinical Monitor will report any inconsistencies found, to be reviewed and corrected by authorized personnel. After quality control, the information will be analyzed with Stata ($^{TM)\ 9}$.1, and the analysis will be performed using the statistical tests indicated according to the type and distribution of the variables. The significance level for statistical tests will be $\alpha = 0.05$. The statistician in charge of data analysis will not have access to the distribution of the groups.

Differences between groups when the variable studied is dichotomous will be analyzed using the Chi^{2}test (Fisher's exact test will be used when the number of data points is less than 5). The comparison of continuous variables between groups will be done using *Student's* t-test, *and* the comparison between several groups will be done using one-way ANOVA (Scheffe's evaluation for *post-hoc* analysis*)*.

8.1. Record keeping

During the study, medical records and FRCs, participants' source documents, ICs, inclusion questionnaires, and all information relevant to volunteers will be stored in a secure location at the ASOCLINIC Ltda. Center (and CIV). Electronic databases will be stored on non-rewritable optical media. Participant records will be transported to the CIV (Km 6 Vía Cali-Puerto Tejada, Corregimiento El Hormiguero, Cali, Colombia) in a portable, secure, waterproof box by authorized research personnel. Once these documents have been used, they will be archived again at the CIV. At the end of the study, all reports, consent forms, questionnaires, and other relevant protocol records will be archived at the CIV for a period of 20 years, after which they will be identified as dead files. (See **Annex 2)** (Data and confidentiality).

9. RISKS TO VOLUNTEERS, THE RESEARCH GROUP, AND THE ENVIRONMENT; PRECAUTIONS TO MINIMIZE RISK

Using the challenge system described with *P. vivax* sporozoites, the CIV group has conducted five (5) controlled human infection trials (*CHMIT*) involving more than 100 participants and more than ten inoculations. In two consecutive trials with more than 35 healthy naïve volunteers, the procedure was shown to be safe with doses between 2-10 infectious bites (Herrera, S. 2009; Herrera, S. 2011; Arévalo-Herrera M, 2016b; Arévalo-Herrera M, 2016a; Arévalo-Herrera, M. 2022). The infections showed pre-patent periods ranging from 9-18 days with an approximate average of 11 days. The duration of symptoms was similar in all volunteers (1.5-4.5 days) and their responses to treatment were rapid and similar in all volunteers. All volunteers eliminated parasitemia within the first 48 hours after starting treatment (Herrera, 2009; Herrera, 2010). In these studies, the pre-patent period was evaluated by GG and PCR from day 7. In some cases, PCR was able to detect parasitemia before GG, but in none of the cases did PCR detect it before 9 days post-challenge. GG was sensitive, detecting parasitemia levels as low as those described above (geometric mean of 46 parasites/µL).

Similarly, with *P. falciparum*, hundreds of volunteers have been safely and reproducibly infected in the USA, with 97% of these volunteers developing moderate and short-lived symptoms (average duration, 3 days) (Hoffman, 1997). These volunteers were able to be treated without complications due to the early initiation of treatment, when parasitemia was still very low (geometric mean of 46 parasites/µL), and because the sensitivity of the parasite to the antimalarials used was known.

Splenic rupture is a very rare event that has only been observed in patients with chronic

established natural infection (Yagmur, 2000). In one of the previous challenge studies, only one of the 17 volunteers presented mild splenomegaly as an AE related to the infection (Herrera, 2010). In the proposed study, volunteers will be thoroughly examined during the admission consultation, and once admitted, they will be closely monitored during the immunization period and even more closely and strictly after the challenge. Therefore, the development of splenomegaly is not expected, beyond minimal splenomegaly, which should respond to antimalarial treatment, which is administered immediately upon detection of the parasite in the bloodstream.

9.1. Risks for volunteer blood donors.

Potential risks associated with blood donation may include redness, itching, infection at the puncture site, or vasovagal symptoms such as dizziness and fainting. Parasite samples will be collected from donors by venipuncture under aseptic and antiseptic conditions, using new, sterile, disposable equipment. A study physician will be present to provide primary medical care, e.g., to treat possible vasovagal episodes (fainting).

A short delay (15-20 minutes) in receiving the first dose of antimalarials may occur for blood donor volunteers; however, this risk will not affect the volunteer's recovery. Every effort will be made to expedite procedures so that antimalarial treatment can be started as quickly as possible. A complete blood count will be performed for the purpose of early detection of hematological abnormalities related to malaria.

There is a potential risk that a positive HIV result in a volunteer may not be handled appropriately and may create negative effects in their personal and/or work environment. This risk will be mitigated by strictly adhering to confidentiality rules. Volunteers will personally receive a copy of the results one week (7 days) after they are performed. , if volunteers test positive for any infectious disease, they will be referred to their healthcare provider in accordance with Law 100, Article 179 of 1993, or, failing that, to the SSDC in accordance with Law 1543 of 1997, Chapter II of the MSP, to provide them with counseling and medical care. If a volunteer already has health insurance, he or she will be referred to their private doctor with the test results. These results will only be given to the volunteer.

9.2. Risks to volunteers associated with the malaria challenge.

Risks associated with the challenge include a very low risk of anaphylaxis, the possible transmission of other infectious agents through mosquito bites, and the risk associated with the use of antimalarial drugs. As mentioned previously, the CIV has conducted 10 experiments with parasite inoculation through direct mosquito bites without any reported cases of anaphylaxis.

Precautions to minimize the risk associated with malaria challenge:

- Anaphylaxis management: Medications for anaphylaxis management, such as epinephrine

1:1000, diphenhydramine, cimetidine, and methylprednisolone, will be available at the challenge site and will be used by the study physician who will remain in the infection area. These medications will be available at the immunization and challenge site from the start of the study. In addition, an ambulance will be available and will be used to transport volunteers who need it from the Entomology Unit to the San Francisco de Asis Hospital in Quibdó, a trip that takes approximately 10-15 minutes. In this case, the patient will be permanently assisted by the study physician and the ambulance paramedical staff. As background, the vaccine has been administered to more than 100 volunteers who have only experienced mild AE, like those of classic vaccines (Expanded Program on Immunization - EPI vaccines). On the other hand, volunteers and the general population remain routinely exposed to mosquito bites without any reported cases of anaphylaxis.

- **Blood screening:** Blood from donors infected with *P. vivax* will be screened for infectious diseases as described above.

Volunteer selection and follow-up: Volunteers will be selected if they meet each of the inclusion criteria and none of the exclusion criteria. They will be monitored clinically and parasitologically, and once infection is documented, the volunteer will begin treatment according to the protocol described above. Early initiation of medication will minimize the risk of developing serious complications, which are usually uncommon in *P. vivax* infections. To ensure adequate follow-up of volunteers, each volunteer will have all the contact details of the research group. Under the carefully controlled conditions for conducting this study, the possibility of a late diagnosis is remote. During infection, some transient signs and symptoms may occur, such as fever, headache, myalgia, nausea, vomiting, mild anemia and/ , asthenia, leukopenia, and/or thrombocytopenia, which are usually mild and short-lived due to close clinical monitoring and early diagnosis. The only serious and direct complication of P. *vivax* infection in healthy adults is splenic rupture (Yagmur, 2000), which occurs in patients with long-term infections. Therefore, it is highly unlikely to occur during the study, since the diagnosis will be made as soon as parasitemia becomes evident (patent in peripheral blood), and treatment will be administered without delay. As mentioned previously, standard treatment results in the disappearance of parasitemia within 48 hours of initiation. However, as a precaution, volunteers will be informed of this risk and advised to avoid contact sports or strenuous activity that could result in abdominal trauma, especially during the two weeks following the start of treatment.

- **Pregnancy and *P. vivax* infection:** Although the effects of P. *vivax* malaria during pregnancy are less severe than those caused by P. *falciparum (*Nosten, 1999), *P. vivax* infection during pregnancy has been associated with high maternal parasitemia (compared to parasitemia in non-pregnant women), maternal anemia, and low birth weight (Nosten, 1999; Singh, 1999). Women will be advised to use contraceptive methods for at least six months after the challenge. Women participating in the study will be advised to inform their physician of their participation in the study so that the physician is aware in the event of pregnancy. If, despite the precautions described any of the women experience a relapse of P. *vivax* while pregnant during the 12-month follow-up period, they will be given immediate treatment, which significantly reduces

the risk of adverse outcomes for both the mother and the fetus during pregnancy. CQ is safe during pregnancy (McGready, 2002), as is amodiaquine as an alternative therapy. Treatment with PQ will be administered after pregnancy.

- **Relapses:** Although no cases of *P. vivax* relapse have been documented with the administration of a supervised combination regimen of PQ and CQ (Baird, 2002; Hoffman, 2002), supervised therapy with CQ and high doses of PQ will be administered. Volunteers will be closely monitored after treatment.

9.3. Risks to volunteers associated with malaria treatment

Potential side effects from the use of antimalarial drugs include nausea, vomiting, diarrhea, abdominal pain, vertigo, headache, sleep disturbances, blurred vision, pruritus, tinnitus, and photosensitivity. The following adverse reactions have been reported by the FDA in connection with the use of these drugs:

- **Chloroquine phosphate (**CQ**):** Gastrointestinal reactions (vomiting, nausea, diarrhea, cramps), mild transient headache, auditory effects such as sensorineural hearing loss, tinnitus, and decreased hearing acuity in those with a history of hearing loss. Visual effects, dermatological reactions (pruritus and alopecia), and cardiovascular reactions (hypotension or changes in the EKG) may also occur. The use of CQ is contraindicated in people with psoriasis or other types of dermatological conditions.

- **Primaquine (PQ):** The most common side effects are related to gastrointestinal disorders such as nausea and abdominal discomfort, especially if the medication is administered on an empty stomach. PQ will be administered with food in this study. PQ has been reported to cause leukopenia, and mild methemoglobinemia occurs in most individuals. The concomitant use of substances that predispose to this side effect, such as sulfonamides, will be avoided. PQ is not recommended for pregnant women. The administration of a 30 mg daily dose of PQ for more than one year in healthy adults has been shown to be well tolerated if it is accompanied by food intake. There are no significant effects related to renal or hepatic damage, as evidenced by serum creatinine, BUN, AST, ALT, LDH, alkaline phosphatase, and the methemoglobinemia that occurs is reversible and asymptomatic (Fryauff, 1995).

- **Falcidar® (Sulfadoxine-Pyrimethamine):** Toxic manifestations are rare and usually attributable to the sulfadoxine component. Severe skin reactions (such as erythema multiforme, Steven-Johnson syndrome, and toxic epidermal necrosis) have been reported in individuals using a weekly regimen as prophylaxis. The safety of the combination during pregnancy has not been established, but the drug has been used to treat many pregnant women.

- **Amodiaquine:** Adverse reactions to amodiaquine are generally like those of CQ, the most common being nausea, vomiting, abdominal pain, diarrhea, and pruritus; a less common effect is bradycardia. There is evidence that pruritus is less common with amodiaquine than with CQ.

- **Treatment precautions:** Volunteers will receive supervised treatment, allowing for close monitoring to observe side effects. Any AEs that arise will be documented, as will any potential associations with the treatment, which will be given a causality score. In the previous trial (Arévalo-Herrera, M., 2022), the AEs most frequently associated with treatment were gastrointestinal in origin (nausea, dizziness, and epigastric pain). The symptoms reported by volunteers did not significantly affect their daily activities.

9.4. Risk to those conducting the study

There is a low risk of accidents with the needles used in procedures with volunteers for researchers responsible for taking and processing blood samples.

o *Precautions for staff*: To reduce the risk, all workers who meet blood or blood products will strictly follow standard precautions. In addition, volunteers with HIV, hepatitis B, or hepatitis C infection and their biological products (blood) will be excluded from the study.

9.5. Risk and precautions associated with the environment.

The risk of accidental transmission of malaria to anyone in the community is negligible; infected mosquitoes will only be found in a secure, restricted area of the insectarium and will not be removed from this location at any time. Infections in volunteers will be treated promptly, before gametocytes can develop (this is generally 10 days after the first appearance of parasites in the blood). Volunteers will only be allowed to stay in the Quibdó area, which, despite being located within an endemic zone, is considered to have natural malaria transmission occurring specifically in localized and recognized areas of the city. If any member of the group is accidentally bitten by an infected mosquito or develops symptoms of malaria, they will be immediately evaluated with GG to confirm the presence of infection. If the result is positive, treatment will be given with standard doses of CQ and PQ under supervised therapy.

10.BENEFITS

10.1.Benefits for volunteer blood donors

There will be no direct benefits for volunteers participating in this study. However, volunteers will receive indirect benefits such as blood testing for infectious diseases. In the event of a positive result for any infectious disease other than malaria, including HIV, the volunteer will receive a copy of their results and will be referred to the appropriate provider under their social security health plan for counseling and medical assistance. The trial team will follow up to ensure that the institution responsible for their social security takes the necessary steps to address the volunteer's needs in this regard.

10.2. Benefits for malaria challenge volunteers

There are no financial benefits for volunteers participating as subjects in the vaccination and

infectious challenge phases or for those participating as parasite donors (infected blood) in this study. However, all volunteers may receive some indirect benefits, such as comprehensive laboratory screening that includes the study of infectious diseases, as well as hematological and blood chemistry tests. If, during the selection phase, a positive result for any infectious disease, including HIV, is found, the volunteer will be referred with a copy of their results to the appropriate health care provider according to the insurance plan to which they are affiliated, for counseling and medical assistance in accordance with Law 100, Article 179 of 1993, or to the SSDC in accordance with Law 1543 of 1997, Chapter II of the MSP.

11.COMPENSATION

11.1.Compensation for blood donors.

As mentioned above and established by the MSP, no monetary compensation will be given to volunteers for their participation in the study. However, they will not incur any financial expenses because of participating in this study, for which reason the study will cover (compensate) the costs of transportation, food, and work absence caused by their attendance at study activities. In addition, the study will cover any additional expenses not covered in the protocol that are incurred because of participation in the study. A complete medical evaluation will be performed, and donors would receive counseling if any pathological conditions associated or not associated with malaria are found. Volunteers will be provided with a snack after blood donation.

11.2. Compensation for volunteers participating in the immunization and infectious challenge phases

There will be no remuneration for volunteers participating in this study during the immunization and infectious challenge phases. However, volunteers will receive indirect benefits such as screening for infectious diseases and other laboratory tests, including screening for infectious diseases, hematological tests, blood chemistry tests, among others. Volunteers will not incur any financial expenses because of participating in this study. Therefore, transportation costs will be reimbursed, and refreshments on the day of the challenge and follow-up days will be covered by the study. Additionally, each time a volunteer is called in for a study-related procedure, they will receive the equivalent of one day's minimum wage as compensation for the time dedicated to the study and any possible loss of wages due to their absence from work.

12.CRITERIA FOR WITHDRAWAL/RETIRMENT OF VOLUNTEERS

Volunteers may withdraw freely at any time during the study. If a volunteer withdraws from the study, they will be treated at the time of withdrawal using the protocols described. If a volunteer is excluded from the study for any reason, a final evaluation (physical and paraclinical examination) will be performed. The reason for the withdrawal of any volunteer

will be reported in an FRC and accompanied by supporting information.
On the other hand, regardless of their withdrawal from the study, they will be provided with timely treatment and medical care in the event of malaria and/or related complications.

13.ADVERSE EVENTS

An AE is any sign, inconvenience, harm, dysfunction, adverse reaction to a medication, or any other undesirable outcome that occurs in any of the volunteers participating in the study, including those that have already been defined as expected risks. Each of these events will be reported in an AE and will be given a degree of severity and causality related to the study activities (e.g., blood donation or malaria challenge). Non-serious AE will be reported to INVIMA in the Investigator's Manual, annually or as required.

The intensity of the AE recorded in the CRF corresponds to the highest degree reached during an episode. For example, if a person has a fever, the intensity is graded according to the highest temperature recorded.

AEs will be divided into two groups: solicited and unsolicited. Solicited AEs: will be asked about by clinical trial staff to volunteers at all contacts and will be recorded in an FRC at the periods determined as described below.

- Local AEs occurring in the region of the body where volunteers were exposed to experimental mosquitoes. These will be verified from the time of the challenge until 7 days after the challenge.

- All local AEs will be recorded in the CRFs, and their severity will be classified according to the following table, adapted from the document *"Guidance for Industry – Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials* (FDA, 2007)".

Local Systemic Adverse Events

Local Reaction	Grade 1	Grade 2	Grade 3	Grade 4
	Does not interfere with activity	Repeated use of NSAIDs >24 hours or interferes with activity	Any use of opioid analgesics or that interferes with daily activity	Emergency care for >12 hours or requiring hospitalization
Sensitivity	Mild discomfort to touch	Discomfort with movement	Significant discomfort at rest	Emergency care for > 12 hours or requiring hospitalization
Erythema	2.5-5 cm	5.1-10 cm	> 10 cm	Necrosis or exfoliative dermatitis
Induration	2.5-5 cm and does not interfere with activity	5.1-10 cm or interferes with activity	> 10 cm or interferes with daily activity	Necrosis

- Systemic AEs will be monitored from the time of challenge until 7 days after completion of antimalarial treatment. AEs may be due to the body's reaction to the challenge or to the administration of antimalarial drugs. Events occurring from the time of challenge until the diagnosis of malaria will be attributed to *P. vivax* infection. Events occurring from the time of administration of antimalarial treatment until 7 days after completion of treatment will be attributed to the drug. A margin of 7 days after treatment is given because subtherapeutic levels of the drug circulate during this time.

- All systemic AEs reported will be recorded in the corresponding CRF according to the following table, adapted from the document "Guidance for Industry – Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials (FDA, 2007)".

Systemic Adverse Events Reported

Systemic Reaction	Grade 1	Grade 2	Grade 3	Grade 4
Adverse clinical event or disease	Does not interfere with activity	Interferes with activity, but does not require medical intervention Medical	Interferes with daily activities and requires medical attention	Emergency care for > 12 hours or requiring hospitalization
Nausea	Does not interfere with activity	Interferes with activity	Interferes with daily activity	Emergency care for > 12 hours or requiring hospitalization
Vomiting	1-2 episodes	> 2 episodes	Requires LEV outpatient	Emergency care for > 12 hours or requiring hospitalization
Diarrhea	2-3 loose stools	4-5 loose stools	6 or more liquid stools or requiring outpatient treatment	Emergency care for > 12 hours or requiring hospitalization
Headache	Does not interfere with activity	Repeated use of NSAIDs >24 hours or interfering with activity	Any use of opioid analgesics or that interferes with daily activity	Emergency care for >12 hours or requiring hospitalization
Fatigue	Does not interfere with activity	Interferes with activity	Significant interferes with daily activity	Emergency care for > 12 hours or requiring hospitalization
Myalgia	Does not interfere with activity	Interferes with activity	Significant interferes with daily activities	Emergency care for > 12 hours or requiring hospitalization

Vital signs

Vital signs	Grade 1	Grade 2	Grade 3	Grade 4
Fever °C	38-38.4	38.5-38.9	39-40	> 40
Tachycardia bpm	101-115	116-130	> 130	Emergency care for > 12 hours or requiring hospitalization
Bradycardia l/m	50-54	45-49	< 45	Emergency care for > 12 hours or requiring Hospitalization
Hypertension (systolic) mm Hg	141-150	151-155	> 155	Emergency care for > 12 hours or requiring hospitalization
Hypertension (diastolic) mm Hg	91-95	96-100	> 100	Emergency care for > 12 hours or requiring hospitalization
Hypotension (systolic) mm Hg	85-89	80-84	< 80	Emergency care for > 12 hours or requiring hospitalization
Respiratory rate r/m	17-20	21-25	> 25	Intubation

Serum	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Glucose Hypoglycemia	65-69	55-64	45-54	> 45
Random glucose Hyperglycemia	110-125	126-200	> 200	Requires insulin or hyperosmolar coma
BUN mg/dL	23-26	27-31	> 31	Requires dialysis
Creatinine 1		1.8-2.0	2.1-2.5	Requires dialysis
ALT, AST increase in factor	1.1-2.5 x ULN	2.6-5.0 x ULN	5.1-10 x ULN	> 10 x ULN
Bilirubin - accompanied by alteration in AST/ALT increase in factor	1.1-1.25 x ULN	1.26-1.5 x ULN	1.51-1.75 x ULN	> 1.75 x ULN
Bilirubin - no change in AST/ALT increase in factor	1.1-1.5 x ULN	1.6-2.0 x ULN	2.0-3.0 x ULN	> 3.0 x ULN

Serum Hematology

Hematology	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Hb Women-g/dL	11-12	9.5-10.9	8.0-9.4	> 8
Hb Men - g/dL	12.5-13.5	10.5-12.4	8.5-10.4	> 8.5
Leukocytosis - cells/mm3	10,800-15,000	15,001-20,000	20,001-25,000	> 25,000
Leukopenia - cells/mm3	2,500-3,500	1,500-2,499	1,000-1,499	> 1,000
Lymphopenia - cells/mm3	750-1000	500-749	250-499	> 205
Neutropenia - cells/mm3	1,000-1,499	500-999	499-300	> 300
Eosinophils - cells/mm3	650-1500	1501-5000	>5000	Hypereosinophilia
Thrombocytopenia cells/mm3	125,000-140,000	100,000-124,000	25,000-99,000	> 25,000
PT - increase in factor	1.0-1.1 x ULN	1.11-1.20 x ULN	1.21-1.25 x ULN	> 1.25 x ULN
PTT - increase in factor	1.0-1.2 x ULN	1.21-1.4 x ULN	1.41-1.5 x ULN	> 1.5 x ULN

Note: The lower cutoff point for neutrophils is set below the reference range due to the association between benign ethnic neutropenia and populations of African descent.

Urine

Urine	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Proteinuria	Traces	1+	2+	Hospitalization or dialysis
Glucosuria	Traces	1+	2+	Hospitalization or hyperglycemia
Hematuria (microscopic) – red blood cells/field	1-10	11-50	>50 or macroscopic	Hospitalization or red blood cell transfusion

Unrequested AEs: These correspond to all AEs reported by volunteers that were not included among the requested AEs. All these events will be recorded in an FRC from the time of the challenge until 7 days after the end of antimalarial treatment. After this time, only events related to treatment or indicating suspected malaria will be recorded in the corresponding medical record. In addition, volunteers who visit a malaria-endemic area will undergo routine GG diagnosis, taking a blood sample on filter paper to confirm the diagnosis by PCR. This sample will help determine whether it is a new malaria infection or a relapse due to the clinical trial.

To grade the severity of unreported AEs, the values assigned for symptoms, signs, and laboratory results in the Common Toxicity Criteria will be applied. The classification of clinical AEs will be made according to the clinical judgment of the evaluating physician and the principal investigator based on the categories specified in the document *"Guidance for Industry - Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials (*FDA, 2007). If there are any abnormalities that require additional clinical or paraclinical studies, these will be performed in addition to the established controls. Follow-up will continue until these parameters return to normal.

The severity of symptoms will be assigned by the physician after evaluating the volunteer and in accordance with the definitions described below:

- Grade 1 = Mild
- Grade 2 = Moderate
- Grade 3 = Severe
- Grade 4 = Potentially life-threatening

Mild: A transient, self-limiting event with minor symptoms that do not interfere with the individual's normal activities (e.g., the volunteer is able to work or study) and does not require any medical intervention. Example: parasite inoculation site (challenge): pain and erythema; malaria: myalgia.

Moderate: Events requiring minimal medical intervention to improve the volunteer's condition. In these cases, once the intervention has been performed, the individual is expected to be able to carry out routine activities; some degree of functional limitation may occur. Example: Bite site: Itching and/or pain sufficient to limit movement; malaria: Fever that improves with nonsteroidal anti-inflammatory drugs.

Severe: Symptoms that require treatment and prevent the individual from effectively carrying out their daily activities. Volunteers with severe AE who are generally unable to work but can be safely managed as outpatients. Example: malaria: flu-like reaction or fever resulting in prostration.

Potentially life-threatening: Any event that results in emergency care for more than 12 hours or requires hospitalization. Example: bronchospasm requiring parenteral medication in the emergency room or seizures evaluated in the emergency room but not resulting in hospitalization.

13.1.1. Serious adverse events (SAE)

SAEs will be reported according to the classification in the Document of the Americas.

According to the following:

1. Results in death.
2. Threatens life, requires hospitalization of the patient, or prolongation of existing hospitalization.
3. Results in persistent or significant disability/invalidity or is a congenital anomaly/birth defect.

13.1.2. Classification of AEs: Relationship to study activities

According to GCP standards, AEs may occur during any interaction with the volunteer, including screening and selection, as well as during study processes or subsequent follow-ups. Each AE will be classified as definitely related, probably unrelated, possibly related, or unrelated to study activities (e.g., blood sampling, challenge, or antimalarial treatment). This classification will be made according to the medical judgment of the principal investigator and in coordination with other physicians evaluating AEs. Any AE that occurs during the study will be reported, whether it is considered to be related to the infectious bites under study. These definitions include intercurrent illnesses, injuries, and exacerbations of preexisting conditions.

Degrees of causality:

4. Unrelated: The event has no temporal relationship to participation in the research and is definitely related to another etiology.

5. Probably unrelated: The timing and nature of the event are not temporally related to the intervention performed in the research.

6. Possibly related: The timing and nature of the AE may be the result of participation in the research, but another explanation may be more likely.

7. Probably related: The timing and nature of the AE suggests that it is related to participation in the study (e.g., arm erythema following mosquito exposure). A different potential etiology is apparent but less likely.

8. Definitely related: Those AEs that have a temporal relationship to the study intervention and cannot be attributed to another etiology.

The occurrence of AEs will be classified as expected or unexpected. Severe or serious AEs are not expected to occur in this study.

13.1.3. AE reporting

Each AE reported by volunteers, whether related to study procedures or not, will be recorded in an CRF, in accordance with GCP standards.

Serious AEs or those that compromise the lives of volunteers and are classified as possibly or probably related to participation in the study will be reported by the investigator immediately to the CIV (sponsor), the Research Ethics Committee, and the clinical monitor by telephone, all SAEs within 24 hours of becoming aware of the event, maintaining the confidentiality of the information. Detailed SAE follow-up reports will be prepared, informing them on the status of the subject participating until the outcome of the SAE. The IRB will report the SAE to INVIMA within seven (7) business days of the first report made by the investigator. They must also prepare a follow-up report on the serious adverse event, which must be submitted to INVIMA within fifteen (15) days of the investigator's initial report. The SAE outcome report must also be sent in the format defined by the institution in accordance with Resolution No. 2011020764 (June 2011).

This report must include the following points:

- Date/time of SA reporting.
- Volunteers code.
- Date of birth, gender, and ethnicity of volunteer.
- Name of the principal investigator.
- Step of the study in which the AEF occurred.
- Procedures performed on the volunteer during the study and their corresponding dates.
- Date/time of SAE occurrence.
- Complete description of the AEE.
- Signs or symptoms of the AEFI and their causality.
- Interventions performed on the volunteer after the AEE, including medications used with their doses, route of administration, and the date of the first and last doses.
- Date/time of resolution of the AEE or death.
- Consequences for the volunteer's health and continued participation in the trial.
- Assessment and categorization of the AEFI in relation to study activities.
- Specific recommendations to ensure the safety of volunteers, which may result in changes to the protocol.

The written report will be reviewed with the local clinical safety monitor and then sent to the chairs of the ethics committees within the first 3 business days after the AE is reported. All AEs and interventions will be recorded in the CRF for each volunteer and included in the reports submitted to the ethics committees.

13.2. AE follow-up period

All AEs will be followed up until the outcome is classified into one of the following options:

1. Fatal
2. Unresolved
3. Resolved
4. Resolved with sequelae

5. In resolution
6. Unknown

Pregnancies that occur in the period between the infectious challenge and the seventh day after completion of antimalarial treatment will be followed by the clinical trial group until their conclusion.

13.3. Reporting of AEs to INVIMA

The CIV, in its capacity as sponsor, will report the SAE to INVIMA's Subdirectorate of Medicines and Biological Products within seven (7) business days of the first report made by the investigator to the CIV and the IRB. SAE reports shall be made through INVIMA's official Vigiflow platform or through its authorized interfaces such as eReporting industria or Vigiflow eForms, in accordance with the provisions of current regulations and external circulars 3000-0526-2021 and 3000-0471-2021. In addition, CIV will submit an EAS follow-up report to INVIMA within fifteen (15) business days of the initial event report and the event outcome report.

In order to ensure the protection of participants, regulatory compliance, and the integrity of the study, a detailed procedure has been established to guide the detection, documentation, reporting, analysis, and follow-up of all SAEs and AEFI that occur during the clinical trial of the vaccine candidate (see **Annex 3**) (EAS and AEFI Management Plan**)** and **Annex 4** (DSMB).

14. ETHICAL CONSIDERATIONS

14.1. Approval by ethics committees and organizational plan

The protocol will be submitted for review to the CIV Human Ethics Committees (CEICIV) in their capacity as sponsor and subsequently to the CEI of the ASOCLINIC Ltda. Clinical Research Center, in its capacity as a GCP-certified Research Center. This protocol contains the IC forms that must be signed by the participants (Informed Consents - IC), which include the conditions regarding the nature and scientific integrity of the research and information on the guarantees provided for volunteers participating in the study. During the study, the PI will be responsible for reporting all events that may affect the safety of individuals and the continuation of the clinical trial. Recruitment activities may not begin until the local Ethics Committees have issued their approval, and the protocol has been endorsed by INVIMA, Colombia's regulatory agency.

14.2. Affiliation of ethics committees with the US FWA

The IPS ASOCLINIC Ltda. research ethics committee is GCP certified by INVIMA (Resolution No. 2025013111 of April 4, 2025), and CECIV is registered with the US Federal Wide Assurance (FWA) for the protection of human subjects of the US Department of Health and Human Services (DHHS), Office for Human Research Protections (OHRP), under the guidelines of regulation 45CFR46.103. (CECIV: FWA00016072). The activities of these institutions with human subjects and all activities of the Ethics Committee will be conducted in accordance with the provisions of the Declaration of Helsinki (as adopted in 1996 or 2000).

14.3. Research-related injuries

Once volunteers are included in steps #1 and #3 of the study, they will be enrolled in the Prepaid Medicine service and a life insurance policy. These services will be provided at no cost to them for the duration of the study.

If the volunteer belongs to the subsidized regime, they will be transferred to the contributory regime and enrolled in the Prepaid Medical Service; if they already belong to the contributory regime, the study will assume the volunteer's payments and enroll them in the Prepaid Medical Service as well. However, if the volunteer is a beneficiary of the "Social Subsidy Beneficiary Identification System - SISBEN," they may decide to remain in it so as not to lose the various benefits and subsidies, waiving the benefit of affiliation to the contributory regime and Prepaid Medicine, which are provided for their participation in the study. In this case, they will only receive the benefit of life insurance.

Participants who are injured because of their cooperation in the study will receive medical care at no cost to them at a level III complexity health institution. They will not receive any other compensation for the injury.

This situation does not suppress the volunteer's right to seek legal assistance to which they are entitled.

15. GOOD PRACTICES CLINICAL (GCP) and GOOD LABORATORY PRACTICES (GLP) AT CIV AND ASOCLINIC LTDA - IPS

ASOCLINIC Ltda., in Quibdó will be the sites for conducting post-challenge follow-up visits with volunteers. IPS ASOCLINIC Ltda. was created in 2022 and authorized as an IPS in 2023. Asoclinic was certified in April 2025 as a GCP institution through **INVIMA** resolution **2025013111.**

Starting in 2000, in partnership with CIV and with the advice of the WHO (World Health Organization), specifically the TDR program (*WHO-Tropical Diseases Research and Training Program*) and under the direction of Dr. Myriam Arévalo-Herrera (PhD), it began implementing Good Laboratory Practices (GLP) in its scientific projects and later established GLP training programs for health professionals in Colombia. Over the last 10 years, the CIV, in partnership with ASOCLINIC Ltda., has conducted a total of seven (7) clinical trials related to this vaccine (Arévalo-Herrera, 2022; 2016a; Herrera, 2005; 2009b; 2011a; 2011b; 2011c). During the same period, members of both institutions participated in GLP and GCP workshops organized and sponsored by the US government through the National Institute of Allergy and Infectious Diseases (NIAID). In the present clinical study, quality controls related to study materials will be ensured by clinical study monitors who will be directly linked to the CIV.

16. CONFIDENTIALITY

All information collected from volunteers will be stored in strict confidence. Each person participating in the selection process will be assigned a 5-digit identification code. Although the names of participants will be available in the inclusion form, as noted above, this information will be filed under lock and key. All volunteer data will be entered into the electronic database in the REDcap program. The

list of volunteer names and codes will have a username and password that only people authorized by the PI will have access to. If any abnormalities are found in the results of the volunteers' tests, they will be contacted as soon as possible to personally deliver the laboratory reports and relevant medical recommendations. The records may be examined by monitors, auditors, and/or regulatory authorities. All individual reviews of the records are subject to strict confidentiality rules.

As mentioned above, to ensure confidentiality and quality of the procedures for collecting, storing, processing, transmitting, and archiving clinical trial data, in accordance with national and international regulations, we have developed a Study Confidentiality and Data Management Plan (see **Annex 2**). National regulations include [Res 8430 of 1993; Res. 2378 of 2008; Law 1581 of 2012 and Decree 1377 of 2013] and international regulations [ICH E6(R3) – BPC (sec. 2.11 and 2.12) on confidentiality and data protection; Helsinki Declaration (principle 24: protection of privacy and personal information); CIOMS Guide to the Protection of Confidentiality and Secondary Use of Data; European Data Protection Regulation (GDPR, where applicable].

17. RULES FOR INTERRUPTION OF THE STUDY

This study will have a total duration of 6 months from the moment the volunteers are included. However, from that moment on, there will be a 12-month post-study follow-up to evaluate the immune response and the rate of reinfection.

The clinical monitor of the study and the PI will review all serious AEs in accordance with GCP standards. The occurrence of an AE possibly related to the study procedures, as determined by the PI and the clinical monitor, will result in the suspension of the study. All reviewing institutions and Ethics Committees will be informed of the development of these serious AEs by the PI and the monitor. The medical records will be reviewed by the clinical monitor and sent to the chairs of the committees within a period of no more than three business days. The ethics committees will review the AEs and decide whether the study can continue. The CIV, in its capacity as sponsor, will also review the reports and the recommendations of the ethics committees and make the final decision regarding the continuation of the study. The PI will be informed of the final decision by the IBC.

18. DEVIATIONS AND MODIFICATIONS TO THE PROTOCOL.

Any inadvertent non-compliance with any part of this protocol will be reported as a deviation from the protocol and will be reported to each of the monitors, as well as to the Ethics Committees. Any modification to the protocol will be reported and submitted for consideration to the Ethics Committees of each of the participating institutions.

19. WITHDRAWAL OF VOLUNTEERS FROM THE STUDY

Volunteers participating in any stage of the clinical trial may withdraw from the study at any time. A memorandum will be written during the study to document the departure or withdrawal of volunteers. There will be an FRC for reporting on the withdrawal of volunteers. Withdrawn volunteers retain the right to receive the results obtained during their participation in the study (see **Annex 5**).

19.1. Follow-up of volunteers who do not continue in the study

If a volunteer is excluded from the study for any reason after the challenge but before infection is detected, they will be treated immediately according to the protocol, and every effort will be made to follow up on the volunteer appropriately. If the volunteer presents clinical manifestations secondary to malaria, every effort will be made to provide appropriate treatment and perform the relevant follow-up evaluations for up to 1 year after the challenge. The reason for the withdrawal of any volunteer will be recorded in the CRF and accompanied by a memorandum supporting such information.

20. POTENTIAL CAUSES OF STUDY FAILURE

Based on national and international standards, we have established three levels of potential failures for this study: logistical, clinical, immunological, and parasitological.

From a logistical point of view, failure could occur due to a) failure to recruit a sample size that allows for statistical analysis, e.g., recruitment of less than 60% of the proposed number of participants. A reduction of up to 40% is considered acceptable, as it would allow for vaccinated groups of 12 volunteers and 6 volunteers in the control groups. b) Abandonment or withdrawal from the study due to these limits, e.g., withdrawal of >40% of volunteers (8) in the vaccine group and (4) in the control group. c) Loss of data or poor data quality. d) Suspension of the study for safety reasons due to public order disturbances.

Clinical failure, a) presence of *P. falciparum* cases after challenge. b) development of complicated malaria due to *P. vivax*. Highly unlikely, due to close monitoring of volunteers after challenge and immediate treatment at minimal parasitemia. c) suspension of the study due to the presence of AES in one or more study volunteers, uncertain factor.

Failure for immunological reasons such as lack or reduction of vaccine immunogenicity, or reduction of the response to a significant level compared to previous studies.

Failure due to **parasitological** reasons, protective efficacy less than 50%, with the presence of parasitemia in >50% of vaccinees, with pre-patent parasitemia periods like or shorter than those of the controls.

21.CIVIL LIABILITY POLICIES

The study will be covered by a clinical trial insurance policy. The insurance covers claims made for damages arising from the clinical trial covered, provided that such claims are made during the period of the study.

22. STUDY MONITORING

To ensure that the CT is conducted in accordance with the protocol approved by INVIMA, in accordance with Good Clinical Practices (GCP), national regulations (Resolution 8430/93 and Resolution

2378/08) and international guidelines (ICH E6(R3), E8(R1), E9, E19). To protect the rights, safety, and well-being of participants and the research team, and to ensure the scientific validity and reliability of the data, the responsibilities of the clinical monitor are described here.

The monitor will be responsible for monitoring activities related to the different stages of the study.

Before the start of the study, they must:

- Verify that ASOCLINIC Ltda. complies with the infrastructure, personnel, and regulations defined for the study.
- Review essential documents related to ethical aspects, contracts, resumes (CVs), and GCP/GCP.
- Evaluate the team's knowledge and familiarity with protocol, safety, and procedures.

During implementation

- Supervise recruitment and informed consent: verification of eligibility and signing of consent forms.
- Clinical and laboratory procedures: compliance with the protocol.
- Control collection, storage, and shipment of biological samples.
- Data recording in CRF/eCRF: accuracy, consistency (CRF/eCRF *vs.* source), and traceability.
- Handling of the investigational product: storage, dispensing, and returns.
- Control vaccine administration and investigational product management.
- Safety report: identification, recording, and reporting of AEs/SAEs/AEFI (Adverse Events Following Immunization)
- Essential file: verify data quality, proper preservation of documents and records.

During monitoring visits

- Review compliance with the visit schedule.
- Ensure consistency in malaria diagnoses (thick blood smear microscopy, PCR, external quality control).
- Reconcile vaccine and reagent inventories.

At the end of the study

- Confirm that all documentation and databases are complete and corrected.
- Reconcile return or destruction of surplus product.
- Prepare final monitoring report and ensure regulatory filing.

To ensure the quality and accuracy of monitoring, a detailed plan has been established called the Clinical Trial Monitoring Plan – Phase II *Pv*CS2/M51), which sets out in more detail the specific activities and responsibilities established to guarantee the quality and safety of the clinical trial. Its implementation will strengthen the credibility of the data generated, as well as the protection of participants, volunteers, and the research team, ensuring compliance with national and international regulations (see **Annex 6)**.

23. USE OF INFORMATION AND PUBLICATIONS ARISING FROM THE

STUDY.

The results of this study are confidential and will be published only after authorization by the PI and the sponsoring institution. It is anticipated that the results of this protocol will be presented to the scientific community through oral presentations at scientific meetings and events and in written publications in specialized international journals. Researchers who are not named from the outset in this protocol will be required to submit to the Researcher Assurance Agreement. The participation of researchers in publications resulting from this study will be determined in accordance with international principles of scientific authorship.

24. TIMELINE

Activities	Year																																		
	2025												2026												2027											
Month	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
Protocol Design																																				
Evaluation of potential study volunteers																																				
Immunizations																																				
Immune response evaluation																																				
Clinical patients follow-up																																				
Mosquito Bite Challenge																																				
Socializing and publication of study results																																				

23. BIBLIOGRAPHY

Alonso P.L., Sacarlal J., Aponte J.J., Leach A., Macete E., Milman J., Mandomando I., Spiessens B., Guinovart C., Espasa M., Bassat Q., Aide P., Ofori-Anyinam O., Navia M.M., Corachan S., Ceuppens M., Dubois M.C., Demoitie M.A., Dubovsky F., Menendez C., Tornieporth N., Ballou W.R., Thompson R., Cohen J. (2004) Efficacy of the RTS,S/AS02A vaccine against *Plasmodium* falciparum infection and disease in young African children: randomised controlled trial. Lancet 364:1411-20. DOI: S0140673604172231 [pii]10.1016/S0140-6736(04)17223-1.

Arévalo-Herrera M., Herrera S. (2001) *Plasmodium vivax* malaria vaccine development. Mol Immunol 38:443-55. DOI: S0161589001000803 [pii].

Arévalo-Herrera M., Roggero M.A., Gonzalez J.M., Vergara J., Corradin G., Lopez J.A., Herrera S. (1998) Mapping and comparison of the B-cell epitopes recognized on the *Plasmodium vivax* circumsporozoite protein by immune Colombians and immunized Aotus monkeys. Ann Trop Med Parasitol 92:539-51.

Arévalo-Herrera M., Vera O., Castellanos A., Cespedes N., Soto L., Corradin G., Herrera S. (2011a) Preclinical vaccine study of *Plasmodium vivax* circumsporozoite protein derived-synthetic polypeptides formulated in montanide ISA 720 and montanide ISA 51 adjuvants. Am J Trop Med Hyg 84:21-7. DOI: 84/2_Suppl/21 [pii] 10.4269/ajtmh.2011.10-0110.

Arévalo-Herrera M., Soto L., Perlaza B.L., Cespedes N., Vera O., Lenis A.M., Bonelo A., Corradin G., Herrera S. (2011b) Antibody-mediated and cellular immune responses induced in naïve volunteers by vaccination with long synthetic peptides derived from the *Plasmodium vivax* circumsporozoite protein. Am J Trop Med Hyg 84:35-42. DOI: 84/2_Suppl/35 [pii]10.4269/ajtmh.2011.09-0507.

Arévalo-Herrera M., Chitnis C., Herrera S. (2010) Current status of *Plasmodium vivax* vaccine. Hum Vaccin 6:124-132.

Arévalo-Herrera M, Valencia AZ, Vergara J. et al., (2002). Identification of HLA-A2 restricted CD8(+) T-lymphocyte responses to Plasmodium vivax circumsporozoite protein in individuals naturally exposed to malaria. Parasite Immunol. 24(3):161-9. doi: 10.1046/j.1365 3024.2002.00449.x. PMID: 12078650.

Arévalo-Herrera M, Vásquez-Jiménez JM, Lopez-Perez M, Vallejo AF, Amado-Garavito AB, Céspedes N, Castellanos A, Molina K, Trejos J, Oñate J, Epstein JE, Richie TL, Herrera S (2016a). Protective Efficacy of Plasmodium vivax Radiation-Attenuated Sporozoites in Colombian Volunteers: A Randomized Controlled Trial. PLoS Negl Trop Dis. 19;10(10):e0005070. doi: 10.1371/journal.pntd.0005070. PMID: 2776014

Arévalo-Herrera M, Lopez-Perez M, Dotsey E, Jain A, Rubiano K, Felgner PL, Davies DH, Herrera S. (2016b). Antibody Profiling in Naïve and Semi-immune Individuals Experimentally Challenged with Plasmodium vivax Sporozoites. PLoS Negl Trop Dis. Mar 25;10(3):e0004563. doi: 10.1371/journal.pntd.0004563. eCollection 2016 Mar. PMID: 27014875.

Arévalo-Herrera M., Forero-Peña D.A., Rubiano K., Gómez-Hincapie J., Martínez N.L., Lopez-Perez M., Castellanos A., Céspedes N., Palacios R., Oñate J.M., Herrera S. Plasmodium vivax sporozoite challenge in malaria-naive and semi-immune Colombian volunteers. (2014). PLoS One. doi: 10.1371/journal.pone.0099754. eCollection 2014. PMID: 24963662

Arévalo-Herrera, M. *et al.* (2022) Randomized clinical trial to assess the protective efficacy of a Plasmodium vivax CS synthetic vaccine. *Nat Commun* 13, 1603. 10.1038/s41467-022-29226-3.

Arévalo-Herrera M, Miura K, Solano E, Sebastián Ramírez J, Long CA, Corradin G, Herrera S. (2022b). Immunogenicity of full-length *P. vivax* rPvs48/45 protein formulations in BALB/c mice. Vaccine. Jan 3;40(1):133-140. doi: 10.1016/j.vaccine.2021.11.036. Epub 2021 Nov 19. PMID: 34802791

Arévalo-Herrera M, Miura K, Cespedes N, Echeverry C, Solano E, Castellanos A, Ramírez JS, Miranda A, Kajava AV, Long C, Corradin G, Herrera S. (2021). Immunoreactivity of Sera From Low to Moderate Malaria-Endemic Areas Against Plasmodium vivax rPvs48/45 Proteins Produced in Escherichia coli and Chinese Hamster Ovary Systems. Front Immunol. Jun 24;12:634738. doi: 10.3389/fimmu.2021.634738. eCollection 2021. PMID: 34248932.

Arévalo-Herrera M, Vallejo AF, Rubiano K, Solarte Y, Marín C, Castellanos A, Céspedes N, Herrera S. (2015). Recombinant Pvs48/45 antigen expressed in E. coli generates antibodies that block malaria transmission in Anopheles albimanus mosquitoes. PLoS One. Mar 16;10(3):e0119335. doi: 10.1371/journal.pone.0119335. eCollection 2015. PMID: 25775466.

Arévalo-Herrera M, Solarte Y, Rocha L, Álvarez D, Beier JC, Herrera S. (2011c). Characterization of Plasmodium vivax transmission-blocking activity in low to moderate malaria transmission settings of the Colombian Pacific coast. Am J Trop Med Hyg. Feb;84(2 Suppl):71-7. doi: 10.4269/ajtmh.2011.10-0085. PMID: 21292881.

Arévalo-Herrera M, Solarte Y, Yasnot MF, Castellanos A, Rincón A, Saul A, Mu J, Long C, Miller L, Herrera S. (2005) Induction of transmission-blocking immunity in Aotus monkeys by vaccination with Plasmodium vivax clinical grade PVS25 recombinant protein. Am J Trop Med Hyg. Nov;73(5 Suppl):32-7. doi: 10.4269/ajtmh.2005.73.32.PMID: 16291764.

Arévalo-Herrera, M., Gaitán, X., Larmat-Delgado, M., Caicedo, M. A., Herrera, S. M., Henao-Giraldo, J., ... & Herrera, S. (2022). Randomized clinical trial to assess the protective efficacy of a Plasmodium vivax CS synthetic vaccine. Nature communications, 13(1), 1603.

Arévalo-Herrera M, Miura K, Solano E, Sebastian Ramirez J, Long CA, Corradin G, et al. (2022). Immunogenicity of full-length P. vivax rPvs48/45 protein formulations in BALB/c mice. Vaccine. doi:

10.1016/j.vaccine.2021.11.036. PubMed PMID: 34802791; PubMed Central PMCID: PMCPMC9109962.

Arnot D.E., Stewart M.J., Barnwell J.W. (1990) Antigenic diversity in Thai *Plasmodium vivax* circumsporozoite proteins. Mol Biochem Parasitol 43:147-9. DOI: 0166- 6851(90)90140-H.

Arnot D.E., Barnwell J.W., Tam J.P., Nussenzweig V., Nussenzweig R.S., Enea V. (1985) Circumsporozoite protein of *Plasmodium vivax*: gene cloning and characterization of the immunodominant epitope. Science 230:815-8.

Barnwell J.W., Galinski M.R. (1995) *Plasmodium vivax*: a glimpse into the unique and shared biology of the merozoite. Ann Trop Med Parasitol 89:113-20.

Barnwell J.W., Galinski M.R., DeSimone S.G., Perler F., Ingravallo P. (1999). *Plasmodium vivax*, P. cynomolgi, and P. knowlesi: identification of homologue proteins associated with the surface of merozoites. Exp Parasitol 91:238-49. DOI: S0014- 4894(98)94372-7 [pii] 10.1006/expr.1998.4372.

Bejon Ph., Lusingu J., Olotu A., Leach A., Lievens M., Vekemans J., Mshamu S., Lang T., Gould J., et al. (2008). Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. N Engl J Med. doi: 10.1056/NEJMoa0807381. PMID: 19064627. PMCID: PMC2655100

Bennett JW, Yadava A, Tosh D, Sattabongkot J, Komisar J, Ware LA, McCarthy WF, Cowden JJ, Regules J, Spring MD, et al. (2016) Phase 1/2a Trial of Plasmodium vivax Malaria Vaccine Candidate VMP001/AS01B in Malaria-Naive Adults: Safety, Immunogenicity, and Efficacy. PLoS Negl Trop Dis . doi: 10.1371/journal.pntd.0004423. .PMID: 26919472

Bloland P., Williams HA. (2002). Malaria Control During Mass Population Movements and Natural Disasters Roundtable on the Demography of Forced Migration, National Research Council, Program on Forced Migration and Health at the Mailman School of Public Health of Columbia University. ISBN: 0-309-50362-0, 184 pages. https://www.ncbi.nlm.nih.gov/books/NBK221163/pdf/Bookshelf_NBK221163.pdf

Bojang K.A., Milligan P.J., Pinder M., Vigneron L., Alloueche A., Kester K.E., BallouW.R., Conway D.J., Reece W.H., Gothard P., Yamuah L., Delchambre M., Voss G., Greenwood B.M., Hill A., McAdam K.P., Tornieporth N., Cohen J.D., Doherty T. (2001) Efficacy of RTS,S/AS02 malaria vaccine against *Plasmodium* falciparum infection in semi-immune adult men in The Gambia: a randomised trial. Lancet 358:1927-34. DOI: S0140- 6736(01)06957-4 [pii] 10.1016/S0140-6736(01)06957-4.

Breman J.G., Egan A., Keusch G.T. (2001) The intolerable burden of malaria: a new look at the numbers. Am J Trop Med Hyg 64:iv-vii.

Burkot T.R., Wirtz R.A., Paru R., Garner P., Alpers M.P. (1992) The population dynamics in mosquitoes and humans of two *Plasmodium vivax* polymorphs distinguished by different circumsporozoite protein repeat regions. Am J Trop Med Hyg 47:778-786.

Céspedes N., Arévalo-Herrera M., Felger I., Reed S., Kajava A.V., Corradin G., Herrera S. Antigenicity and immunogenicity of a novel chimeric peptide antigen based on the P. *vivax* circumsporozoite protein (2013).doi: 10.1016/j.vaccine.2013.05.082. PMID: 23954378.

Céspedes N, Habel C, Lopez-Perez M, Castellanos A, Kajava AV, Servis C, Felger I, Moret R, Arévalo-Herrera M, Corradin G, Herrera S. (2014). Plasmodium vivax antigen discovery based on alpha-helical coiled coil protein motif. PLoS One. Jun 24;9(6):e100440. doi: 10.1371/journal.pone.0100440. eCollection 2014. PMID: 24959747.

Céspedes N, Li Wai Suen CSN, Koepfli C, França CT, Felger I, Nebie I, Arévalo-Herrera M, Mueller I, Corradin G, Herrera S. (2017). Natural immune response to Plasmodium vivax alpha-helical coiled coil protein motifs and its association with the risk of *P. vivax* malaria. PLoS One. Jun 26;12(6):e0179863. doi: 10.1371/journal.pone.0179863. eCollection 2017. PMID: 28651021.

Céspedes, N., Vallejo, A., Arévalo-Herrera, M., & Herrera, S. (2013). Malaria vaccines: high-throughput tools for antigen discovery with potential for their development. Colombia Médica, 44(2), 121-128.

Cattani J.A., Tulloch J.L., Vrbova H., Jolley D., Gibson F.D., Moir J.S., Heywood P.F., Alpers M.P., Stevenson A., Clancy R. (1986a) The epidemiology of malaria in a population surrounding Madang, Papua New Guinea. Am J Trop Med Hyg 35:3-15.

Cattani J.A., Tulloch J.L., Vrbova H., Jolley D., Gibson F.D., Moir J.S., Heywood P.F., Alpers M.P., Stevenson A., Clancy R. (1986b) The epidemiology of malaria in a population surrounding Madang, Papua New Guinea. Am J Trop Med Hyg 35:3-15.

Cerami C., Frevert U., Sinnis P., Takacs B., Clavijo P., Santos M.J., Nussenzweig V. (1992) The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium* falciparum sporozoites. Cell 70:1021- 33. DOI: 0092-8674(92)90251-7.

Clyde D.F. (1975) Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. Am J Trop Med Hyg 24:397-401.

Clyde D.F., McCarthy V.C., Miller R.M., Hornick R.B. (1973) Specificity of protection of man immunized against sporozoite-induced falciparum malaria. Am J Med Sci 266:398-403.

Cochrane A.H., Aikawa M., Jeng M., Nussenzweig R.S. (1976) Antibody-induced ultrastructural changes of malarial sporozoites. Journal of Immunology 116:859-67.

Cochrane A.H., Nardin E.H., de Arruda M., Maracic M., Clavijo P., Collins W.E., Nussenzweig R.S. (1990) Widespread reactivity of human sera with a variant repeat of the circumsporozoite protein of *Plasmodium vivax*. Am J Trop Med Hyg 43:446-51.

Collins W.E., Jeffery G.M. (1999) A retrospective examination of sporozoite- and trophozoite-induced infections with *Plasmodium* falciparum in patients previously infected with heterologous species of *Plasmodium*: effect on development of parasitologic and clinical immunity. Am J Trop Med Hyg

61:36-43.

Collins W.E., Sullivan J.S., Morris C.L., Galland G.G., Richardson B.B. (1996) Observations on the biological nature of *Plasmodium vivax* sporozoites. J Parasitol 82:216-9.

Comer R.D., Young M.D., Porter J.A., Jr., Gauld J.R., Merritt W. (1968) Chloroquine resistance in *Plasmodium* falciparum malaria on the Pacific coast of Colombia. Am J Trop Med Hyg 17:795-9.

Charoenvit Y., Collins W.E., Jones T.R., Millet P., Yuan L., Campbell G.H., Beaudoin R.L., Broderson J.R., Hoffman S.L. (1991) Inability of malaria vaccine to induce antibodies to a protective epitope within its sequence. Science 251:668-71.

Chitnis C.E. (2001) Molecular insights into receptors used by malaria parasites for erythrocyte invasion. Curr Opin Hematol 8:85-91.

Chulay J.D., Schneider I., Cosgriff T.M., Hoffman S.L., Ballou W.R., Quakyi I.A., Carter R., Trosper J.H., Hockmeyer W.T. (1986) Malaria transmitted to humans by mosquitoes infected from cultured *Plasmodium* falciparum. Am J Trop Med Hyg 35:66-8.

Datoo, M. M., Dicko, A., Tinto, H., Ouédraogo, J. B., Hamaluba, M., Olotu, A., ... & Hill, A. V. A Phase III Randomized Controlled Trial Evaluating the Malaria Vaccine Candidate R21/Matrix-M™ in African Children.

Del Portillo H.A., Longacre S., Khouri E., David P.H. (1991) Primary structure of the merozoite surface antigen 1 of *Plasmodium vivax* reveals sequences conserved between different *Plasmodium* species. Proc Natl Acad Sci U S A 88:4030-4.

Dhiman, S. (2019) Are malaria elimination efforts on the right track? An analysis of gains achieved and challenges ahead. *Infect Dis Poverty* 8, 14.10.1186/s40249-019-0524x.

Doolan DL, Dobaño C, Baird JK. (2009). Acquired immunity to malaria.Clin Microbiol Rev. 22(1):13-36, doi: 10.1128/CMR.00025-08. PMID: 19136431.

Druihle P., Reina L., Fidock D.A. (1998) Immunity to liver stages, in I. Sherman (Ed.), Malaria: Parasite biology, pathogenesis and protection Washington DC. pp. 513-543.

Druilhe P, Pérignon JL (1994) Mechanisms of defense against *P. falciparum* asexual blood stages in humans. Immunol Lett. 41(2-3):115-20. doi: 10.1016/0165- 2478(94)90118-x. PMID: 8002025.

Egan J.E., Hoffman S.L., Haynes J.D., Sadoff J.C., Schneider I., Grau G.E., Hollingdale M.R., Ballou W.R., Gordon D.M. (1993) Humoral immune responses in volunteers immunized with irradiated *Plasmodium* falciparum sporozoites. Am J Trop Med Hyg 49:166-73.

Espinosa, D.A., Yadava, A., Angov, E., Maurizio, P.L., Ockenhouse, Ch.F., Zavala, F. Development of a chimeric Plasmodium berghei strain expressing the repeat region of the P. vivax circumsporozoite

protein for in vivo evaluation of vaccine efficacy. (2013) Infect Immun. doi: 10.1128/IAI.00461-13. PMID: 23716612. PMCID: PMC3719583. DOI: 10.1128/IAI.00461-13

Eyles D.E. (1950) A stain for malarial oocysts in temporary preparations. J Parasitol 36:501.

Fairley N.H. (1947) Sidelights on malaria in man obtained by sub-inoculation experiments. Trans R Soc Trop Med Hyg 40:621-76.

Franke E.D., Lucas C.M., San Roman E., Wirtz R.A. (1992a) Prevalence of antibody to the variant repeat of the circumsporozoite protein of *Plasmodium vivax* in Peru. Am J Trop Med Hyg 46:708-10.

Franke E.D., Lucas C.M., Chauca G., Wirtz R.A., Hinostroza S. (1992b) Antibody response to the circumsporozoite protein of *Plasmodium vivax* in naturally infected humans. Am J Trop Med Hyg 46:320-6.

Frevert U., Sinnis P., Cerami C., Shreffler W., Takacs B., Nussenzweig V. (1993) Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. J. Exp. Med. 177:1287-1298.

Galappaththy, G.N. *et al.* (2013) Primaquine for preventing relapse in people with Plasmodium vivax malaria treated with chloroquine. *The Cochrane database of systematic reviews*, CD004389. 10.1002/14651858.CD004389.pub3

Galinski M.R., Corredor-Medina C., Povoa M., Crosby J., Ingravallo P., Barnwell J.W. (1999) *Plasmodium vivax* merozoite surface protein-3 contains coiled-coil motifs in an alanine-rich central domain. Mol Biochem Parasitol 101:131-47. DOI: S0166- 6851(99)00063-8.

Galinski M.R., Ingravallo P., Corredor-Medina C., Al-Khedery B., Povoa M., Barnwell J.W. (2001) *Plasmodium vivax* merozoite surface proteins-3beta and-3gamma share structural similarities with *P. vivax* merozoite surface protein-3alpha and define a new gene family. Mol Biochem Parasitol 115:41-53. DOI: S0166-6851(01)00267-5 [pii].

Genton B., Corradin G. (2002) Malaria vaccines: from the laboratory to the field. Curr Drug Targets Immune Endocr Metabol Disord 2:255-67.

Glynn J.R., Collins W.E., Jeffery G.M., Bradley D.J. (1995) Infecting dose and severity of falciparum malaria. Trans R Soc Trop Med Hyg 89:281-3.

Gonzalez J.M., Hurtado S., Arévalo-Herrera M., Herrera S. (2001) Variants of the *Plasmodium vivax* circumsporozoite protein (VK210 and VK247) in Colombian isolates. Mem Inst Oswaldo Cruz 96:709-12. DOI: S0074-02762001000500023.

Grassi B.A., Bignami, Bastianelli G. (1899) Further research on the cycle of human malaria parasites in the body of the mosquito. Atti Reale Accad. Lincei 5:8-21.

Guerra C.A., Howes R.E., Patil A.P., Gething P.W., Van Boeckel T.P., Temperley W.H., Kabaria C.W.,

Tatem A.J., Manh B.H., Elyazar I.R., Baird J.K., Snow R.W., Hay S.I. (2010) The international limits and population at risk of *Plasmodium vivax* transmission in 2009. PLoS Negl Trop Dis 4:e774. DOI: 10.1371/journal.pntd.0000774.

Guinovart C., Aponte J.J., Sacarlal J., Aide P., Leach A., Bassat Q., Macete E., Dobano C., Lievens M., Loucq C., Ballou W.R., Cohen J., Alonso P.L. (2009) Insights into long-lasting protection induced by RTS, S/AS02A malaria vaccine: further results from a phase IIb trial in Mozambican children. PLoS One 4:e5165. DOI: 10.1371/journal.pone.0005165.

Gunewardena D.M., Carter R., Mendis K.N. (1994) Patterns of acquired anti-malarial immunity in Sri Lanka. Mem Inst Oswaldo Cruz 89:63-5.

Gysin J., Moisson P., Pereira da Silva L., Druilhe P. (1996) Antibodies from immune African donors with a protective effect in *Plasmodium* falciparum human infection are also able to control asexual blood forms of the parasite in Saimiri monkeys. Res Immunol 147:397-401. DOI: 0923249496820487 [pii].

Herrera S., Escobar P., de Plata C., Avila GI., Corradin G., Herrera MA. (1992) Human recognition of T cell epitopes on the Plasmodium vivax circumsporozoite protein. J Immunol1992 Jun 15;148(12):3986-90 PMID: 1376347

Herrera M.A., de Plata C., Gonzalez J.M., Corradin G., Herrera S. (1994) Immunogenicity of multiple antigen peptides containing *Plasmodium vivax* CS epitopes in BALB/c mice. Mem Inst Oswaldo Cruz 89:71-6.

Herrera S., De Plata C., Gonzalez M., Perlaza B.L., Bettens F., Corradin G., Arévalo Herrera M. (1997) Antigenicity and immunogenicity of multiple antigen peptides (MAP) containing *P. vivax* CS epitopes in Aotus monkeys. Parasite Immunol 19:161-70.

Herrera S., Fernandez O.L., Vera O., Cardenas W., Ramirez O., Palacios R., Chen Mok M., Corradin G., Arévalo-Herrera M. (2011a) Phase I safety and immunogenicity trial of *Plasmodium vivax* CS derived long synthetic peptides adjuvanted with montanide ISA 720 or montanide ISA 51. Am J Trop Med Hyg 84:12-20. DOI: 84/2_Suppl/12 [pii] 10.4269/ajtmh.2011.09-0516.

Herrera S., Solarte Y., Jordan-Villegas A., Echavarria J.F., Rocha L., Palacios R., Ramirez O., Velez J.D., Epstein J.E., Richie T.L., Arévalo-Herrera M. (2011b) Consistent safety and infectivity in sporozoite challenge model of *Plasmodium vivax* in malaria-naïve human volunteers. Am J Trop Med Hyg 84:4-11. DOI: 84/2_Suppl/4 [pii] 10.4269/ajtmh.2011.09-0498.

Herrera S., Fernandez O., Manzano M.R., Murrain B., Vergara J., Blanco P., Palacios R., Velez J.D., Epstein J.E., Chen-Mok M., Reed Z.H., Arévalo-Herrera M. (2009c) Successful sporozoite challenge model in human volunteers with *Plasmodium vivax* strain derived from human donors. Am J Trop Med Hyg 81:740-6. DOI: 81/5/740 [pii] 10.4269/ajtmh.2009.09-0194.

Herrera S., Bonelo A., Perlaza B.L., Fernandez O.L., Victoria L., Lenis A.M., Soto L., Hurtado H., Acuna L.M., Velez J.D., Palacios R., Chen-Mok M., Corradin G., Arévalo Herrera M. (2005) Safety and e -

elicitation of humoral and cellular responses in Colombian malaria-naïve volunteers by a *Plasmodium vivax* circumsporozoite protein-derived synthetic vaccine. Am J Trop Med Hyg 73:3-9. DOI: 73/5_suppl/3.

Herrera, S., Fernández, O., Manzano, M.R., Murrain, B., Vergara, J., Blanco, P., Palacios, R., Vélez, J.D., Epstein, J., Chen-Mok, M., Reed, Z., Arévalo-Herrera, M. 2009. Case report: Successful sporozoite challenge model in human volunteers with Plasmodium vivax strain derived from human donors. Am. J. Trop. Med. Hyg, 81(5):740-746. doi: 10.4269/ajtmh.2009.09-0194. PMID: 19861603

Herrera, S., Solarte, Y., Jordán-Villegas, A., Echavarría, J.F., Rocha, L., Palacios, R., Ramírez, O., Vélez, J.D., Epstein, J.E., Richie, T.L. and Arévalo-Herrera, M. 2011. Consistent safety and infectivity in sporozoite challenge model of Plasmodium vivax in malaria-naïve human volunteers. Am J. Trop. Med. Hyg, 84(2):4-11 doi: 10.4269/ajtmh.2011.09-0498. PMID: 21292872

Herrera, S., De Plata C., González M., Perlaza BL., Bettens F., Corradin G., Arévalo-Herrera M. (1997). Antigenicity and immunogenicity of multiple antigen peptides (MAP) containing *P. vivax* CS epitopes in Aotus monkeys. Parasite Immunol., 19(4): p. 161-70. doi: 10.1046/j.1365-3024.1997.d01-193.x. PMID: 9149283

Herrera, SM; Flores Y; Corradin G; Arevalo-Herrera M; Herrera S; Zavala F, (2025; Submitted). Validation of the human protective efficacy of a Plasmodium vivax CS synthetic vaccine candidate in a rodent model

Herrington D.A., Nardin E.H., Losonsky G., Bathurst I.C., Barr P.J., Hollingdale M.R., Edelman R., Levine M.M. (1991) Safety and immunogenicity of a recombinant sporozoite malaria vaccine against *Plasmodium vivax*. Am J Trop Med Hyg 45:695-701.

Hill AV. (2011) Vaccines against malaria. Philos Trans R Soc Lond B Biol Sci. 366(1579):2806–2814. doi: 10.1098/rstb.2011.009

Hoffman S.L., Doolan D.L. (2000) Malaria vaccines-targeting infected hepatocytes. Nat Med 6:1218- 9. DOI: 10.1038/81315.

Hoffman S.L., Goh L.M., Luke T.C., Schneider I., Le T.P., Doolan D.L., Sacci J., de la Vega P., Dowler M., Paul C., Gordon D.M., Stoute J.A., Church L.W., Sedegah M., Heppner D.G., Ballou W.R., Richie T.L. (2002) Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium* falciparum sporozoites. J Infect Dis 185:1155-64. DOI: JID010922 [pii] 10.1086/339409.

Hoffman, S. L., Vekemans, J., Richie, T. L., & Duffy, P. E. (2015). The march toward malaria vaccines. Vaccine, 33, D13-D23.

Hurtado S., Salas M.L., Romero J.F., Zapata J.C., Ortiz H., Arévalo-Herrera M., Herrera S. (1997) Regular production of infective sporozoites of *Plasmodium* falciparum and *P. vivax* in laboratory-bred *Anopheles albimanus*. Ann Trop Med Parasitol 91:49-60.

Kain K.C., Brown A.E., Webster H.K., Wirtz R.A., Keystone J.S., Rodriguez M.H., Kinahan J., Rowland M., Lanar D.E. (1992) Circumsporozoite genotyping of global isolates of *Plasmodium vivax* from dried blood specimens. J Clin Microbiol 30:1863-6.

Karch Ch.P., Doll T., Paulillo S.M., Nebie I., Lanar D.E., Corradin G., Peter Burkhard. The use of a *P. falciparum* specific coiled-coil domain to construct a self-assembling protein nanoparticle vaccine to prevent malaria. J Nanobiotechnology. PMID: 28877692. doi: 10.1186/s12951-017-0295-0.

Kublin, J. G., Mikolajczak, S. A., Sack, B. K., Fishbaugher, M. E., Seilie, A., Shelton, L., ... & Kappe, S. H. (2017). Complete attenuation of genetically engineered Plasmodium falciparum sporozoites in human subjects. Science translational medicine, 9(371), eaad9099.

Kumar S., Epstein J.E., Richie T.L., Nkrumah F.K., Soisson L., Carucci D.J., Hoffman S.L. (2002) A multilateral effort to develop DNA vaccines against falciparum malaria. Trends Parasitol 18:129-35. DOI: S1471492201022073 [pii].

Lata De S., Ntumngia F.B., Nicholas J., Adams J.H. (2022). Expert Rev Vaccines Progress towards the development of a P. vivax vaccine. PMC7994195 NIHMSID: NIHMS1667293 PMID: 33481638 doi: 10.1080/14760584.2021.1880898

Lopez-Perez, M., Jain, A., Davies, D. H., Vásquez-Jiménez, J. M., Herrera, S. M., Oñate, J., ... & Arévalo-Herrera, M. (2023). Human sterile immunity to Plasmodium vivax malaria correlates with specific antibody response elicited by immunization with radiation-attenuated sporozoites.

Lopez-Perez M, Jain A, Davies DH, Vasquez-Jimenez JM, Herrera SM, Onate J, Felgner PL, Herrera S, Arevalo-Herrera M: Profiling the antibody response of humans protected by immunization with Plasmodium vivax radiation-attenuated sporozoites. *Sci Rep* 2024, 14:2790.

Malkin, E. M. et al. Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria. Vaccine 23, 3131–3138, doi:10.1016/j.vaccine.2004.12.019 (2005).

Manjurano, A. *et al.* (2015) African glucose-6-phosphate dehydrogenase alleles associated with protection from severe malaria in heterozygous females in Tanzania. *PLoS Genetics* 11, e1004960

Macete E., Aponte J.J., Guinovart C., Sacarlal J., Ofori-Anyinam O., Mandomando I., Espasa M., Bevilacqua C., Leach A., Dubois M.C., Heppner D.G., Tello L., Milman J., Cohen J., Dubovsky F., Tornieporth N., Thompson R., Alonso P.L. (2007) Safety and immunogenicity of the RTS, S/AS02A candidate malaria vaccine in children aged 1-4 in Mozambique. Trop Med Int Health 12:37-46. DOI: TMI1754 [pii]
10.1111/j.1365-3156.2006.01754.x.

Machado R.L., Povoa M.M. (2000) Distribution of *Plasmodium vivax* variants (VK210, VK247 and *P.* vivax-like) in three endemic areas of the Amazon region of Brazil and their correlation with chloroquine treatment. Trans R Soc Trop Med Hyg 94:377-81

Maheswary N.P., Perpanich B., Rosenberg R. (1992) Presence of antibody to a heterologous circumsporozoite protein of *Plasmodium vivax* (VK247) in southeastern Bangladesh. Trans R Soc Trop Med Hyg 86:28.

Mann V.H., Huang T., Cheng Q., Saul A. (1994) Sequence variation in the circumsporozoite protein gene of *Plasmodium vivax* appears to be regionally biased. Mol Biochem Parasitol 68:45-52. DOI: 0166-6851(94)00148-0 [pii].

Mendis K., Sina B.J., Marchesini P., Carter R. (2001) The neglected burden of *Plasmodium vivax* malaria. Am J Trop Med Hyg 64:97-106.

Miller L.H., McAuliffe F.M., Mason S.J. (1977) Erythrocyte receptors for malaria merozoites. Am J Trop Med Hyg 26:204-8.

Mo, AX, McGugan. Understanding the Liver-Stage Biology of Malaria Parasites: Insights to Enable and Accelerate the Development of a Highly Efficacious Vaccine (2018). Am J Trop Med Hyg. Oct;99(4):827-832. doi:10.4269/ajtmh.17-0895. PMID: 30141395. PMCID: PMC6159572 DOI: 10.4269/ajtmh.17-0895

Molina DM, Finney OC, Arévalo-Herrera M, Herrera S, Felgner PL, Gardner MJ, Liang X, Wang R. (2012) Plasmodium vivax pre-erythrocytic-stage antigen discovery: exploiting naturally acquired humoral responses. Am J Trop Med Hyg. Sep;87(3):460-9. doi: 10.4269/ajtmh.2012.12-0222. Epub 2012 Jul 23. PMID: 22826492.

Molina-Cruz A, Gonzalez-Ceron L, Dwivedi A, Torres TZB, Raytselis N, Young M, Kamath N, McNinch C, Su X, Ford A, Ferreira MU, Arévalo-Herrera M, Herrera S, Lo E, Silva JC, Barillas-Mury C. (2025). Worldwide genetic diversity of Plasmodium vivax Pv47 is consistent with natural selection by anopheline mosquitoes. Nat Commun. doi: 10.1038/s41467-025-62680-3. PMID: 40783499.

Nardin E., Clavijo P., Mons B., van Belkum A., Ponnudurai T., Nussenzweig R.S. (1991) T cell epitopes of the circumsporozoite protein of *Plasmodium vivax*. Recognition by lymphocytes of a sporozoite-immunized chimpanzee. J Immunol 146:1674-8.

Nussenzweig R., Vanderberg J., Most H. (1969) Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium* berghei. IV. Dose response, specificity and humoral immunity. Mil Med 134:1176-82.

Nussenzweig RS, Vanderberg J, Most H, Orton C Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei. (1967). Nature. doi: 10.1038/216160a0. PMID: 6057225

PAHO (2022). Malaria. In Salud, O.P.d.l., ed. https://www.paho.org/es/temas/malaria

Penny MA, Verity R, Bever CA, et al. (2016). Public health impact and cost-effectiveness of the RTS, S/AS01 malaria vaccine: a systematic comparison of predictions from four mathematical models. *Lancet*; 387:367–375. doi: 10.1016/S0140-6736(15)00725-4

Qari S.H., Goldman I.F., Povoa M.M., di Santi S., Alpers M.P., Lal A.A. (1992) Polymorphism in the circumsporozoite protein of the human malaria parasite *Plasmodium vivax*. Mol. Biochem. Parasitol. 55:105-114

Ramasamy R., Nagendran K., Ramasamy M.S. (1994) Antibodies to epitopes on merozoite and sporozoite surface antigens as serologic markers of malaria transmission: studies at a site in the dry zone of Sri Lanka. Am J Trop Med Hyg 50:537- 47.

Richie T.L., Saul A. (2002) Progress and challenges for malaria vaccines. Nature 415:694-701. DOI: 10.1038/415694a415694a [pii].

Richie TL, Church LWP, Murshedkar T, Billingsley PF, James ER, …, Hoffman SL. Expert Rev Vaccines. 2023 Jan-Dec;22(1):964-1007. Sporozoite immunization: innovative translational science to support the fight against malaria. doi: 10.1080/14760584.2023.2245890. Epub 2023 Aug 11.

Rieckmann K.H., Beaudoin R.L., Cassells J.S., Sell K.W. (1979) Use of attenuated sporozoites in immunizing human volunteers against falciparum malaria. Bull World Health Organ 57:261-5.

Rodriguez M.H., Betanzos-Reyes A.F., Hernandez-Avila J.E., Mendez-Galvan J.F., Danis-Lozano R., Altamirano-Jimenez A. (2009) The participation of secondary clinical episodes in the epidemiology of vivax malaria during pre-and post-implementation of focal control in the state of Oaxaca, Mexico. Am J Trop Med Hyg 80:889-95. DOI: 80/6/889 [pii].

Rogers W.O., Malik A., Mellouk S., Nakamura K., Rogers M.D., Szarfman A., Gordon D.M., Nussler A.K., Aikawa M., Hoffman S.L. (1992) Characterization of *Plasmodium* falciparum sporozoite surface protein 2. Proc Natl Acad Sci U S A 89:9176-80.

Rosenberg R., Wirtz R.A., Lanar D.E., Sattabongkot J., Hall T., Waters A.P., Prasittisuk C. (1989) Circumsporozoite protein heterogeneity in the human malaria parasite. *Plasmodium vivax*. Science 245:973-6.

Roestenberg M, Bijker EM, Sim BKL, et al. *Controlled human malaria infections by intradermal injection of cryopreserved Plasmodium falciparum sporozoites.* (2013). Am J Trop Med Hyg. doi:10.4269/ajtmh.2012.12-0613.

Rougemont M., Van Saanen M., Sahli R., Hinrikson H.P., Bille J., Jaton K. (2004) Detection of four *Plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. J Clin Microbiol 42:5636-43. DOI: 42/12/5636 [pii] 10.1128/JCM.42.12.5636-5643.2004.

Raghunandan R, Mayer BT, Flores-Garcia Y, Gerber MW, Gottardo R, Jhun H, Herrera SM, Perez-Ramos DW, Locke E, King CR, Zavala F. (2020). Characterization of two in vivo challenge models to measure functional activity of monoclonal antibodies to Plasmodium falciparum circumsporozoite protein. Malar J. doi: 10.1186/s12936-020-03181-0. PMID: 32183833.

Sachs J., Malaney P. (2002) The economic and social burden of malaria. Nature 415:680- 5. DOI: 10.1038/415680a 415680a [pii].

Samuels, A. M., Ansong, D., Kariuki, S. K., Adjei, S., Bollaerts, A., Ockenhouse, C., ... & Ivinson, K. (2022). Efficacy of RTS, S/AS01E malaria vaccine administered according to different full, fractional, and delayed third or early fourth dose regimens in children aged 5–17 months in Ghana and Kenya: an open-label, phase 2b, randomized controlled trial. The Lancet Infectious Diseases, 22(9), 1329-1342.

Stanisic DI, McCarthy JS, Good MF. Controlled Human Malaria Infection: Applications, Advances, and Challenges. Infect Immun. 2017 Dec 19;86(1):e00479-17. doi: 10.1128/IAI.00479-17. PMID: 28923897; PMCID: PMC5736798.

Sattabongkot J., Tsuboi T., Zollner G.E., Sirichaisinthop J., Cui L. (2004) *Plasmodium vivax* transmission: chances for control? Trends Parasitol 20:192-8. DOI: 10.1016/j.pt.2004.02.001 S1471492204000327 [pii].

Sinnis P, Clavijo P, Fenyö D, Chait BT, Cerami C, Nussenzweig V.J Structural and functional properties of region II-plus of the malaria circumsporozoite protein. (1994). Exp Med. doi: 10.1084/jem.180.1.297.PMID: 8006589

Sinnis P., Nussenzweig V. (1996) Preventing sporozoite invasion of hepatocytes, in: S.L. Hoffman (Ed.), Malaria vaccine development. A multi-immune response approach, ASM Press, Washington, D.C. pp. 15-34.

Soares da Veiga G.T., Ribeiro Moriggi M., Vettorazzi J.F., Müller-Santos M., Albrecht L. Plasmodium vivax vaccine: What is the best way to go? (2023). Front Immunol. PMID: 36726991 PMCID: PMC9885200. DOI: 10.3389/fimmu.2022.910236.

Solarte Y., Manzano M.R., Rocha L., Hurtado H., James M.A., Arévalo-Herrera M., Herrera S. (2011) *Plasmodium vivax* sporozoite production in *Anopheles albimanu*

Mosquitoes for vaccine clinical trials. The American journal of tropical medicine and hygiene 84:28-34.

Soto J., Toledo J., Gutierrez P., Luzz M., Llinas N., Cedeno N., Dunne M., Berman J. (2001) *Plasmodium vivax* clinically resistant to chloroquine in Colombia. Am J Trop Med Hyg 65:90-3.

Stoute J.A., Slaoui M., Heppner D.G., Momin P., Kester K.E., Desmons P., Wellde B.T., Garcon N., Krzych U., Marchand M. (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium* falciparum malaria. RTS, S Malaria Vaccine Evaluation Group. N Engl J Med 336:86-91. DOI: 10.1056/NEJM199701093360202.

Stoute J.A., Heppner D.G., Jr., Mason C.J., Siangla J., Opollo M.O., Kester K.E., Vigneron L., Voss G., Walter M.J., Tornieporth N., Cohen J.D., Ballou W.R. (2006) Phase One safety and immunogenicity

trial of malaria vaccine RTS, S/AS02A in adults in a hyperendemic region of western Kenya. Am J Trop Med Hyg 75:166-70. DOI: 75/1/166.

Stoute J.A., Kester K.E., Krzych U., Wellde B.T., Hall T., White K., Glenn G., Ockenhouse C.F., Garcon N., Schwenk R., Lanar D.E., Sun P., Momin P., Wirtz R.A., Golenda C., Slaoui M., Wortmann G., Holland C., Dowler M., Cohen J., Ballou W.R. (1998) Long-term efficacy and immune responses following immunization with the RTS, S malaria vaccine. J Infect Dis 178:1139-44.

Tang J., Amin M.A., Campian J.L. Past, Present, and Future of Viral Vector Vaccine Platforms: A Comprehensive Review. (2025). Vaccines (Basel). doi: 10.3390/vaccines 13050524. PMID: 40432133

Templeton T.J., Kaslow D.C. (1997) Cloning and cross-species comparison of the thrombospondin-related anonymous protein (TRAP) gene from *Plasmodium* knowlesi, *Plasmodium vivax* and *Plasmodium* gallinaceum. Mol Biochem Parasitol 84:13- 24. DOI: S0166-6851(96)02775-2 [pii].

Thomas A.W., Trape J.F., Rogier C., Goncalves A., Rosario V.E., Narum D.L. (1994) High prevalence of natural antibodies against *Plasmodium falciparum* 83-kilodalton apical membrane antigen (PF83/AMA-1) as detected by capture-enzyme-linked immunosorbent assay using full-length baculovirus recombinant PF83/AMA-1. Am J Trop Med Hyg 51:730-40.

Tsoumani M.E., Voyiatzaki C., Efstathiou A. Malaria Vaccines: From the Past towards the mRNA Vaccine Era. (2023) Vaccines (Basel). doi: 10.3390/ vaccines 11091452. PMID: 37766129

Tsuji M., Zavala F. (2001) Peptide-based subunit vaccines against pre-erythrocytic stages of malaria parasites. Mol Immunol 38:433-42. DOI: S0161589001000797 [pii].

Vallejo AF, García J, Amado-Garavito AB, Arévalo-Herrera M, Herrera S. (2016) Plasmodium vivax gametocyte infectivity in sub-microscopic infections. Malar J. Jan 28;15:48. Doi: 10.1186/s12936-016-1104-1. PMID: 26822406

Van der Boor SC, Alkema M, van Gemert GJ, Teelen K, van de Vegte-Bolmer M, Walk J, van Crevel R, de Mast Q, Ockenhouse CF, Sauerwein RW, McCall MBB. BMC Med. 2023 Apr 7;21(1):137. Whole sporozoite immunization with Plasmodium falciparum strain NF135 in a randomized trial. doi: 10.1186/s12916-023-02788-9

Vaughan AM, Kappe SHI. Expert Rev Vaccines. 2017 Aug;16(8):765-767. Genetically attenuated malaria parasites as vaccines. doi: 10.1080/14760584.2017.1341835. Epub 2017 Jun 16.

Walther M., Dunachie S., Keating S., Vuola J.M., Berthoud T., Schmidt A., Maier C., Andrews L., Andersen R.F., Gilbert S., Poulton I., Webster D., Dubovsky F., Tierney E., Sarpotdar P., Correa S., Huntcooke A., Butcher G., Williams J., Sinden R.E., Thornton G.B., Hill A.V. (2005) Safety, immunogenicity and efficacy of a pre-erythrocytic malaria candidate vaccine, ICC-1132 formulated in Seppic ISA 720—vaccine 23:857-64. DOI: S0264-410X(04)00642-5 [pii] 10.1016/j.vaccine.2004.08.020.

Webster, R., Sekuloski, S., Odedra, A., Woolley, S., Jennings, H., Amante, F., ... & McCarthy, J. S. (2021). Safety, infectivity and immunogenicity of a genetically attenuated blood-stage malaria vaccine. BMC medicine, 19(1), 1-13.

WHO. (2010) World Malaria Report 2010, World Health Organization, Washington.

WHO. (2021) World Malaria Report 2021: regional data and trends. https://www.who.int/es/publications/m/item/WHO-UCN-GMP-2021.09

WHO, (2023) WHO recommends R21/Matrix-M vaccine for malaria prevention in updated advice on immunization. https://www.who.int/news/item/02-10-2023-who-recommends-r21-matrix-m-vaccine-for-malaria-prevention-in-updated-advice-on-immunization

Williams T.N., Maitland K., Bennett S., Ganczakowski M., Peto T.E., Newbold C.I., Bowden D.K., Weatherall D.J., Clegg J.B. (1996) High incidence of malaria in alpha thalassaemic children. Nature 383:522-5. DOI: 10.1038/383522a0.

Wirtz R.A., Burkot T.R., Graves P.M., Andre R.G. (1987) Field evaluation of enzyme-linked immunosorbent assays for *Plasmodium* falciparum and *Plasmodium vivax* sporozoites in mosquitoes (Diptera: Culicidae) from Papua New Guinea. J Med Entomol 24:433-7.

Wirtz R.A., Rosenberg R., Sattabongkot J., Webster H.K. (1990) Prevalence of antibody to heterologous circumsporozoite protein of *Plasmodium vivax* in Thailand. Lancet 336:593-5. DOI: 0140-6736(90)93393-4 [pii].

Wombou Toukam CM, Solano P, Bengaly Z, Jamonneau V, Bucheton B. (2011) Experimental evaluation of xenodiagnosis to detect trypanosomes at low parasitaemia levels in infected hosts. Parasite 18(4):295-302

Yadava A., Sattabongkot J., Washington M.A., Ware L.A., Majam V., Zheng H., Kumar S., Ockenhouse C.F. (2007) A novel chimeric *Plasmodium vivax* circumsporozoite protein induces biologically functional antibodies that recognize both VK210 and VK247 sporozoites. Infect Immun 75:1177-85. DOI: IAI.01667-06 [pii] 10.1128/IAI.01667-06.

Zhuo Y., Zeng H., Su C., Lv Q., Cheng., Lei L. Tailoring biomaterials for vaccine delivery. (2024). J Nanobiotechnology. doi: 10.1186/s12951-024-02758-0. PMID: 39135073