Universidade Nova de Lisboa
Instituto de Higiene e Medicina Tropical

Characterisation of *Plasmodium falciparum* antigens for the development of serological assay or vaccine candidates for malaria

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Resumo

A malária causada por *Plasmodium falciparum* continua a ser um grande problema de saúde nas regiões tropicais e subtropicais em todo o mundo. Várias proteínas do parasita expressas durante o ciclo replicativo no hospedeiro vertebrado, foram descritas nos últimos anos e estão sendo consideradas como potenciais candidatos à vacina anti-malária; no entanto ainda existem algumas proteínas que não foram identificadas como potenciais alvos de diagnostico, terapêuticos e/ou candidatos a vacina. Este estudo retrospetivo descreve a identificação e caracterização de antigénios imunogénicos de *P. falciparum* como possíveis candidatos para o desenvolvimento de novos testes serológicos ou candidatos a vacina para malaria. Os métodos: Um extrato total de *P. falciparum* (PfET) do clone 3D7, foi selecionado como o nosso alvo de estudo. A antigenicidade do PfET foi avaliada usando soro humano de indivíduos anteriormente expostos à malária. Foram analisadas 436 amostras de soro, das quais 419 soros de indivíduos, com história clínica de malária, oriundos de alguns países de Africa, América e Asia, previamente diagnosticados na Unidade de Clínica Tropical do IHMT no 2012; e 17 soros de indivíduos sem história clínica de malária foram usados como controle negativo. Foram determinados anticorpos Totais, IgM, IgG, IgG1, IgG2, IgG3, IgG4 anti-*P. falciparum* por ELISA. Foi feita a caracterização imunoquímica de antigénios de *P. falciparum* responsáveis por tais reatividades serológicas por *immunoblotting*. Foi efetuada uma análise *in silico* de frações proteicas de *P. falciparum* previamente identificadas por espectrometria de massa. Resultados: Anticorpos anti-*P. falciparum* reconheceram os antigénios do PfET, com maior contribuição de anticorpos tipo IgG (72%), IgG1 (43%) e IgG3 (64%). O *immunoblotting* revelou frações proteicas de *P. falciparum* com massa molecular entre 20 -190 kDa, das quais bandas com massa molecular de 25, 35, 40, 45, 75, 80, 100 e 150 kDa apresentaram maior reatividade antigénica. A análise *in silico* identificou 29 epitopes correspondentes a seis proteínas de *P. falciparum* previamente identificadas: *Elongation factor 1 alpha; Protein disulphide isomerase (PDI), Phosphoglycerate kinase; Glucose regulated protein homologue; Rhoptry associated protein 2 (RAP-2); Rhoptry-associated protein 3 (RAP-3*); a análise *in silico* sugere que *RAP-2, RAP-3, PDI*, apresentam características imunogénicas essenciais como epitopes consensos, signal peptidica, domínio transmembrana. Conclusão: Este estudo mostra a caracterização de antigénios do PfET, tendo em conta que algumas das suas proteínas apresentaram várias características imunogénicas importantes, como aqui se mostra, estas proteínas podem ser consideradas como um bom candidato à vacina. São assim recomendados estudos complementares destinados a avaliar a sua capacidade de imunogenicidade e indução de proteção contra a malaria. Futuramente espera-se potencializar a ação destas proteínas e contribuir na caracterização de potenciais novos alvos antigénicos de *P. falciparum* importantes para o diagnóstico sorológico ou candidatos a vacina para malaria.

Palavras-chave: Malaria, *Plasmodium falciparum*, Serologia, ELISA, Diagnóstico, Reatividade antigénica, Predição de epitopes.

Abstract

Plasmodium falciparum malaria remains a major health problem in tropical and subtropical regions worldwide. Several parasite proteins expressed during the replicative cycle in the vertebrate host have been described in recent years, are being considered as potential candidates for anti-malaria vaccine; there are still some proteins that have not been identified as potential diagnostic, therapeutic and/or vaccine candidates. This retrospective study describes the identification and characterization of immunogenic *P. falciparum* antigens as possible candidates for the development of new serological tests or candidates for the malaria vaccine. Methods: A of *P. falciparum* total extract 3D7 (PfTE) was selected as our study target. The antigenicity of PfTE was evaluated using human sera from individuals previously exposed to malaria. We analyzed 436 serum samples, of which 419 sera from individuals with a clinical history of malaria, from some countries of Africa, America and Asia, previously diagnosed at the Tropical Clinic Unit of the IHMT in 2012; and 17 sera from healthy Portuguese individuals, who had never been in malaria-endemic area, were used as negative control. Total antibodies, IgM, IgG, IgG1, IgG2, IgG3, IgG4 anti-*P. falciparum* were determined by ELISA. Immunochemical characterization of *P. falciparum* antigens responsible for such serological reactivities was performed by *immunoblotting*. An *in silico* analysis of protein fractions of *P. falciparum* previously identified by mass spectrometry was performed. Results: Anti-*P. falciparum* antibodies recognised antigens of PfET, with a higher contribution of IgG (72%), IgG1 (43%) and IgG3 (64%) antibodies. Immunoblotting revealed protein fractions of *P. falciparum* with molecular mass between 20 -190 kDa, of which bands with molecular mass of 25, 35, 40, 45, 75, 80, 100 and 150 kDa showed higher antigenic reactivity. *In silico* analysis identified 29 epitopes corresponding to six *P. falciparum* proteins previously identified: Elongation factor 1 alpha; Protein disulphide isomerase (PDI), Phosphoglycerate kinase; Glucose regulated protein homologue; Rhoptry associated protein 2 (RAP-2); Rhoptry-associated protein 3 (RAP-3); *in silico* analysis suggests that RAP-2, RAP-3, PDI, present essential immunogenic characteristics as consensus epitopes, signal peptide, transmembrane domain. Conclusion: This study shows the characterization of PfTE antigens, taking into account that some of its proteins presented several important immunogenic characteristics, as shown here, it could be considered as a good vaccine candidate. Further studies aimed at assessing its immunogenicity and protection-inducing ability against malaria are thus recommended. It is expected to potentiate the action of these proteins and contribute to the characterization of potential new antigenic targets of *P. falciparum* important for the serological diagnosis or vaccine candidates for malaria.

Keywords: Malaria, *Plasmodium falciparum*, Serology, ELISA, Diagnosis, Antigenic reactivity, Epitopes prediction.

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List of Symbols, Acronyms and Abbreviations

Abs	Absorbance
ACT	Artemisinin-based combination therapy
ADCC	Cytotoxicity processes mediated by antibody-dependent cells
AMA1	Apical membrane antigen 1
APCs	Antigen presenting cells
AUC	Area Under Curve
BepiPred	Sequential B-Cell Epitope Predictor
BcePred	Prediction of linear B-cell epitopes, using physicochemical properties
BCPred	B-cell epitope prediction server
cDC	Conventional Dentritic Cell
CI	Confidence Intervals
CSP	Circumsporozoite Protein
DAB	(3,3'-Diaminobenzidine)
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EBA-175	Erythrocyte 19 binding antigen-175
EDTA	Etilenodiaminotetra acetic acid
EF- 1α	Elongation factor- 1 alpha
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FCM	Flow Cytometry
GCS	Glasgow Coma Scale

GRP- 78	glucose regulated protein homologue
HPLC	High-Performance Liquid Chromatography
HRP II	Histidine-Rich Protein II
IFAT	Indirect immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IHMT - UNL	Institute of Hygiene and Tropical Medicine – New University of Lisbon
IL	Interleukin
IPT	Intermittent preventive treatment during pregnancy
iRCBs	Infected Red Blood Cells
J	Youden index
LAMP	Loop-mediated isothermal amplification
LDH	Dehydrogenase lactic
LSA- 1, 3	Liver stage antigen -1, 3
LT	T Lymphocyte
MHC I, II	Major Histocompatibility System class I and class II
MPL	Monophosphorylate Lipid A
MSP1	Merozoite Surface Protein 1
N	Total individuals
n	Number
NC	Negative Control
NK cells	Natural killer cells
NO	Nitric Oxide
OD	Optical density
OPD	O-Phenylenediamine dihydrochloride
PAMPs	Pathogen-associated molecular pattern
PALOP	Paises Africanos de Lingua Oficial Portuguessa- Portuguese-speaking African countries

PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pDCs	Plasmacytoid Dendritic Cells
Pf	*Plasmodium falciparum*
PfALD	*Plasmodium falciparum aldolase*
PfHGPRT	*Plasmodium falciparum* Hypoxanthine-guanine phosphoribosyl transferase
PfRH5	*P. falciparum* Reticulocyte Binding Protein Homologue 5
Pf PP	*P. falciparum* parasite prevalence
PFRIPR	*P. falciparum* Cysteine-rich protective antigen
PfTE	*P. falciparum* total extract
PGK	Phosphoglycerate kinase
PGluDH	*Plasmodium Glutamate Dehydrogense*
Plasmodium DsDNA	Doubles stranded DNA *Plasmodium*
P. chabaudi	*Plasmodium chabaudi*
P. falciparum	*Plasmodium falciparum*
P. knowlesi	*Plasmodium knowlesi*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
PLDH	*Plasmodium* Lactate dehydrogenase quantum dots
POCT	Point-of-care test
PRBCs	Parasite-infected red blood cell
QDs	Quillaja Saponaria Molina
RAP-2	Rhoptry associated protein 2
RAP-3	Rhoptry-associated protein 3
RBCs	Red Blood Cells
RDTs	Rapid Diagnostic Tests

RESA 1	Reticulocyte-binding-like homologue 1
Rh1	RNA helicase 1
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RT	Room Temperature
RTS, S/AS01	recombinant protein-based malaria vaccine
SALSA	Sporozoite And Liver-Stage Antigen
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium-Dodecil Sulfate- Polyacrylamide Gel Electrophoresis
Se	Sensibility
SE	Standard Error
Signal P4	Server predicts the presence and location of signal peptide cleavage sites
Sp	Specificity
SP	Sulfadoxine-pyrimethamine
STARP	*Sporozoite threonine and asparagine-rich protein* statistical significance
TEMED	Tetramethylethylenediamine
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TLRs	Toll-Like Receptors
TNF	Tumour Necrosis Factor
TRAP	Thrombospondin-related adhesive protein or thrombospondin-related anonymous protein
Tris	Tetrametiletilene diamine
UniProt	Universal Protein Resource

WB	Western blotting reaction

1. Introduction

Malaria is a disease caused by parasites of the *Plasmodium* genus. Five primate malaria parasites are regularly transmitted to humans, including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi* (White *et al.*, 2014). *P. falciparum* is the most virulent, being responsible for the majority morbidity from disease. The understanding of the mechanisms that mediate both the innate and adaptive immune response against this disease is crucial for developing effective prophylactic tolls able to prevent malaria, hence avoiding the morbidity and mortality caused by this disease (Frevert *et al.*, 2014).

Protection against malaria is a world challenge and a research priority. It is crucial to investigate the molecular and immune interactions that this parasite establishes with the mammal host (Fretes, Kemmerling and Sarr, 2012). More than 5.000 proteins are expressed during *Plasmodium* sp life cycle, of which only a small fraction is characterised (Gardner *et al.*, 2002; Birkholtz *et al.*, 2008). However, approximately 2500 *Plasmodium* sp. proteins have been identified, among them, 124 were found in four stages in the life cycle of the malaria parasite (sporozoite, merozoite, trophozoite, and gametocytes) (Florens *et al.*, 2004).

Proteome studies of *Plasmodium* sp. are therefore extremely important for the identification and characterisation of biomarkers that allow developing better diagnosis, able to detect the disease as early as possible, allowing infection monitoring. Trustworthy early diagnosis tools are essential for malaria control and eradication.

1.1. Epidemiology of malaria

Malaria is endemic in tropical and sub-tropical areas, including Africa, Asia, and South America. The incidence rate of malaria declined globally between 2010 and 2018, from 71 to 57 cases per 1000 population at risk. Figure 1 shows the geographical distribution of indigenous malaria cases. In 2018, 228 million malaria cases and 405.000 of malaria deaths worldwide. The African region accounted for 94% of all deaths, caused mainly by *P. falciparum* (WHO, 2019).

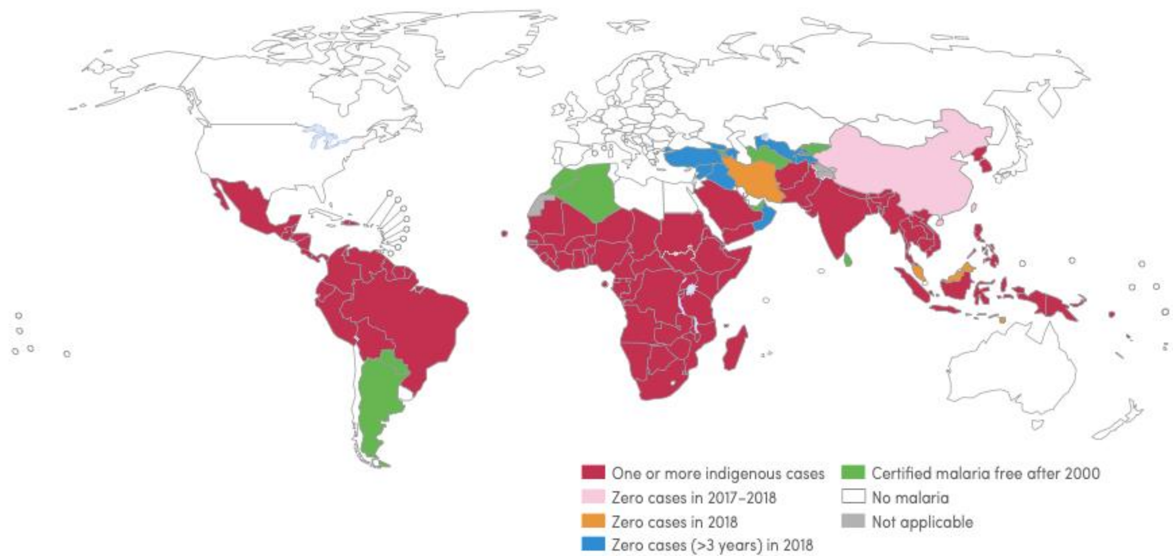

Figure 1. Countries that reported indigenous malaria cases in 2000 and their status by 2018. Adapted from WHO-Malaria Report 2019.

Between 2014 and 2016, the incidence of malaria cases increased substantially in the Americas, and marginally in the South-western, Western Pacific, and Africa areas (WHO, 2018b). Even so, in 2018, most malaria cases were recorded in the African Region (93% or 213 million), followed by the South-East Asia Region (3.4%) and the Eastern Mediterranean Region (2.1%) (WHO, 2019). *P. falciparum* is the most prevalent malaria parasite in the WHO African Region, accounting for 99.7% of estimated malaria cases in 2018, as well as in the WHO South-East Asia Region (50%), the WHO Eastern Mediterranean Region (71%) and the WHO Western Pacific Region (65%) (WHO, 2019). Figure 2 shows the geographical distribution of *P. falciparum* parasite prevalence in African countries.

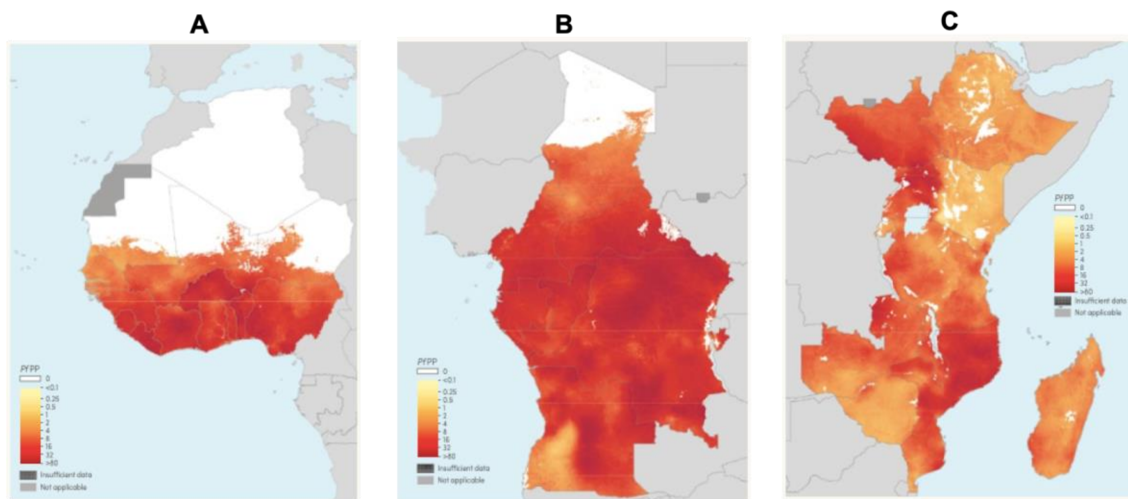

Figure 2. ***Plasmodium falciparum* parasite prevalence in the African continent**. **(A)** *P. falciparum* parasite prevalence (Pf PP), 2018 in West Africa. **(B)** *P. falciparum* parasite prevalence (Pf PP), 2018 in Central Africa. **(C)** *P. falciparum* parasite prevalence (Pf PP), 2018; countries with high transmission in East and Southern Africa. Adapted from WHO-Malaria Report 2019.

1.2. *Plasmodium* sp. transmission

Plasmodium sp. transmission occurs in areas characterized by geographic diversity, climatic variation, and diverse environmental factors. The relationship established between the parasite and its host is an important issue to be addressed in malaria research. Approximately 200 species of *Plasmodium* sp. have already been identified from different hosts, including mammals, birds, and reptiles (Martinsen and Perkins, 2013). *P. falciparum* and *P. vivax* represent the most significant threat in public health to the human population, and these species differ in morphology, virulence, geographical distribution, disease patterns, susceptibility mechanisms and drug resistance (Murray, Rosenthal and Pffaler, 2009).

Malaria parasites have complex life cycles involving female mosquitoes of the Anopheles genus, the primary host where parasites reproduce sexually, and in the intermediate mammalian host (King, 1883; Manson, 1894). There are about 400 species of *Anopheles*

mosquitoes, of these, 60 are recognised as malaria vectors under natural conditions, and 30 usually present higher infection rates (Meigen, 1818; Meigen, 2011).

The parasite is transmitted mainly through the bite of a *Plasmodium*-infected female mosquito. However, malaria also can be transmitted through other routes, such as, direct inoculation of infected blood of asymptomatic donors with low parasitemia, organ transplants, or by sharing needles infected with *Plasmodium* sp. (Bolad *et al.*, 2005; Kitchen and Chiodini, 2006; Falade *et al.*, 2007; Miura *et al.*, 2008; Mali, Tan and Arguin, 2011; Faddy *et al.*, 2013). Congenital malaria can also occur when infected pregnant women transmit the parasite to their children during pregnancy or childbirth (Menendez, 1995; Mali, Tan and Arguin, 2011; Fretes, Kemmerling and Sarr, 2012).

Malaria cases by organ donation have been reported. A study revealed that a patient transplanted that received liver and kidney developed a febrile disease with the appearance of *P. vivax*, without clinical signs of active malaria infection, the donor emigrated from Cameroon to Germany 18 months before the transplant (Fischer *et al.*, 1999).
Malaria transmission through blood transfusion from asymptomatic individuals is a problem in Africa because *Plasmodium* sp. research in blood donors is not performed (Owusu - Ofori, Parry and Bates, 2010; Askling *et al.*, 2012). WHO recommends that the entire blood donation process (procurement, laboratory assessment, blood component processing, storage, and distribution) be governed by national policies that ensure the quality and blood safety (WHO, 2014).

1.3. *Plasmodium* sp. life cycle

The malaria parasite life cycle involves two hosts: an invertebrate (female *Anopheles* mosquito) and a vertebrate (human). It is a very complex life cycle involving a sequence of different phases in both the mosquito and the human being. In figure 3 is schematized *Plasmodium* sp. life cycle.

Human *Plasmodium* infection begins when the malaria vector, a female *Anopheles* mosquito, inoculates sporozoites from salivary gland parasites into the humans during a blood meal. Once in the host, sporozoite form first invade the liver where it mature, and replicates asexually producing thousands of merozoites, that proceed to infect mature human erythrocytes. At this stage, the infection is not clinically evident. Inside of the erythrocytes, the parasite grows and multiplies cyclically. In each cycle this growth, the parasite infects the erythrocyte as a merozoite. After rupture, up to 30,000 merozoites are released in the bloodstream (Crompton, Pierce and Miller, 2010; White *et al.*, 2014). Merozoites invade the red blood cells (RBCs), mature and develop the ring forms (trophozoites) and replicate as a schizonts. Infective merozoites are formed by mitosis in the schizont stage (each of which contains 16-20 merozoites) in a period of 24 - 72 hours, depending on parasite species: *P. malariae* (72 hours); *P. falciparum* (48 hours); , *P. vivax* (48 hours); , *P. ovale* (48 hours); and *P. knowlesi* (24 hours) (White *et al.*, 2014).

Eventually, the schizonts are fragmented to release more merozoites into the bloodstream that invade more RBCs, starting the cycle again. Approximately 16 to 32 new merozoites of a schizont are released during the subsequent rupture, providing an increase in parasitaemia density. Consequently, this procedure causes a rapid loss of RBCs and directs to a spectrum of mild to severe symptoms (Bledsoe, 2005). Merozoites remain to be discharged into the bloodstream, infecting new RBCs (Cook and Zumla, 2009). After several erythrocytes' asexual replication cycles, the parasite differentiates into gametocytes. These are the infectious forms for the mosquito vector and, therefore, are the forms that enable parasite perpetuation (Figure 3). Gametocytogonia starts as soon as the parasite is installed in the erythrocyte and takes 7 to 10 days in *P. falciparum* and about 4 hours in *P. vivax*. After being ingested by the mosquito vector, gametocytes initiate the replicative sexual cycle in the mosquito (White *et al.*, 2009).
All species *Plasmodium* sp. invade according to the mechanism described below, but *P. falciparum* reaches higher levels of parasitemia, due to greater flexibility in the receptors pathways that this parasite can use to invade red blood cells (Miller *et al.*, 2002; Weatherall *et al.*, 2002).

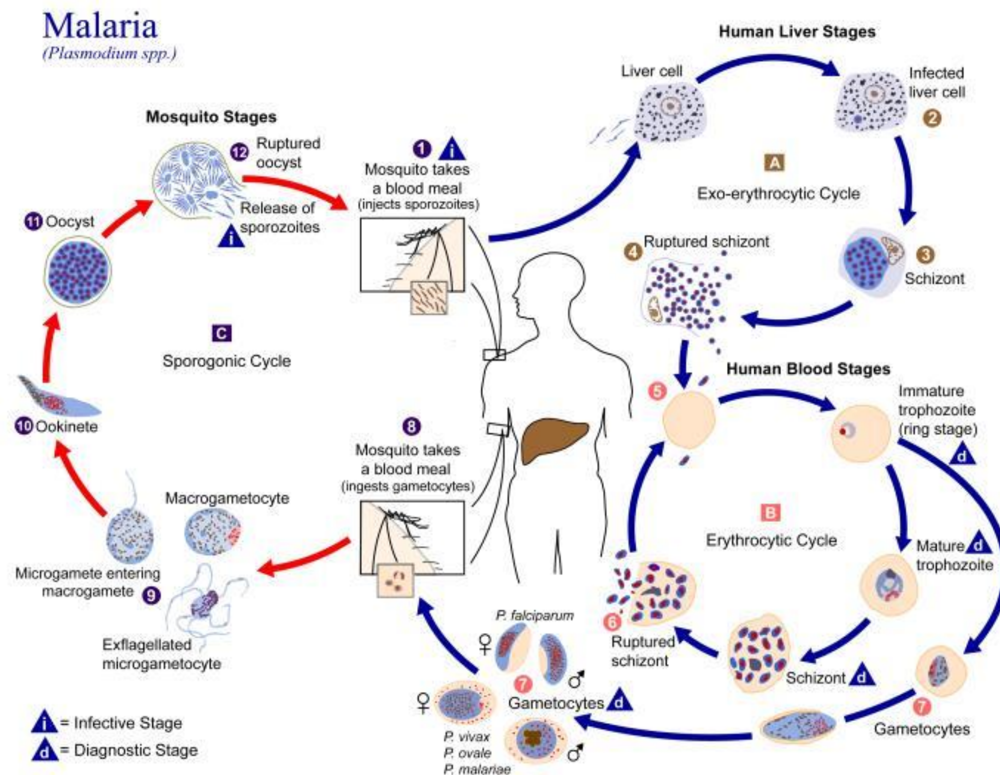

Figure 3: Schematic representation of *Plasmodium* sp. life cycle. *Plasmodium* parasite life cycle represents (A) Exo-erythrocytic cycle, (B) Erythrocytic cycle and (C) Sporogonic cycle. During the bite (blood meal), the malaria-infected female *Anopheles* mosquito inoculates the sporozoites in the human host (1), sporozoites migrate to hepatocytes, where multiple rounds of cell division occur (2-3). Many thousands of merozoites then released (4), merozoites invade erythrocytes (5), within erythrocytes, merozoites develop into immature trophozoites, then mature into schizonts (6), which rupture releasing merozoites, some parasite develop into male or female gametocytes (7), are ingested by the mosquito during a blood meal (8). In the mosquito's stomach, these sexual forms of the parasite develop into zygotes (9). The zygotes in turn ookinetes (10) and then develop into an oocyst (11). The oocysts grow, rupture, and release sporozoite (12). The sporozoites travel to the insect salivary glands and are re-introduced into a human host during the mosquito's next blood meal. Diagram from Centers for Disease Control and Prevention at (https://www.cdc.gov/malaria/about/biology/life_cycle.html).

1.4. Immune response during *Plasmodium* sp. infection

Fully understanding the immune mechanisms activated by the vertebrate host during *Plasmodium* sp. infection represents the main challenge and priority for human malaria research studies. *Plasmodium* sp. life cycle includes two phases and several parasites stages, which complicates their study (Tuteja, 2007). Immune response analysis has been difficult due to the presence of different parasites forms, a large number of malaria antigens, and the antigenic variation that takes place during the genetic recombination in the sexual phase of *Plasmodium* sp. life cycle (Toro and Castaño, 1992; Yazdanbakhsh and Sacks, 2010; He *et al.*, 2018).

Malaria immunity seems to require cellular and humoral mechanisms, probably in co-operation, although its importance remains unclear (Drew *et al*., 2016; Beeson *et al*., 2008). Figure 4 shows a schematic representation of some immunological mechanisms unchained during the *Plasmodium* infection.

The host immune system is crucial in determining the outcome of infection, especially among adults in areas of moderate or intense malaria transmission. Protective immunity against *Plasmodium* sp. can develop after prolonged parasite exposure (WHO, 2015). *P. falciparum* is responsible for most malaria-related deaths global in children under five years (Doolan, Dobaño and Baird, 2009).

The interaction between humoral and cellular immune responses produces protective immunity against malaria infection (Beeson, Osier and Engwerda, 2008). Naturally acquired immunity in people living in malaria-endemic regions is mostly dependent on the attainment of a protective repertoire of specific antibodies directed against the blood-stage antigens. These antibodies are related to the control of parasitemia levels by directly inhibiting invasion or merozoite opsonization (Kalra, Mukherjee and Chauhan, 2016). The immune response induced by malaria protects against both parasitic phases: the pre-erythrocytic and the erythrocyte stage.

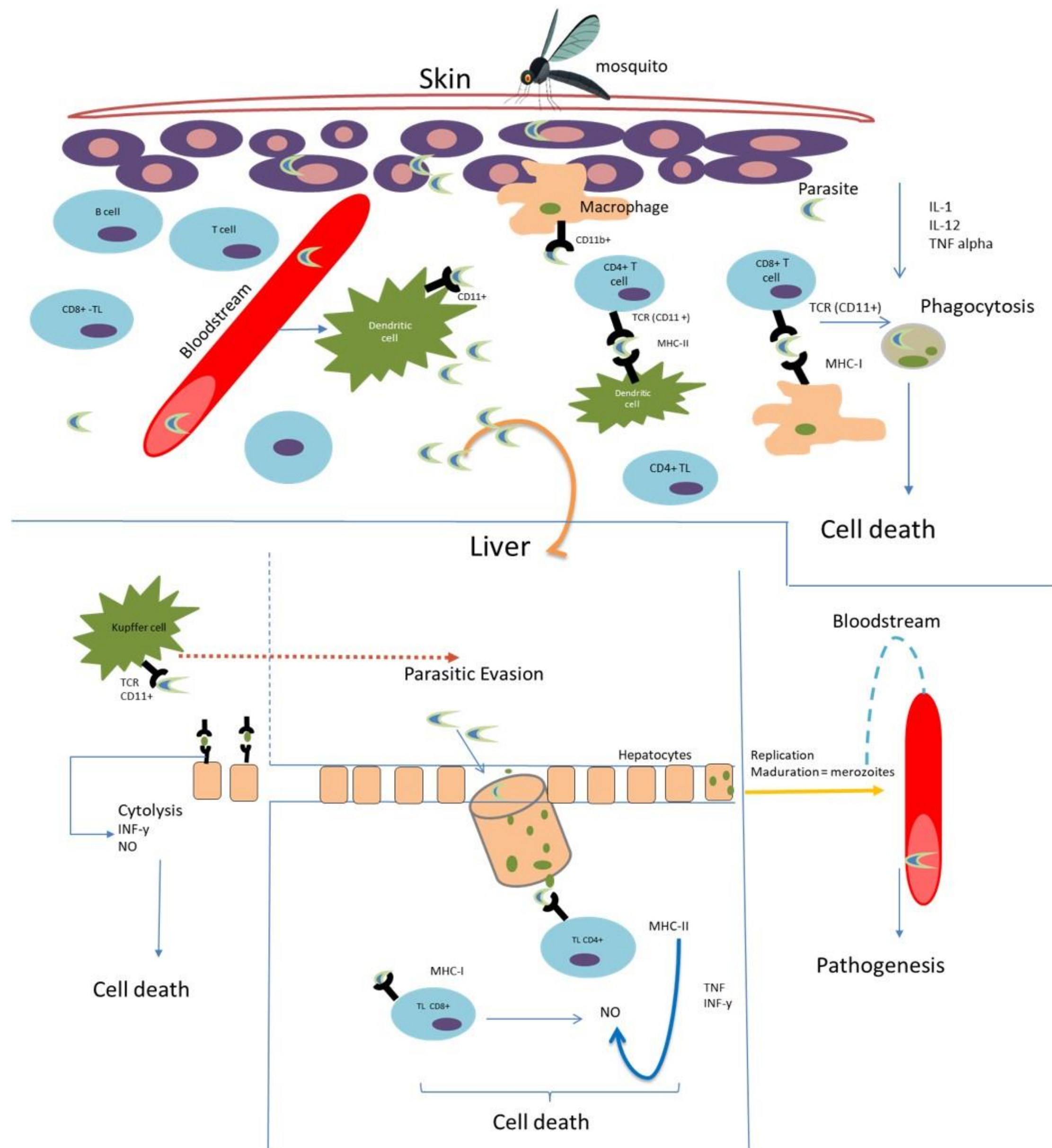

Figure 4. Representative scheme of adaptative and cellular immune responses against *Plasmodium* infection. *Plasmodium* antigens and parasite-infected red blood cell (PRBCs) are phagocytosed by active dendritic cells, and their antigens are presented to T cells. After, the secretion of cytokines is initiated, and promoting the inflammation that underlies malaria pathogenesis. The B cells are differentiated, antibodies are secreted, also is secreted interferon gamma (IFN-γ), which actives macrophages. The macrophages are alerted and phagocytose the parasites and PRBCs opsonized, after kill them by nitric oxide (NO) pathways. The inflammation is limited by the secretion of anti-inflammatory cytokines from T cell receptor (TCR), macrophages. Adapted from (Cowman and Crabb, 2006; Riley and Stewart, 2013).

1.4.1. Innate immune response

The innate immune response manages to decrease the infection, beginning by restricting the maximum parasite density. The innate immunity in malaria comprises the participation of the major histocompatibility complex (MHC), macrophages, monocytes, dendritic cells (DCs), natural killer (NK) cells, and natural killer cytotoxic (NKT). The MHC molecules cause direct parasitic lysis, macrophages induces phagocytose, and the NK and NKT lymphocytes induce the lysis of the parasitized cells (Engwerda and Good, 2005).

Dendritic cells

Dendritic cells (DCs) play a central role in the initiation of adaptive immune responses, efficiently shown to have the capacity to present malaria antigens to T cells (Amorim *et al.*, 2016). The DCs recognise relatively conserved molecular patterns among multiple pathogenic organisms through a range of Pathogens Associated Molecular Patterns (PAMPs) receptors. Among the most representative are the Toll-Like Receptors (TLRs). Upon recognition of the PAMPs, the TLRs induce a signal in cascade of proinflammatory cytokines, chemokines and interferons, inducing the DCs maturation (Plebanski and Hill, 2000; Marsh and Kinyanjui, 2006; Okwa, 2012).
The DCs are specialized in capturing, processing, and presenting *Plasmodium* antigens to T cells (Wykes, 2012). The DCs are divided into two different subtypes based on their biological functions: plasmacytoid DCs (pDCs) and conventional DCs (cDCs); the latter are cells responsible for presenting antigens to effective T cells. In contrast, pDCs are considered cells of immune modulation through the secretion of large amounts of IFN- α (Wykes, 2012).

Macrophages and Natural Killer cells

The macrophages and NK cells are responsible for the initial cytokine's secretion. The NK cells, through cytokines secretion and not due to their cytolytic activity, have important participation serving as a bridge between the mechanisms of innate resistance and adaptive immunity during the erythrocytic infection stage (Kitaguchi *et al.*, 1996; Mohan, Moulin and Stevenson, 1997).

Macrophages act in the secretion of cytokines (IL-12, IL-1 and TNF-α) and the production of reactive metabolites of oxygen and nitric oxide. Although macrophages cannot avoid the increase in parasitemia through the initial phase of the infection, during the crisis phase they have fundamental participation in the elimination of malaria-parasitized erythrocytes (Stevenson *et al.*, 1989). Natural killer cells (NK) have been implicated as the source of early proinflammatory responses such as IFN-γ and TNF-α against malaria parasites (Artavanis-Tsakonas and Riley 2003)

Cytokines and malaria

Several studies in mouse and humans have shown that erythrocytic infections are characterized by a response of pro-inflammatory cytokines that involve IL-1β, IL-6, IFN-γ, TNF-α and IL-12 (Angulo and Fresno, 2002; Mary M Stevenson *et al.*, 2011); these pro-inflammatory have been found to be elevated in sera or plasma of *P. falciparum*-infected patients and TNF as well as IL-1α and IL-1β were described to be associated with cerebral malaria severity, increase the phagocytosis and destruction of infected RBCs (iRBCs) through macrophages (Osier *et al.*, 2014). Nevertheless, parasite antigens are highly polymorphic and undergo a large clonal antigenic variation, which means that effective opsonization, preventing iRBCs accumulation in blood vessels (Winter *et al.*, 2005).

Recent studies about the interaction of *P. falciparum* with *Homo sapiens sapiens* shows that pro-inflammatory cytokines (TNF-α, IL-1, IL 6, IL-12 and IFN-γ) help control parasitemia (Janeway, York and Science, 2001). Efforts have been made to increase the knowledge of this interaction in an attempt to elucidate the involvement of the innate immune system, especially of DCs and TLRs in pathophysiology and immunity (Perez *et al.*, 2015).

Experimental data show the role of hemozoin as a ligand of TRL-9, which favours the subsequent secretion of IL-6, IL-12 and TNF-α (Perlmann and Troye-Blomberg, 2002; Perez-Mazliah and Langhorne, 2015). Malaria parasite glycophosphoinostol (GPI) has been shown to interact with TLR2 and to some extent TLR4 (Mary M Stevenson *et al.*, 2011), favouring the cytokines production (Spence and Langhorne, 2012) playing a role in the genesis of symptoms - fever, chills, headache, asthenia and shock - as well as hypoglycemia and the expression of adhesion molecules.

Interferon

In both described phases, there is a subset of IFN-γ producing T cells, that are present during the early phase of malaria infection and could, therefore, contribute to the innate response. Likewise, it has been shown to inhibit the replication of the parasite *in vitro* in cells from donors that have never been exposed to malaria (Perlmann and Troye-Blomberg, 2002).

There is evidence that IFN-γ play a protective role during cerebral malaria, and IFN-γ response has been shown to be important in protecting against development of severe disease symptoms (Winkler *et al.*, 1999). For individuals exposed to malaria, Dodoo and colleagues reported an association ammong IFN-γ production and a reduced risk of fever and clinical malaria (Dodoo *et al.*, 2002). In the pre-erythrocyte stage, IFN-γ also has an inhibitory effect *in vitro* on the development of hepatic forms of the parasite (Schofield *et al.*, 1987).

Interleukins

Interleukin-10 (IL-10) shown a protective role in *P. falciparum* infection; IL-10 production in response to Liver Stage Antigen 1 (LSA-1) predicts resistance to *P. falciparum* after eradication therapy in human (Kurtis *et al.*, 1999). Studies in children indicate that high concentrations of TNF and IL-10 are associated to cases of anemia in severe malaria with high parasitemia levels (Shaffer *et al.*, 1991; Othoro *et al.*, 1999). IL-10 promotes the inhibition of TNF production (Ho *et al.*, 1998), which may suggest that a low IL-10 response allows high TNF levels, promoting anemia in malaria cases.

In malaria caused by *P. falciparum*, high levels of IL-4 have been detected in children with severe malaria, when compared with acute malaria cases (Cabantous *et al.*, 2009). Troye-Blomberg and colleagues demonstrated that IL-4 has been shown to be crucial in the induction of *P. falciparum*-specific antibody production during blood-stage malaria (Troye-Blomberg *et al.*, 1990); Weid and colleagues (1994) showed that IL-4-deficient murine mice are able to control and eliminate the erythrocyte phase of *P. chabaudi* infection with similar efficiency to controls, suggesting that IL-4 is not required for elimination of the erythrocyte phases (von der Weid *et al.*, 1994). High levels of IL-6 were detected in subjects with severe malaria (Kern *et al.*, 1989), and high levels of this interleukin were associated with fatal outcomes (Day *et al.*, 1999). The production of IL-12 appears to be associated with a reduced of parasitemia in malaria (Dodoo *et al.*, 2002).

1.4.2. Adaptive immune response

The adaptive immunity greatly influences how malaria affects an individual and, consequently, the community. After repeated malaria infections, an individual may develop partially protective immunity, these *semi-immune* individual can be infected by malaria parasites but not develop severe disease, and often do not exhibit any typical symptoms of the disease (Mary M. Stevenson *et al.*, 2011). The adaptive immune response against malaria depends of the activation of T lymphocyte (Kalra, Mukherjee and Chauhan, 2016). The T cells stimulate cytokines production, such as interleukin IL-4, IL-5, and IL-6, that favour the differentiation of B lymphocytes in plasmatic cells and consequently the production of specific antibodies (Vazquez, Catalan-Dibene and Zlotnik, 2015). On the other hand, T lymphocytes also release pro-inflammatory cytokines, such as IFN-γ and TNF-α important to activate macrophages, to synthesize reactive metabolites of oxygen and nitrogen, which are involved in the parasite destruction (Legorreta-herrera and Sánchez-cruz, 1998).

The adaptive immune response arises approximately 24 hours after infection and is activated by the innate immune response for the complete parasite elimination (Janeway, York and Science, 2001). Therefore, adaptive immune mechanisms are made by T cells, such as $CD8^+$ T, $CD4^+$ T "helper", T regulatory (Tregs), memory T cells, and memory B cells; during these processes, the innate immune cells response will also be present since

they are necessary to activate and differentiate T and B cells, which could be defined as positive feedback; T cells activation leads to macrophages activation, phagocytosis of infected red blood cells, NK cells activation, cytokine production, and pro-inflammatory molecules production, such as nitric oxide (NO) and oxygen radicals (Wykes and Good, 2008).

In the exo-erythrocytic phase, $CD8^+$ T cells actively participate due to their cytotoxic capacity, since they prevent parasite development in the liver. These can kill parasites in the hepatocyte by lysis mediated for perforins and granzymes, induce apoptosis, and secrete cytokines when they recognise antigens transfer of MHC-I. The $CD4^+$ T cell is activated by antigens that exhibit molecules of the major histocompatibility complex class II (MHC); however, $CD8^+$ T cell is activated by antigens that exhibit molecules of the MHC- I. The $CD4^+$ T cells induct immunity in the erythrocytic phase, whereas $CD8^+$ T cells have been shown to have cytolytic action against hepatic parasite forms (Troye-Blomberg, Berzins and Perlmann, 1994). Feel and colleagues *et al*. (1994) demonstrated that $CD4^+$ and $CD8^+$ T cells, from patients not previously exposed to malaria, might inhibit parasite growth *in vitro* (H. Fell, Currier and F. Good, 1994).

The $CD8^+$ T cells, eliminate infected cells through various cytolytic mechanisms, in addition to lymphokine release (Trimnell *et al.*, 2009; Cockburn *et al.*, 2013).
The protective immunity conferred by $CD8^+$ T cells against the erythrocyte phase is controversial, as the infected erythrocytes do not exhibit MHC molecules. However, Imai and colleagues showed that the *P. yoelii* could infect erythroblasts that express MHC-I and are thus recognised by $CD8^+$ T cells. Subsequently, the same investigators suggest that the cytotoxic activity of these cells involves the collaboration of phagocytes (Imai *et al.*, 2015).
The difficulties that immunologists frequently face when studying the host's responses to *P. falciparum* lead to the use of experimental models - mainly murine; thus, some murine studies with *P. berghei* and *P. yoelii* have demonstrated that the adoptive transfer of $CD8^+$ T cells specific for the epitopes located in CSP has protected murine models non-immune against infections by these parasites (Romero *et al.*, 1989; Rodrigues *et al.*,

1991). Weiss *et al*. (Weiss *et al.*, 1988) demonstrated in murine that $CD8^+$ T cells are the primary mediators in the protection after immunization with attenuated sporozoites.

The adoptive transfer of specific $CD8^+$ T cell clones to epitopes expressed on sporozoites and hepatic forms, strongly inhibit the intracellular development of the parasite (Khusmith, Sedegah and Hoffman, 1994). Other studies indicate that immunization with vaccines expressing antigens from this parasite induces a $CD8^+$ T cell response capable of eliminating intrahepatic development (Li *et al.*, 1993; Schneider *et al.*, 1998). Furthermore, studies with chimpanzees infected with *P. falciparum* have been used to identify the antigenic targets of $CD8^+$ T cell immunity, such as LSA-3 (BenMohamed, Thomas and Druilhe, 2004).

Although the vast majority of studies on $CD8^+$ T cells are performed in non-human models, some knowledge has been reached in order to understand the role of these cells in malaria infection in humans. However, there is evidence that lymphocytes play a role in the pathogenesis of malaria, in relation to the development of severe *P. falciparum* malaria, the most critical complication and the leading cause of death in humans (Belnoue *et al.*, 2002; Nitcheu *et al.*, 2003). Thus, human studies on the response of $CD8^+$ T cells to the erythrocyte phase are scarce due to ethical issues, with most of the knowledge based on murine models.

Nevertheless, Aidoo and colleagues showed that the $CD8^+$ T cells of individuals l from malaria-endemic areas, recognise epitopes of *P. falciparum* and *P. vivax* pre-erythrocyte; these antigens include CSP, TRAP, LSA-1 and LSA-3 (Aidoo *et al.*, 1995). Some clinical trials with vaccines have shown to induce good $CD8^+$ T cell responses against pre-erythrocyte antigen in humans (Webster *et al.*, 2005; Ewer *et al.*, 2013).

1.4.3. Role of Immunoglobulins in *Plasmodium* sp. infection

Antibodies are believed to play a crucial role in naturally acquired immunity against malaria. Studies have shown that continuous and frequent exposure to *P. falciparum* favour the development of specific humoral immune response against multiple antigens, lowering the risk of disease. The immunogenic complexity of *P. falciparum* proteins

makes challenging the identification of biomarkers associated with disease protection. There are mechanisms of protection provided by high immune responses after exposure to parasite antigens that have not been studied in detail.

Due to the increasing evidence of the protective role of anti-*Plasmodium* antibodies (IgM, IgD, IgG, IgE, and IgA) and subclasses (IgG1, IgG2, IgG3, and IgG4) your role has been studied. It is expected that some of the IgG subclasses, produced against the *Plasmodium*-specific antigens, have a striking influence on the effectiveness of the immune response.

The malaria immunity is complex and specific for each parasite's stage, the development of protective immunity can be slow and only is evidenced after the individual has been exposed, over the years, to repeated infections (Bull and Marsh, 2002). The importance of antibodies in malaria was demonstrated by Cohen and colleagues when passively transferred purified IgG antibodies from immune individuals to children with acute malaria, showing a rapid decrease of parasite density in peripheral blood, accompanied by a fever resolution (Cohen, McGregor and Carrington, 1961; Crompton *et al.*, 2014).

Recently, many studies have been developed to identify the parasite target protein of *Plasmodium* sp., to understand the exact mechanism by which protection is achieved, and the reason for such protection only to be acquired after repeated exposures over a long time (Crompton *et al.*, 2014). The passive transfer of anti-*P. falciparum* antibodies contributed to the reduction of parasite density and protection against infection (Grant *et al.*, 2015; Dobbs and Dent, 2016).

During parasite infection, it is possible to detect antibodies classes and subclasses, which can be used as a useful diagnostic tool for the screening of parasite antibodies (Fotedar *et al.*, 2007). Rowe (2002) showed that IgM binds to the surface of infected erythrocytes, neutralizing interactions between infected and uninfected erythrocytes (Rowe *et al.*, 2002). Also, Clough and colleagues reported the connection of IgM to erythrocytes forming rosettes (Clough *et al.*, 1998). A study performed in a population of individuals in Mali suggested that IgM antibodies have a protective action (Maiga *et al.*, 2005). Boudin *et al.* (Boudin *et al.*, 1993) also had suggested a protective function for IgM

antibodies in limiting malaria severity (Brasseur, Ballet and Druilhe, 1990). A further study performed with *P. chabaudi* suggested that IgM prevents parasitic replication during the erythrocyte phase (Kevin N. Couper *et al.*, 2005).

IgM antibodies have an intimate relationship with *P. falciparum*, which can affect the survival of the parasite within the infected cell (Czajkowsky *et al.*, 2010). Other authors have shown that IgM antibodies acting first than IgG antibodies are effective in neutralizing intracellular parasites by inhibiting their transfer from cell to cell (Boes *et al.*, 1998; Ochsenbein *et al.*, 1999). Furthermore, IgM appears to agglutinate intracellular parasites in a very effective way by linking the iRBC to the IgM Fc (Czajkowsky *et al.*, 2010). Another role of the IgM is to limit the parasite replication and to produce the first generation of cells B (K. N. Couper *et al.*, 2005). Regardless of the low detection of IgM antibodies in relation to the values of IgG antibodies in serum, the IgM antibody also has an impact on malaria immunity (Racine and Winslow, 2009).
Cytophilic IgG1 and IgG3 antibodies are the most common subclasses (Nhabomba *et al.*, 2014), but the doubt remains on which they are initially produced or have a greater preponderance in the humoral immune response. Nevertheless, it seems to vary depending on the *Plasmodium* antigens (Medeiros *et al.*, 2013).

Kana *et al.*, (Kana *et al.*, 2018) evidenced the role of IgG in protection against malaria, specifically IgG1 and IgG3. In New Guinea, it is established that the presence of antibodies of IgG subclasses is associated with malaria protection. In a group of children infected with *Plasmodium*, it was found that subclasses of cytophilic IgG1 and IgG3 antibodies were predominant, suggesting that IgG1 and IgG3 play a significant role in the acquired malaria immunity (Weaver *et al.*, 2016).

Cytophilic antibodies are mainly produced against merozoite antigens and play an important role in opsonization and lysis of these forms, thus reducing the risk of developing malaria (Polley *et al.*, 2006; Tongren *et al.*, 2006; Nebie *et al.*, 2008). Another study has shown that non-cytophilic antibodies IgG2 and IgG4 have been associated with the susceptibility to *P. falciparum,* because they do not mediate opsonization of infected erythrocytes (Groux and Gysin, 1990). Aucan (2000) demonstrated a protective role of

IgG2 by displaying cytophilic Non-cytophilic antibodies such as IgG2, IgG4, and IgM (Aucan *et al.*, 2000). It was also described in a study carried out in Brazil in symptomatic individuals with infection by *P. falciparum* (Medeiros *et al.*, 2013).

Aribot and colleagues demonstrated that on children and adults in Senegal, antibodies IgG3 antibodies are associated with reduced risk of malaria (Aribot *et al.*, 1996). It has been shown that IgG2 and IgG4 antibodies can also block the effect of cytophilic antibodies, causing an imbalance in the humoral immune response, which can culminate in resistance or susceptibility to the disease (Groux and Gysin, 1990).
In addition, experimental trials against MSP3 have shown that IgG3 antibodies can be strongly associated with clinical protection against *P. falciparum* infection (Roussilhon *et al.*, 2007; Wang *et al.*, 2016). In malaria-endemic areas, the predominance of cytophilic antibodies is associated with low parasitemia (Shi *et al.*, 1996) and low malaria risk (Sarthou *et al.*, 1997; Polley *et al.*, 2006).

1.4.4. *Plasmodium* sp. proteome

Proteomics is the study of the proteins of an organism, in terms of its expression, location, interaction and post-transcriptional modifications, allowing a global and integrated perspective of biological (Graves and Haystead, 2002).
Proteomic studies can be performed to identify proteins, as well as to identify different protein expression profiles, post-transcriptional modifications and regulation, alternative splicing mechanisms, subcellular localization, and the interconnectivity among all these different processes (Graves and Haystead, 2002).

Thus, being an excellent tool for the study of host-parasite interactions. Identification and understanding of the expressed parasite proteins, as well as their expression levels during the life cycle may aid in the identification of new diagnostic targets, treatment drugs, or antimalarial vaccines, bearing in mind that the proteomics of an organism can be considered dynamic, since it reflects the environment in which it is studied (Graves and Haystead, 2002).

Due to the need for proposing possible diagnosis targets and vaccine candidates for malaria, many *Plasmodium* sp. proteins expressed in the invasion processes of the sporozoite and merozoite have been studied, in table 1 some these proteins are shown.

Table 1. Characteristics of some *Plasmodium* proteins.

Protein	Characteristics	References
Histidine-Rich Protein II (HRP II)	The protein is secreted by *P. falciparum* to the blood, allowing its detection by antigenic capture with specific antibodies and immunochromatography techniques. Is used as a target in the diagnosis of *P. falciparum* parasites.	(Lee *et al.*, 2006)
Lactate dehydrogenase (PLDH)	Localised inside of iRBCs. Is a homotetrameric protein with monomer of 34 kDa. Common in the four species of *Plasmodium* (*P. vivax, P. ovale, P. falciparum* and *P. malariae*).	(Moody and Chiodini, 2002; Barber *et al.*, 2013)
Aldose (PfALD *Plasmodium falciparum* aldolase)	This protein is localised inside of iRBCs. It has high sequence diversity and potential as a drug target. It plays a significant role in energy generation through the parasite's glycolytic pathway by converting fructose-1.6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.	(Barber *et al.*, 2013)
Glutamate dehydrogenase *Plasmodium* (PGluDH)	Play a role in parasite redox metabolism. It plays an essential role in parasite survival and growth by linking carbon and nitrogen metabolism.	(Mathema and Na-Bangchang, 2015)

Table 1. Characteristics of some *Plasmodium* proteins.

Protein	Characteristics	References
Hypoxanthine-guanine phosphoribosyl transferase (PfHGPRT)	It is an import enzyme involved in the purine metabolism of the parasite. Even though a similar protein is found in bacteria and humans, it can serve as a reliable target for malaria diagnosis and therapeutics development.	(Raman *et al.*, 2005)
Circumsporozoite protein (CSP)	Plays a critical role in the invasion of sporozoites at hepatocytes, as some antibodies against these inhibit the invasion of the hepatocytes and the expression on CSP. Correlates with the infectivity of the sporozoite.	(Nussenzweig and Nussenzweig, 1985; Pancake *et al.*, 1992)
Liver-stage antigen-1 (LSA-1)	The role of this parasitic protein is not yet completely determined. Still, it is suggested that LSA-1 is involved in the parasite's interaction with the erythrocytes, contributing to the efficient invasion of hepatic merozoites.	(Nicoll *et al.*, 2011)
Liver-stage antigen-3 (LSA-3)	It is expressed in sporozoites and mature hepatic merozoites.	(Brahimi *et al.*, 2001; Garcia, Puentes and Patarroyo, 2006)

Table 1. Characteristics of some *Plasmodium* proteins.

Protein	Characteristics	References
Thrombospondin-related adhesive protein (or thrombospondin-related anonymous protein) - (TRAP)	A type 1 transmembrane protein with multiple adhesive domains in its extracellular region. This protein is involved in sporozoite motility and the invasion of hepatic cells.	(Kappe *et al.*, 1999; Nicoll *et al.*, 2011)
Sporozoite threonine and asparagine-rich protein- (STARP)	Is expressed in the sporozoite membrane. This protein is involved in the invasion of the sporozoites towards hepatocytes.	(Fidock *et al.*, 1997; Bozdech *et al.*, 2003; Jongwutiwes *et al.*, 2008)
Sporozoite and liver-stage antigen (SALSA)	Located in the sporozoite membrane, is expressed in the hepatic phase. This protein may have a role in the invasion of the hepatic cells and the invasion of the merozoites towards erythrocytes.	(Bottius *et al.*, 1996; Garcia, Puentes and Patarroyo, 2006)
Merozoite surface protein 1- (MSP-1)	It is synthesized by intracellular schizonts and hepatic forms and is expressed on the merozoites surface. After being released at the rupture of the iRBC, it passes through a series of proteolytic cleavage, and it is thought to be involved in the initial interaction of the parasite with RBC, being relevant for the invasion process.	(Holder *et al.*, 1992; Kauth *et al.*, 2003; Kadekoppala and Holder, 2010)

Table 1. Characteristics of some *Plasmodium* proteins.

Protein	Characteristics	References
Erythrocyte 19 binding antigen-175 - EBA-175	Located in the microneme, it is a *P. falciparum* protein that binds its receptor glycophorin A (GpA) on human erythrocytes during invasion.	(Adams *et al.*, 1992; Dittrich *et al.*, 2003; Duraisingh *et al.*, 2003; Tolia *et al.*, 2005)
Apical membrane antigen 1 - (AMA1)	It is a type 1 membrane protein present in the apical complex of the merozoites (accumulates in the micronemes). This protein is assumed to be involved in erythrocyte invasion.	(Peterson *et al.*, 1989; Triglia *et al.*, 2000; Bannister *et al.*, 2003; Healer *et al.*, 2005)
Reticulocyte-binding-like homologue 1 - (RH1)	This protein binds to a receiver resistant to trypsin dependent on sialic acid on the surface, showing that it is relevant in recognition and host cell invasion.	(Rayner *et al.*, 2001; Triglia *et al.*, 2005, 2009; Arévalo-Pinzón *et al.*, 2013)
Ring-infected erythrocyte surface antigen (RESA-1)	Interacts with the protein spectrin of the erythrocyte cytoskeleton, stabilizing the cytoskeleton of iRBC and conferring increased rigidity of the erythrocyte's membrane.	(Badaut *et al.*, 2015)

1.5. Clinical manifestations of malaria disease

According to WHO criteria, malaria can be classified as uncomplicated and severe, depending on the clinical manifestations present and their severity (WHO, 2016). The clinical manifestations depend primarily on the patient's immune status, the stage of infection, and the parasite species involved (Pasvol, 2005). Malaria diagnosis must be opportune to allow adequate antimalarial therapy to be quickly provided, and to prevent severe disease from developing (Palma *et al.*, 2012).

Common clinical manifestations of uncomplicated malaria (acute malaria) are fever, sweating, abdominal pain, headache, cough, nausea, and joint pain (WHO, 2018). These symptoms usually appear about 15 days after the parasite inoculation. In severe malaria caused by *P. falciparum*, hyperparasitemia, severe anemia, hydroelectrolytic imbalance hemorrhagic disorders, pulmonary edema, acute renal failure, and hepatic dysfunction (Table 2), can be found, among others (WHO, 2016).

Table 2. Clinical and laboratory characteristics of severe malaria, according to WHO 2012.

Clinical characteristics	Alteration of consciousness, prostration. Convulsions: More than 2 episodes in 24 hours. Confusion or agitation, with Glasgow Coma Scale (GCS) » 11. Coma (GCS ≤ 11 or Blantyre coma Scale < 3 in children). Respiratory difficulty or Dispnoea. Shock: Extended Capillary Recharge time (2 s), with or without systolic blood pressure < 80 mm Hg in adults (< 70 in children). Pulmonary Edema (must be confirmed radiologically). Abnormal bleeding, Jaundice, Anuria, Repeated vomiting.
Laboratory features	Severe normocytic Anemia: hemoglobin <7 g/dl in adults, <5 g/dl in children. Hemoglobinuria. Hypoglycemia= (blood glucose <2.2 mmol/L or <40 mg/dl). Metabolic acidosis = (deficit of base >8 meq/L or plasma bicarbonate <15. Pulmonary edema (by radiology). Acute renal lesion (creatinine > 3 mg/dL or urea > 20 mmol/L). Hyperlactaemia (Lactate > 5mmol/L). Asexual Parasitemia > 10% of infected red blood.

1.6. Surveillance, prevention and control of malaria

Measures to control malaria transmission involve epidemiological, genetic, statistical, bioinformatic and immunological aspects that are linked to the elimination and eradication of malaria. It is known that the humoral immune response plays a crucial role in developing immunity against malaria. The main objectives of malaria control programs are control transmission, protect against. In order to comply with this, some fundamental objectives were defined, such as early diagnosis and immediate appropriate treatment of the disease, as well as selective control of vectors, which includes adequate control of the environment, and chemical treatment of households and open spaces (WHO, 2005).

1.6.1. Malaria diagnosis

The need for useful and practical diagnostic methods is real since an effective diagnosis would reduce complications and mortality associated with the disease. Malaria infection cannot be diagnosed clinically, as the clinical signs and symptoms presented can be confused with other tropical infections, therefore, the diagnosis must be confirmed by the. The following are malaria diagnostic methods recommended by WHO.

1.6.1.1. Parasitological diagnosis

Precise parasitological diagnosis is a prerequisite for the proper management of malaria. This has made parasitological diagnosis a cost-effective strategy for malaria management (WHO, 2010a) (WHO, 2009;). In 2009, WHO established that the main method of malaria diagnosis should be through parasitological analyses, using microscopy and/or rapid diagnostic tests (TDRs); however, determines that empirical treatment should only be done in conditions where it is not possible to make a parasitological analyses (D'Acremont, Lengeler and Genton, 2007). Some studies suggest that in Africa, in some areas of high malaria endemicity with low resources, it is justified to treat children under 5 only with a clinical diagnosis. However, the WHO currently does not support this strategy (WHO, 2015).

1.6.1.2. Optical microscopy

Malaria microscopy is the "gold standard" technique for malaria diagnosis. Giemsa is the classical stain used for malaria microscopy, however, Field or Wright stains also are used (Moody and Chiodini, 2002). This technique allows the identification of different parasites that cause malaria, their multiple parasitic stages, including gametocytes, and to quantify parasite density to control response to treatment. Under optical microscope, the parasitic morphology is visualized differently depending on the infection stage and the infecting species (Cook and Zumla, 2009).

It is a simple and low-cost technique with the possibility of identifying the infecting species, quantifying the parasite density, and monitoring the response to therapy. However, it requires a proper staining technique, it is time-consuming, and requires experienced professionals to correctly identify the species and quantify the parasitemia, especially when there are low parasitemia or mixed infections. Its use is not possible in remote areas without access to electricity and laboratory resources. Maintenance of high performance by health professionals may also be a problem in non-endemic areas where the disease is rarely diagnosed (Tangpukdee *et al.*, 2009).

The sensitivity and specificity of the method depend on the microscopist experience, quality of the slides and of the microscope, time spent for the examination of the thick drop, and the peripheral blood smear and reagent quality (White, Cook and Zumla, 2009). Figure 5 shows an example of a thick drop of an individual infected with *Plasmodium falciparum,* which was stained with Giemsa.

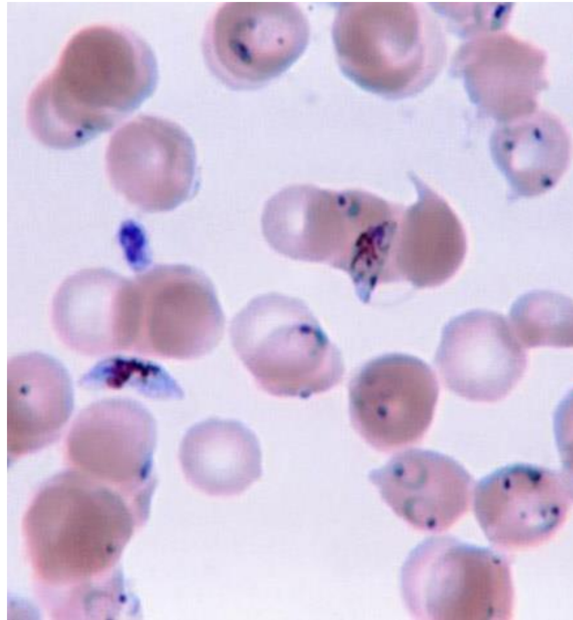

Figure 5. Example of morphology *Plasmodium falciparum*. A thin-film Giemsa stained micrograph of trophozoites, and gametocytes of *Plasmodium falciparum* infecting some of the patient's red blood cells. Image taken from CDC. http://phil.cdc.gov/phil/home.asp

1.6.1.3. Rapid Diagnostic Tests

Rapid Diagnostic Test (RDT) is an alternative way to quickly establish the diagnosis of malaria infection by detecting malaria antigens that are present in the blood of the infected individual. Tests are usually based on the detection of histidine-rich protein 2 (HRP-2), or parasite-specific lactate dehydrogenase (LDH) - the glycolytic pathway of the parasite found in *Plasmodium* (Moody and Chiodini, 2002). The use of RDT does not eliminate the need for analysis of samples by microscopy, since RDT sometimes does not detect infections with low parasitemia that circulates in the patient's bloodstream (Ashley *et al.*, 2006).

Rapid Diagnostic Tests are immunochromatographic tests, wherein a patient blood sample is applied to the test plate, which migrates through a nitrocellulose membrane together with a buffer containing a conjugated antibody. Later, the antigens bind to specific antibodies, forming immune complexes.

The conjugate allows the visualization of this antibody-antigen bond forming a colored line in the capture zone (Moody and Chiodini, 2002), an example of a mode of action of common malaria RDT format is presented in figure 6.

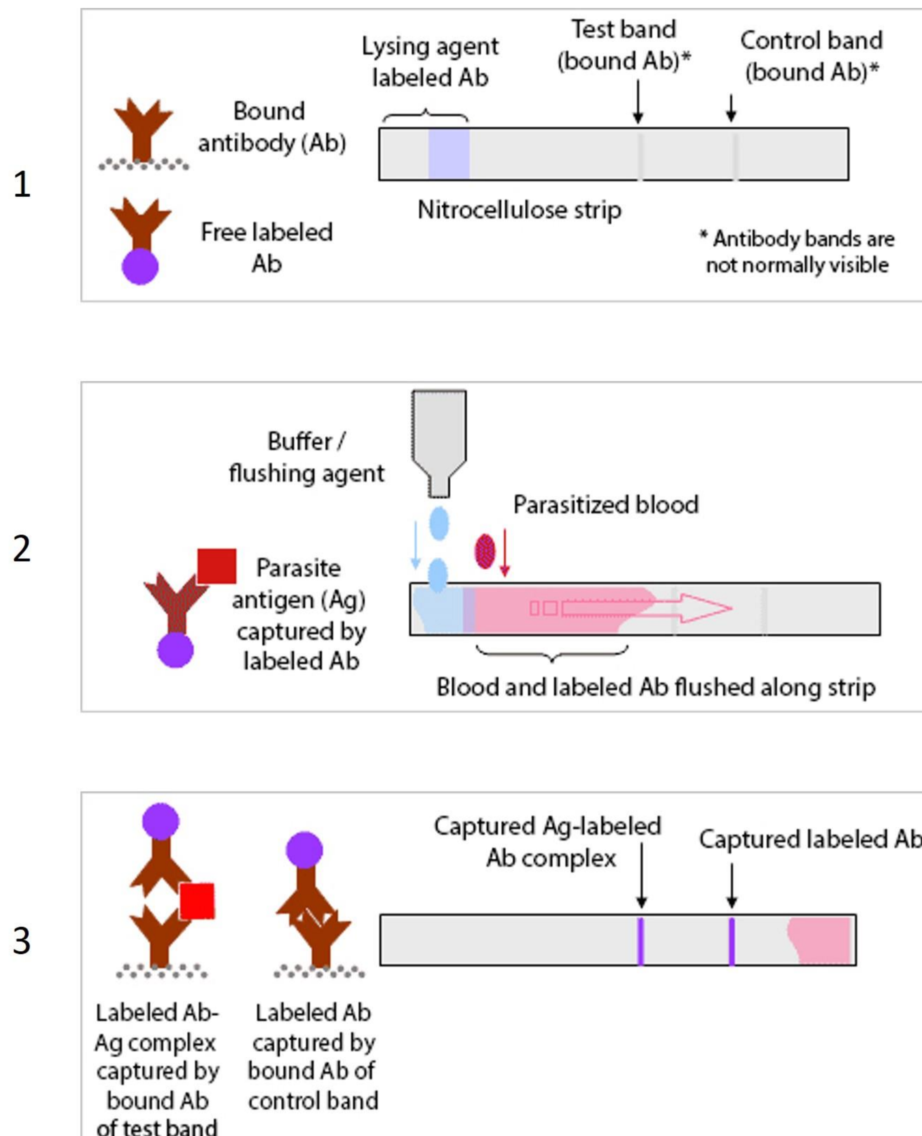

Figure 6. **Mode of action of common malaria RDT format.** (1) Dye labelled antibody (Ab), specific for a target antigen, is present in a well provided with nitrocellulose strip. Antibody, also specific for the target antigen, is bound to the strip in a thin (test band) line, and either antibody specific for the labelled antibody, or antigen, is bound at the control line. (2) Blood and buffer, which have been placed in the well, are mixed with the labelled antibody and are drawn up the strip across the lines of bound antibody. (3) If the antigen is present, some labelled antibody will be trapped on the test line. Other labelled antibody is trapped on the control line. Taken from CDC Malaria (WHO, 2006).

1.6.1.4. Molecular Techniques: Nucleic Acid Amplification (NAATs)

Nucleic acid amplification tests (NAATs) allow the sensitive detection of malaria infections with low-density (below one parasite/µL). Among the principal NAATs developed to detect malaria are: Polymerase chain reaction (PCR) – including nested (n), quantitative (q) or real-time reverse transcription (RT) PCR), loop-mediated isothermal amplification (LAMP), and quantitative nucleic acid sequence-based amplification (QT-NASBA).

The parasite's nucleic acids are detected through PCR and specific amplification of a selected region of the genome. Although this technique is slightly more sensitive than microscopy, its use is limited, since it is a technique that requires well-trained and specialized personnel. Most of the time, PCR results are also not quickly available. PCR analysis is most useful for confirming the parasite species after the diagnosis established by microscopy or RDT (Chotivanich, Silamut and Day, 2007). In cases of low parasitemia and "mixed" infections, the PCR is considered a good diagnosis method (Morassin *et al.*, 2002; Johnston *et al.*, 2006); and the PCR allows the detection of parasitic drug resistance (Imwong *et al.*, 2001, 2003).

Loop-mediated isothermal amplification (LAMP) can be conducted under isothermal conditions and does not need expensive thermocyclers; this technique consists in making the detection of turbidity after DNA sequences amplification (Lau *et al.*, 2011). LAMP detects the conserved 18S ribosome RNA gene of *P. falciparum* (Poon *et al.*, 2006). It can be used to detect hemozoin or *Plasmodium* dsDNA in infected erythrocytes (Jain *et al.*, 2014). LAMP is a simple and inexpensive molecular malaria-diagnostic test. Other studies have shown high sensitivity and specificity, not only for *P. falciparum* but also for *P. vivax*, for *P. malariae*, and for *P. ovale* (Han *et al.*, 2007; Aonuma *et al.*, 2008). LAMP seems be easy, sensitive, quick and cheaper than PCR (Erdman and Kain, 2008).

1.6.1.5. Serological diagnosis

Malaria diagnosis can also be indirectly performed by serological techniques that allow the detection of malaria antigen or antiparasitic antibodies in the blood. This can be done using indirect immunofluorescence antibody assay (IFAT), enzyme-linked immunosorbent assay (ELISA), or enzyme immunoassay (EIA) (Tangpukdee *et al.*, 2009).

Serological diagnoses are generally used in epidemiological studies, for screening blood donors, to check infection in individuals from malaria-endemic areas with a possible chronic infection or repeated infections, or to clarify a diagnosis that remains doubtful after treatment of the individual (Tangpukdee *et al.*, 2009).

EIA and ELISA are qualitative and quantitative techniques that rely on antigen-antibody binding, allowing the detection of small amounts of proteins, peptides, hormones or antibodies in a liquid sample. These techniques use antibodies bound to an enzyme (conjugate), which, in the presence of a compound metabolized by the enzyme, produces a chromogenic reaction indicating the presence of the antigen (Gan and Patel, 2013).

IFAT remains the reference method for serological techniques and consists in the formation of immunocomplexes that occurs when the serum of individuals with malaria is added to a slide whose parasite is adsorbed. The immunocomplexes formed are visualized under fluorescence microscopy (Tangpukdee *et al.*, 2009).

In malaria diagnosis, all the techniques mentioned above have advantages and disadvantages. Therefore, the choice of the technique must be adapted to each context considering the individual, the species of *Plasmodium* epidemiologically dominant, and the material and human resources of each medical service.

It is important to remember that diagnosis based only on clinical evidence is inaccurate, but continues to be accepted for patients (especially children), who are febrile, in areas where malaria is the major cause of fever, and where laboratory support is lacking (Niruthisard *et al.*, 2007; Cook and Zumla, 2009). In that case, the decision to administer antimalarials should be based on the likelihood that the febrile illness is malaria. However, whenever possible, WHO recommends confirmation of clinical suspicion by laboratory methods (WHO, 2010).

Table 3. Summary of the malaria diagnostic test. Comparative analysis of malaria diagnostic test. NA, not applicable

Test	Principle of the method	Instrument used	Time consumed (min)	Sensitivity and specificity	Detection limit (parasites/µL)	Expertise required	Instrument cost
Clinical diagnosis	Based on presenting malarial symptoms and signs.	Medical doctor.	Depends on physician´s skill.	Depends on malaria endemic.	NA	High in non-endemic areas.	NA
Microscopic	Morphological distinguishable stage of the parasite under microscope by thick and thin blood smear.	Optical microscope.	30-60	Depends on the instrumental quality, technique, reagents and microscopist´s skill.	5-10	High in non-endemic areas.	Low
RDT	Detection based on antigen-antibody complex and enzyme assay.	Disposable dipsticks.	10-15	Moderate at higher parasitemia (>100 parasite/µL).	50-100	Low	Moderate
Polymerase chain reaction (PCR)	Specific amplification of malaria DNA.	Thermocycler.	45-360 depends of the methods	High	≥1	High	Expensive
Loop-mediated isothermal amplification (LAMP)	Detection of turbidity after amplifying DNA sequences.	Turbidity meter.	<60	High	>5	High	Moderate

Adapted from (Tangpukdee *et al.*, 2009; Jain *et al.*, 2014; Ashley, Pyae Phyo and Woodrow, 2018).

1.6.2. Pharmacological treatment of human malaria

The treatment of uncomplicated malaria aims to cure the individual (i.e. eliminate the parasite from the individual's organism) as soon as possible, to prevent the progression of infection to severe malaria. Overall, the treatment of infected individuals aims to reduce the transmission of infection to other individuals, if we consider the individual infected as a reservoir, and to prevent the spread of resistance to antimalarials (WHO, 2010).

A wide variety of drugs have been developed to fight, control malaria; several drugs are available for the treatment of malaria and are divided into three families: quinolones, antifolates and artemisinins. Numerous antibacterial with antimalarial activity are also available, although their time of action is slow; these relate to the families of sulphonamides and sulfones, tetracyclines, clindamycin, macrolides and chloramphenicol (Cook and Zumla, 2009). The antibacterial should be chosen taking into account several factors: age of the individual, infecting species, existence of resistance of the parasite to the antimalarials, stage of infection, adverse effects and the kinetics of the drug. Table 4 describes some characteristics of antimalarial drugs.

Timely treatment with appropriate antimalarial drugs, such as Artemisinin-based Combination Therapies (ACTs), is currently the most effective treatment for infections caused by *P. falciparum* (WHO, 2015; 2018) which can be seen in the table 5. However, *Plasmodium* sp. resistance to antimalarial drugs, hitherto described in *P. falciparum* and *P. vivax*, remains a serious problem concerning therapeutic alternatives available for malaria control. Indiscriminate administration of antimalarial drugs, such as chloroquine and sulfadoxine-pyrimethamine (SP), led the *Plasmodium* parasite to develop resistance to antimalarial, and is now becoming the biggest obstacle to combat the disease (Helb *et al.*, 2015; WHO, 2016).

At the beginning of the 20th century, resistance to quinine was first described. Also, drug-resistance to antimalarials has been extended to represent a global problem in the fight and control of malaria. Understanding the mechanisms, geography, and control tools that can act against resistance to antimalarials are of fundamental importance to prevent its expansion.

Since the 1940s, chloroquine has been the most used for the prevention and treatment of malaria. Its misuse as an uncontrolled administration has led to widespread resistance over the years (Hall and Wilks, 1967). From its first appearance in the 1950s in Latin America and Southeast Asia, it spread to the Indian subcontinent in the 19770s and then to Africa in the 1980s (Wellems and Plowe, 2001).

Table 4. Characteristic of antimalarial drugs**.** Adapted from (Tracy and Webster, 2005; WHO, 2010b; Mojab, 2012).

Drugs	Commercial name	Mechanism of action	Anti malaria activity	Doses	Presentation
Chloroquine	Aralen Avlocor Nivaquina Resochin	Inhibits ferriprotoporphyrin IX detoxification	Blood schizonticide	Oral: 10mg/Kg stat, then three doses of 5mg/Kg/over 36-48 h	Tablets of 100 or 150 mg
Artemether	___	Altered membrane transport properties and inhibits nutrient flow to the parasite	Blood schizonticide	3.2 mg/Kg intra muscularly as a loading doses, followed 1.6 mg/Kg/daily or oral 4mg/kg on first day followed by 2 ml ampoule	80mg/ml injection and 40mg per capsule
Artemehter/Lumefantrine	Coartem, Riamet	Preventing detoxification to hemozoin	Blood schizonticide	3 day treatment and 6 oral doses: 5- <15Kg 1 tablet/doses, 15 - <25 Kg 2 tablets/doses, 25- <35Kg 3 tablets/dose and ≥ 35Kg 4 tablets/doses.	Tablets of 20mg artemether/120mg lumefantrine
Mefloquine	Lariam	Inhibits ferriprotoporphyrin IX detoxification	Blood schizonticide	Oral: 10mg/Kg stat, then three doses of 5mg/Kg/over 36-48 h	250mg tablets
Quinine	Qualaquin	Inhibits ferriprotoporphyrin IX detoxification	Blood schizonticide	Oral: 10mg/Kg 8 h for 4 d and 5 mg/Kg 8 h for 3 days Intra muscular: 20 mg/Kg stat, followed by 10 mg/Kg 8 h by deep intra muscular injections for 5-7 days	Tablets of 300 or 600 mg base
Primaquine	___	Maybe mitocondrial electron transport	Tissue schizonticide Gametocytocide, Sporontocide	0.25 at 0.75 mg base/Kg/d x 14 days	2.5, 7.5 and 15 mf of the salt (Tablests)
Artesunate	___	Inhibits malaria protein EXP1, a glutathione S-transferase, responsible for breaking down cytotoxic hematin	Blood schizonticide	4 mg/Kg (oral) 2.4 mg/kg followed by 2.4mg/Kg after 24h.(parenteral)	50mg tablets, 60mg/ml injection, 100mg suppository, 200mg rectocap
Atovaquone	Melarone	It has a highly lipophilic molecule that supposedly interferes with the mitochondrial electron transport	Blood schizonticide	250mg atovaquone/100mg proguanil/day	Tablets 250mg actovaquone+100mg proguanil
Proguanil	Paludrine	Acts Inhibiting parasitic dihydrofolate reductase enzyme	Tissue schizonticide	3 mg/Kg/day	Tablets of 100mg
Clindamycin	Cleocin	Acts by inhibiting the protein synthesis by binding to the 50s subunit of ribosomes	Blood schizonticide	20 mg base/Kg/day per 7 days	Tablets of 100mg
Doxycycline	Vibracine	Inhibits apicoplast functions	Blood schizonticide	1.5 – 2 mg/Kg/day	Tablets of 100mg

Table 5. Malaria treatment.

Parasite	Choice teratmnet	Alternative treatment
Treatment uncomplicated malaria		
P. falciparum	DIHYDROARTEMISINE-PIPERAQUINE® (EURARTESIM® 40mg / 320mg) 36 to 74 kg 3 comp/d x 3 days, 75 to 100 kg 4 comp/d x 3 days. Take on an empty stomach (3 h before or after eating) - ATOVACUONAE + PROGUANILO (MALARONE® 250 mg / 100 mg) 4 comp /d x 3 days. Take with food rich in fat.	[QUININE SULFATE 10 mg (salt) / kg / 8h. Quinine sulfate capsule prepared in the hospital with 300 mg of salt: 2 comp / 8h + DOXICICLINE 100 mg / 12h x7 days.
P. malariae	CHLOROQUINE (RESOCHIN® 150 mg base) 4 comp + 2 comp at 6, 24 and 28h (4+2+2+2=10 comp).	Treatment of uncomplicated *P. falciparum* .
P. vivax	CHLOROQUINE (RESOCHIN® 150 mg base) 4 comp + 2 comp at 6, 24 and 28h (4+2+2+2=10).	Treatment of uncomplicated *P. falciparum*
P. ovale	PRIMAQUINA (PRIMAQUINE 7.5 mg base) 30 mg base = 4 comp / d x2 sem in *P. vivax* and 15 mg base = 2comp / d x2 week in *P. ovale*	PRIMAQUINA (PRIMAQUINE 7.5 mg base) 30 mg base = 4 comp/d x2 week in *P. vivax* and 15 mg base = 2comp / d x 2 week in *P. ovale*
Treatment of malaria during pregnancy		
P. falciparum	[QUININE SULFATE 2 comp / 8h + CLINDAMYCIN 450 mg / 8h] x7 days or ARTEMETHER-LUMEFANTRINE (RIAMET® 20 mg / 120 mg) 4 comp + 4 comp at 8h + 4 comp / 12h x 2 more days (total 24 comp) in second and third trimesters	MEFLOQUINE (LARIAM 250 mg) 3 comp + 2 comp at 12 o'clock
P. vivax	CHLOROQUINE (RESOCHIN 150 mg base) 4 comp + 2 comp at 6, 24 and 48h	Treatment of *P. falciparum* during pregnancy
P. ovale	CHLOROQUINE (RESOCHIN 150 mg base) 4 comp + 2 comp at 6, 24 and 48h	Treatment of *P. falciparum* during pregnancy
	Maintain further prophylaxis with CHLOROQUINE (RESOCHIN 150 mg base) 2 comp / weekly until delivery. After delivery, PRIMAQUINE (PRIMAQUINE 7.5 mg base) 4 comp / day x 2 week	
Treatment of severe malaria		
	[ARTESUNATE 2.4 mg / kg IV initial, at 12 and 24h. Then every 24h + DOXYCYCLINE 100 mg IV / 12h] x7 days	In situations of non-response to the indicated regimens, exanguinotransfusion can be considered

Adapted from WHO, 2010.

WHO has established the following parameters to define a suspicion of resistance to artemisin. Some of these parameters are presented in table 6.

Table 6. Some parameter used to define resistance to artemisinin drugs in malaria human.

Parameters	Resistance determination	Reference
Parasitic clearance half-life ≥5 hours after starting treatment with ACTs or artemisinin in monotherapy.	It is determined when the parasitic load in the patient's blood does not drop below 50% at ≥5 hours after the start of treatment	(Ashley *et al.*, 2014)
Persistent parasitism on day> 3 after treatment with ACTs or artemisinins in monotherapy	Although no longer used, it can also be determined *in vitro* when> 1% of parasites survive after 6 hours of exposure to therapeutic doses of dihydroartemisinin and 66 hours of culture	(Witkowski *et al.*, 2013)
Presence of parasitic genetic markers:	*K13 mutation: It is determined when mutations are found in the PF3D7_1343700 gene of chromosome 13 of *P. falciparum* and that it encodes the kelch13 protein. *The nucleotide substitution in position M476IT. *Mutations associated with the delay of parasitic clearance in vitro, being the most important mutations: C580Y, Y493H, R539T, I543T and F446I. *Mutations of pfmdr1 (1034C and 1042D polymorphisms) described for other drugs appear to be less important in the case of artemisinin.	(Ariey *et al.*, 2014; Dogovski *et al.*, 2015)

1.6.3. Vector control in malaria

Vector control is another recommended measure for effective malaria control; the guidelines for the application of appropriate vector control interventions include the use of insecticide-treated mosquito nets (MTI/lightweight) and residual insecticidal spraying. Bed nets treated with long-term insecticide are more effective in high-transmission areas,

where vectors rest in closed-night environment, whereas the protective effect provided using topic and special repellents were not convincingly demonstrated. Still, there is evidence of resistance to insecticides, an aggravating factor to achieve malaria elimination (WHO, 2016).

1.6.4. Malaria vaccine development

There are different approaches to try to solve this public health problem, but none has been an ideal solution. Despite the many available approaches to prevent and treat malaria, an effective vaccination would likewise be considered as an essential tool for the global decrease of malaria. The goal of the vaccination strategy is to induce immune memory responses that provide protection on *Plasmodium* sp. to individuals after immunization (Arama and Troye-Blomberg, 2014).

The search for a vaccine against malaria has been focused primarily on the one produced by *P. falciparum*, mainly due to the severity that this species produces in humans, compared to the clinically less severe and rarely lethal species of this disease produced by *P. vivax, P. ovale* and *P. malariae*. There is still no 100% effective vaccine for malaria, although there are some in the development phase and others in study of its effectiveness (Reyes-Sandoval *et al.*, 2010; Olotu *et al.*, 2013; Van Den Berg *et al.*, 2019).

Plasmodium is a complex parasite, composed for a large number of antigenic (proteins) targets. *Plasmodium* parasite has an extremely complicated biology, throughout its life cycle, the parasite produces different stage-specific antigens that establish sophisticated interactions between the parasite and the host's immune system (Doolan *et al.*, 2003; Crompton, Pierce and Miller, 2010).

Some *Plasmodium* proteins were identified as targets of natural immunity response many years ago; however, several studies have been carried out in search of new vaccine candidates. Vaccine candidates are expressed in the stages of the parasite life cycle; they were classified in three different groups according to their lifecycle stage (pre-erythrocytic vaccines; blood stage vaccines and transmission-blocking vaccines) (Vaughan *et al.*, 2012; Birkett *et al.*, 2013). Figure 7 shows some malaria vaccine candidates.

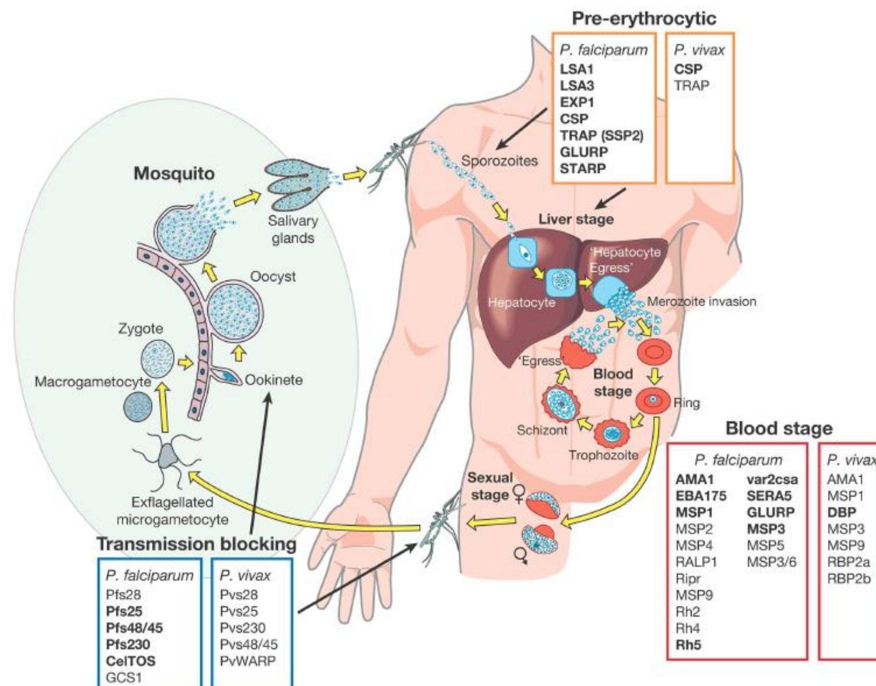

Figure 7. Some malaria vaccine candidates. These graphics represent some candidate antigens for *Plasmodium falciparum* and *Plasmodium vivax* superimposed on the *Plasmodium* lifecycle. Antigens indicated in bold (Pre-erythrocytic: LSA1, LSA3, EXP1, CSP, TRAP, GLURP, STARP; Blood stage: AMA1, EBA175, MSP1, MSP3, Rh5, SERA5, GLURP, Var2csa, DBP; Transmission blocking: Pfs25, Pfs48/45, Pfs230, CelTOS) are those that are currently being evaluated in pre-clinical trials or have entered at least on Phase 1 clinical trials according to the WHO (WHO, 2013). The *P. vivax* latent stages known as "hyponozoites" are not shown, but these occur in the liver stage. Extracted from (Barry and Arnott, 2014).

Pre-erythrocytic stage vaccines

In the pre-erythrocyte stage, the aim is to contain the infection at early stage, and the vaccine target may be whole sporozoites or antigenic subunits of the sporozoite proteins (Arama and Troye-Blomberg, 2014). Circumsporozoite protein (CSP), is the most used protein for the development of potential vaccines against malaria, being the main protein on the sporozoite surface, as it generates a higher immune response (Yoshida *et al.*, 1980).

This membrane-associated protein is well characterized in terms of its primary structure and its variability, in a good number of *Plasmodium* strains (Singh *et al.*, 2007; Herrera *et al.*, 2015). RTS S/AS01 malaria vaccine (MosqurixTM), is currently the most promising vaccine, and the only existing vaccine against malaria licensed, approved by the European Medicines Agency (EMA) in 2015, and implemented in young African children; recently, a pilot implementation study has beeng initiated in Kenya, Malawi, and Ghana (Van Den Berg *et al.*, 2019).

RTS, S is a vaccine based on the combination of the hepatitis B surface antigen (HBsAg) with the thrombospondin domain of the CSP protein, using adjuvants (Sacarlal *et al.*, 2009; RTS, 2015). This vaccine is administered with an adjuvant AS01, a liposomal mixture containing two immunostimulants 3-O-desacyl-4'-monophosphoryl lipid A (MPL) and the saponin QS-21, this adjuvant helps to trigger the cellular immune response mediated by T lymphocytes (Neafsey *et al.*, 2015; RTS, 2015).

RTS, S vaccine induces protection against clinical malaria by reducing of the number of merozoites exiting in the liver by extending exposure to asexual forms existing at subclinical levels, and thereby giving at the immune system more time to develop the naturally acquired immune response (Crompton, Pierce and Miller, 2010). Phase II trials of the RTS, S vaccine that were conducted in children in African countries, also confirmed that the vaccine is safe and capable of inducing high antibody response, which was associated with reduced time to new infections (Alonso *et al.*, 2005). Early results from the Phase III study showed that RTS, S / AS01 was able to reduce clinical malaria and halve it in small children, during the 12 months following vaccination. RTS, S has been shown to be most effective in children aged 5–17 months, who receive three doses of the vaccine and then a booster at 20 months of age, reducing the cases of severe malaria by 36% (RTS, 2015).

In addition to the CSP-based antigen, other antigens, such as LSA1 (Liver Antigen Phase 1) LSA3 (Liver Antigen Phase 3), SPf66, TRAP protein (thrombospondin-related Adhesive Protein), SALSA, and STARP, were identified and studied for their potential as vaccines. Its development is in phase II clinical after having passed phase 1 and having been shown to be safe (Duffy *et al.*, 2012; Hodgson *et al.*, 2014).

Blood (Erythrocytic) stage vaccine

The most advanced vaccines against parasitic forms of the erythrocyte phase are targeted to AMA1 and MSP1. These vaccines are under development with new adjuvants (Crompton, Pierce and Miller, 2010).

Another proposal, still under development, is FALVAC-1 (*P. falciparum* Vaccine 1) containing 21 cells B and T epitopes of different stages (pre-erythrocytic, erythrocyte and sexual) among them: CSP (circumsporozoite) LSA1 (liver Antigen 1 Phase), MSP1 (Protein 2 surface protein), SSP2 (sporozoite surface Protein 2) MSP2 (merozoites surface Protein 2), AMA1 (apical membrane Antigen 1), RAP1 (Proteins associated rhoptry 1), EBA- 175 (Erythrocyte Binding Antigen 175) and Pfg27 (*Plasmodium falciparum* gametocyte 27) (Collins *et al.*, 2005).

Transmission-blocking vaccine

Vaccines against sexed stages are vaccines intended to block the transmission of infection; its targets are the gametes, oocyte or zygote (Crompton, Pierce and Miller, 2010). The most advanced candidate antigen for a transmission-blocking malaria vaccine is Pfs25 (Rener, Graves and Carter, 1983; Nikolaeva, Draper and Biswas, 2015); Antigens such as Pfs25 - the oocyte antigen - were submitted to Phase I clinical trials (Malkin, Durbin and Diemert, 2005; Collins, Barnwell and Sullivan, 2006). As vector antigens are not naturally presented to the human immune system means that the induction of a natural immune response, resulting from immunization, does not occur and is a limitation on the efficacy of this vaccine (Arama and Troye-Blomberg, 2014). This vaccine is a good strategy to include in malaria elimination measures, although it is difficult to implement, either because of the need to immunize the entire population of a given community or because individual benefits are not observable in the community (Crompton, Pierce and Miller, 2010; Vaughan *et al.*, 2012). A study reported that mice models immunized with Pfs25 induce an antibody response that completely blocks parasite transmission to mosquitos (Nikolaeva, Draper and Biswas, 2015).

The combination of a vaccine targeting the pre-erythrocyte cycle with a a vaccine targeting of transmission-blocking vaccine can constitute a vital strategy to potentially reduce transmission and promote individual protection (Crompton, Pierce and Miller, 2010). Another study showed that Pfs25-IMX313 (transmission blocking vaccine candidate - nanoparticle platform) increases antibody responses against Pfs25 in mice (Li *et al.*, 2016).

A recent study showed that the combination of RTS, S and Pfs25-IMX313 induces a functional antibody response against malaria infection and transmission in mice. The immunogenicity of both vaccines was maintained, and functional assessment of the induced antibody response by standard membrane feeding assays (Brod *et al.*, 2018). Despite the existence of some promising prototypes in the development of malaria vaccines, it is necessary to consider that human malaria is caused principally by five species of *Plasmodium*, and, therefore, the vaccines should provide protection against the different species. This condition is more easily achieved using the parasite as an immunogenic element rather than the protein subunit (Vaughan *et al.*, 2012).

While a vaccine against malaria that confers complete immunity is not achieved, malaria control depends on vector control and the use of antimalarials, but as there is resistance to insecticides and resistance to the antimalarials in use today, it becomes more urgent to invest in early diagnosis and an effective vaccine. An important starting point is the search and identification of immunogenic epitopes of *Plasmodium* sp. antigens (proteins) that, after confirmation of their role by laboratory, could be postulated as possible targets of diagnosis or vaccine against malaria.

2. Objectives

2.1. Main objective:

The present study aims to identify immunogenic *P. falciparum* antigens that could be used for the development of more efficient diagnostic tests or as vaccine candidates for malaria.

Thus, the main question of this study is: Which *P. falciparum* proteins are involved in the humoral immune response displayed by the infected host and what is their role in human acute malaria?

And the hypothesis raised is *P. falciparum* proteins play a key role in the humoral immune response against malaria could be used for the development of new diagnostic tests or as the candidate of a prophylactic vaccine for malaria.

2.2. Specific objectives:

The specific objectives of the current study are as follows:

2.2.1. To evaluate the antigenic activity of total extract of *P. falciparum*, through the detection of IgM, IgG and IgG subtypes (1, 2, 3 and 4) antibodies in human sera from individuals with a clinical history of malaria using ELISA assay.

2.2.2. To identify *P. falciparum* protein fractions by immunoblot using human sera from individuals with a clinical history of malaria.

2.2.3. To characterize highly reactive *P. falciparum* proteins by *in silico* prediction of immunogenic exposed epitopes.

3. Material and Methods

3.1. Study design

To acknowledge the objectives of this retrospective study, an experimental design was elaborated, represented in figure 9, which included: (i) reactivity evaluation of *P. falciparum* antigens by ELISA using a human serum from malaria-endemic countries, (ii) detection of protein fractions of *P. falciparum* that were recognised by IgG antibodies using immunoblotting and (iii) identify immunogenic epitopes in reactive *P. falciparum* proteins by *in silico* analysis.

3.2. Sampling and ethical considerations

In this study, sera from 436 individuals were selected and analysed through *in-house* ELISA for detection of isotypes (IgM, IgG and IgG subtypes 1- 4) during human malaria; of which, 419 from individuals with a clinical history of malaria potentially exposed to *P. falciparum* infection. As a negative control, a group of 17 sera from healthy Portuguese individuals, who had never been in malaria-endemic area, were used. Sera samples recruited for this study of patients with malaria clinical history, as well as negative sera (healthy Portuguese individuals who have never been in malaria-endemic area) were obtained at the Clinical Unit for Tropical Diseases of the Institute of Hygiene and Tropical Medicine (IHMT) from New University of Lisbon (UNL) (Portugal). The study's protocol was approved by The Institutional Ethics Committee at the Instituto de Higiene e Medicina Tropical (Lisbon, Portugal - approval ref 4-2012-PN of CE-IHMT-UNL, February 22, 2012, see Annex A) and waived informed consent as a retrospective observational study. All serum donors gave their permission to the interview and answered an inquiry for the collection of socio-demographic data including age, gender, and country residency (Annex B), Samples and survey data were obtained during the year 2012. Samples were obtained from individuals diagnosed with a clinical history of malaria and subsequently subjected to a serological diagnosis of malaria at the IHMT-UNL. All serum samples were used exclusively for laboratory diagnosis of malaria by immunoassays.

3.3. Collection and storage of blood samples

Samples and survey data were obtained during the year 2012. The blood samples used in this study were collected by venous puncture and temporarily stored in a dry tube. After blood centrifugation (centrifuge 5810r, Eppendorf, Germany) at 4000 *g* for 15 min, the serums were collected to eppendorfs. Serum samples were anonymized, coded and cryopreserved at -20ºC. The representative diagram of the steps to obtain the total extract of *P. falciparum* is shown in figure 8.

3.3.1. Parasite

To perform serological and immunoblotting analyses, *P. falciparum* 3D7 strain blood-stage parasites were used to produce soluble *P. falciparum* total extract (PfTE).

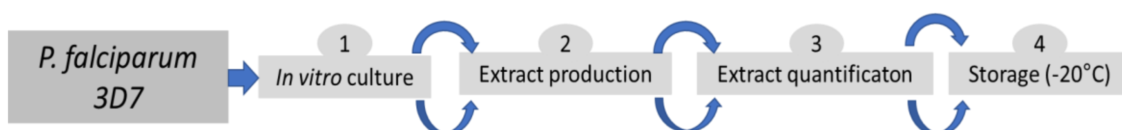

Figure 8. **Parasite protein extraction**. A diagram representing the steps necessary to obtain *P. falciparum* extract was created to be used in immunoassays (ELISA and immunoblotting).

3.3.2. *Plasmodium falciparum in vitro* culture

P. falciparum parasites were cultured using modifications to the method described by Trager and Jensen (1976). Briefly, parasites were grown in human erythrocytes in RPMI 1640 medium containing L-glutamine (ThermoFisher Scientific, Carlsbad, CA), supplemented with 5% (*v*/*v*) human serum and 5% (*v*/*v*) Albumax II (ThermoFisher Scientific, Carlsbad, CA), and incubated at 35 °C in a humid atmosphere with 5% CO_2.

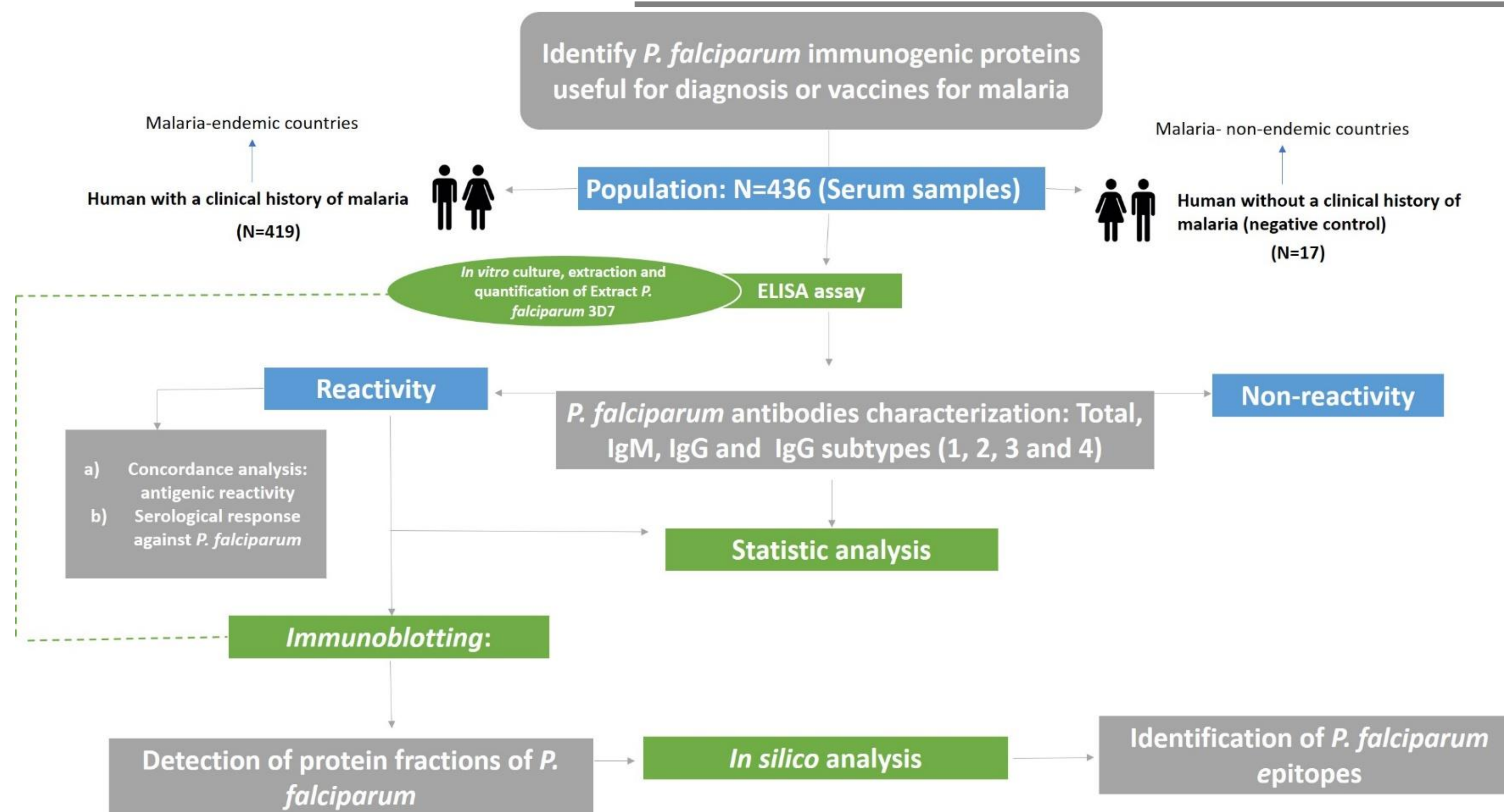

Figure 9. **Experimental design of the current study**. A flowchart representative of the methodological process used to accomplish the objective of this study. Sera from individuals living in malaria-endemic area and with a clinical history of malaria were used to determine the reactivity of *P. falciparum* antigens to specific antibodies and identify reactive protein fractions. *In silico* of the highly reactive *P. falciparum* proteins were performed to identify immunogenic epitopes of interest for the development of a serological immune assay for malaria diagnosis or for the production of a vaccine able to confer efficient protection against malaria.

3.3.3. *Plasmodium falciparum* 3D7 total extract

With some adjustments, of *P. falciparum* total extract (PfTE) was determined according to the method described by Medina (2013). When erythrocytes evidenced parasitemia above 10%, and showed a predominance of merozoites and schizonts, cultures were transferred to 15 mL tubes, homogenized, and centrifuged (centrifuge 5810r, Eppendorf, Damstadt, Germany) at 2000× *g* for 5 min at room temperature (RT). The supernatant was discarded, and the pellet was resuspended in a volume of saponine 0.05% that corresponded to 5 times the pellet volume. Tubes were placed on ice for 15 min and then centrifuged at 3220× *g* at 4 °C for 20 min. Supernatant was discarded and the pellet was washed three times with cold phosphate buffered saline (PBS) and centrifuged at 18,000× *g* for 10 min at 4 °C. For the lysis of the parasites, 4 times the pellet volume of lysis buffer with inhibitor was added (0.1% Triton X-100 in PBS with protease inhibitor (complete ULTRA Tablets, Roche, Switzerland)). The tubes were placed on ice for 30 min under agitation, homogenizing every 10 min in the vortex. Pellets containing parasites were frozen at −70 °C for 5 min and then heated at 37 °C for 5 min. After three freeze–thaw cycles, samples were then centrifuged at 18,000× *g* for 10 min at 4 °C. The supernatant was recovered, and the pellets containing hemozoin were discarded. Soluble protein extract of *P. falciparum* was cryopreserved at −20 °C. Figure 10 shows an image representative of the first step to obtain the total extract of *P. falciparum*. (Medina Costa *et al.*, 2013).

Figure 10. **Centrifugation: *Plasmodium falciparum* protein extraction**. Tubes containing *P. falciparum* cultured parasites were centrifuged. The obtained pellet was used to produce *P. falciparum* extract.

3.3.4. Protein quantification of *Plasmodium falciparum* extract

The MicroBCA method was used for protein quantification of *P. falciparum* extract. The entire procedure was performed according to the manufacturer's instructions (MicroBCATM Protein Assay Reagent Kit, ThermoFisher Scientific, Rockford, USA), using Brand plates®-pureGrade TM (Brand, Germany), (Portugal-Calisto *et al.*, 2016). Figure 11 shows a schematic representation of the protocol used in this study for the quantification of proteins.

This test is based on bicinconínico acid (BCA) for colour detection and quantification of total proteins. This method combines the reduction of Cu^{+2} to Cu^{+1} in alkaline medium (Biuret reaction) with the selective detection of the cation $Cu^{+1,}$ using bicinconínico acid. The reaction product has a purple colour formed by the BCA- Cu^{+1} complex. The hydrosoluble complex absorbs at 562 nm in a linear way with the increase of protein in a range of 20-2000 µg/mL. The calibration curve used in this assay was performed with bovine serum albumin (BSA), at concentrations ranging from 200 to 1000 µg/mL. For the calibration curve, 225 µL of the prepared solution was added into the wells of the plate (BRANDplates microplates, Brand, Germany) according to the desired concentration. For the samples to be quantified, 200 µL of the working reagent (supplied by the manufacturer) and 25 µL of the sample were added and homogenized. The plate was incubated for 30 min at 37 °C. Results were read on a microplate reader (microplate reader model 680, BioRad) at 562 nm.

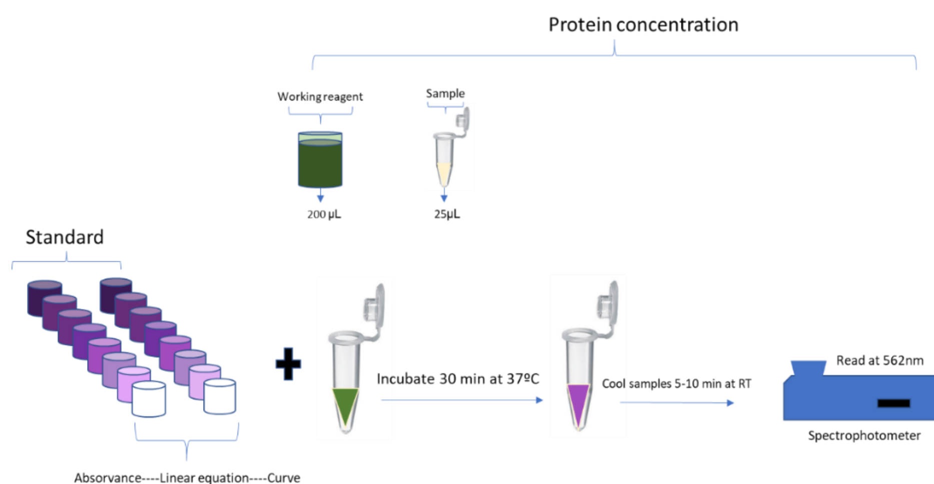

Figure 11. **Protein extract quantification**. Representative diagram of the steps taken to quantify the concentration of *P. falciparum* extract.

3.4. Detection/quantification of anti-*Plasmodium falciparum* antibodies in human serum by *in-house* ELISA

Enzyme-linked immunosorbent test (ELISA) is a colorimetric immunoassay that allows the detection of specific antibodies in serum or plasma. This assay is often used to determine antibody levels in patients with parasitic infections (Wahlgren *et al.*, 1983). The enzymatic activity is quantified using an appropriated substrate that changes colour when altered by the enzyme. The level of light absorbed by the product formed is measured by spectrophotometry, and results are presented by optical density or absorbance values. Figure 12 shows a schematic representation of the ELISA protocol.

In this study, the ELISA was optimized for detection of Ig total, IgM, IgG and IgG subclasses antibodies anti-*P. falciparum* in sera from individuals with a clinical history of malaria. First, 96 well microtiter plates (Thermo Fisher Scientific, Rochester, NY, USA) were coated with different concentrations (from 10 to 100 ng/well) of *P. falciparum* 3D7 crude extract, diluted in 0.1 M bicarbonate buffer, pH 8.5, and incubated overnight at 4 °C. Plates were washed three times with 200 µL/well of washing buffer (PBS with 0.05% Tween-20), blocked with 200 µL/well of blocking buffer (PBS with 5% of skimmed milk), and incubated at room temperature for 1 h. Plates were then washed thrice. The serum samples (1:200 dilution) were diluted in antibody buffer (washing buffer with 1% of skimmed milk) and added to the plates (100 µL/well). Plates were incubated for 1 h at room temperature in an orbital shaker to avoid non-specific binding. Afterwards, plates were again washed three times and the secondary anti-human antibody conjugated to alkaline phosphatase (Calbiochem) was added to determinate total and IgM antibodies; for the determination of total IgG antibodies anti-*P. falciparum*, 1:10000 (v/v) dilutions of peroxidase-conjugated monoclonal anti-human IgG (Sigma Aldrich Corporation) was added; for the determination of isotypes IgG antibodies anti-*P. falciparum*, 1:1000 (v/v) dilutions of biotin-conjugated goat anti-human IgG1, IgG2, IgG3 and IgG4 (Sigma Aldrich Corporation) were added to each case. Conjugated antibody was diluted 1:10,000 (*v/v*) in antibody buffer according to the manufacturer's recommendations, and 100 µL/well added to plate. The plates were incubated for 1 h at

RT in orbital stirring to avoid non-specific binding. After incubation, the plates were washed five times with washing buffer to remove any remaining secondary antibodies. Then, to detect the presence of Ig total and IgM secondary antibodies, 100 µL/well of substrate [1 pellet (20 mg) of 4-nitrophenyl phosphate disodium salt hexahydrate, diluted in 20mL of water] was added to the wells, and the plate was incubated for 30 min, in the dark, at room temperature with orbital shaking. After incubation, this reaction was stopped adding 50 µL/well of 3 N sodium hydroxide (NaOH).

To detect the presence of IgG secondary antibodies, 100 µL of substrate solution [10 mg O-Phenylenediamine dihydrochloride (OPD), Sigma-Aldrich] diluted in 10 mL of citrate buffer [0.1 M citric acid, 0.1 M disodium phosphate, 0.0001% (v/v) H_20_2, pH 5.0] and 10 µl of 30 % H_2O_2 (Sigma-Aldrich) were added; to reveal the presence of conjugated of IgG subclass, 100µl / well of peroxidase-streptavidin conjugate diluted in antibody buffer, was added. The plates from IgG total and IgG subclasses were incubated for 30 minutes at room temperature and protected from light. After incubation, this reaction was stopped adding 50 µl/well of 4.0 N H_2SO_4.

Finally, the optical densities (OD) were measured at 405 nm for Ig total and IgM; at 490 nm for IgG and at 450 nm for IgG subclass with an automatic microplater reader (Bio-Rad Model 680 Microplate Reader). The cut off value was defined as the mean of the negatives plus 2 SD (Standard Deviation). OD index was calculated for each immunoglobulin as OD/cut-off ratio. A sample presenting OD index > 1.0 was considered positive (Reactive). In this study, positive results indicate that antibodies detected in sera recognised anti-*P. falciparum* antigens.

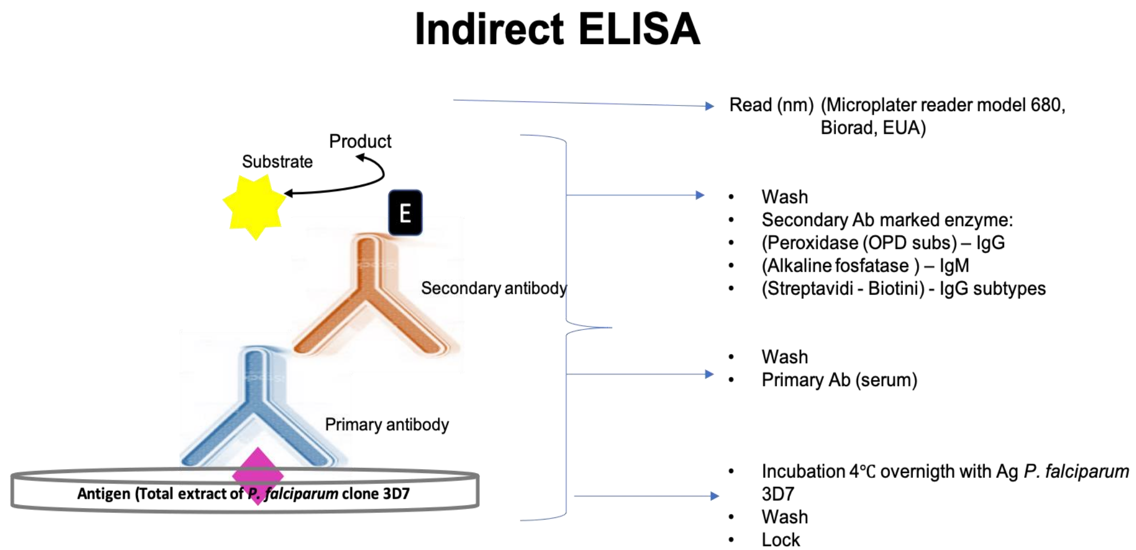

Figure 12. Graphic representation of *in-house* ELISA steps used to evaluate the reactivity of soluble antigen *P. falciparum* to serum specific antibodies.

3.5. Validation of *in-house* ELISA used for detection of human antibodies anti-*Plasmodium falciparum*

In this work, a concordance analysis was performed between two ELISA tests, aiming to made *in-house* ELISA validation, and demonstrate that antibodies present in the sera were capable to reacting with *P. falciparum* antigens. To perform this analysis, 365 serum samples from individuals with a clinical history of malaria were analysed by an *in-house* ELISA and also by a commercial ELISA test (ELISA EIA Kit, Bio-Rad, Marnes-la-Coquette, France) used for serological diagnosis of human malaria. Serum samples from individuals without a clinical history of malaria (*n* = 17) were used as a negative control. The results were categorised as reactive and non-reactive (Medina *et al.,* 2013). Figure 13 shows the representative design of the methodology used to evaluate the level of agreement between ELISAs.

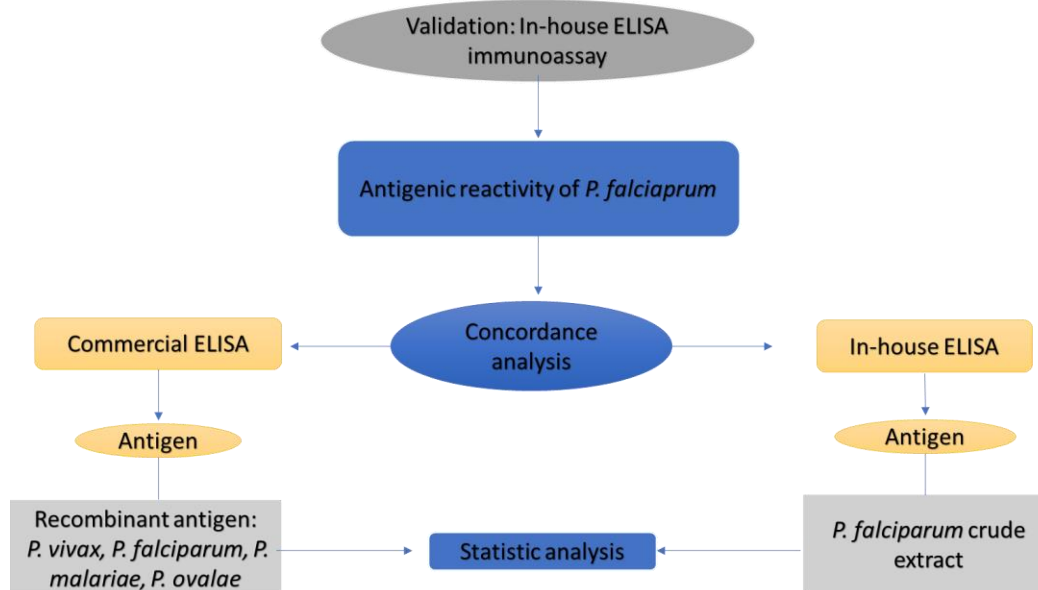

Figure 13. Graphic representation of the methodology used to evaluate the level of Concordance between *in-house* ELISA and commercial ELISA assay.

3.5.1. Commercial ELISA: detection of human antibodies anti-*Plasmodium falciparum*

A commercial ELISA was performed to detect total antibodies anti-*Plasmodium* sp. (Malaria EIA kit, BioRad). This commercial assay is an indirect ELISA that identifies, in human serum or plasma, specific anti-*P. falciparum, -P. vivax, -P. ovale*, and -*P. malariae* IgM, IgG, and IgA antibodies at any stage of the parasitic life cycle. This assay uses recombinant antigens of *P. vivax*, *P. falciparum, P. ovale*, and *P. malariae*. All sample processing was carried out according to the manufacturer's instructions. Briefly, 50 µL of undiluted of serum samples was deposited in the wells of a 96 well plate (Microtiter 96 well plates, Thermo Fisher Scientific, Rochester, NY, USA); the plate was incubated for 30 min at 37ºC in an orbital shaker (Bio-Rad Microtiter/Plate Shaker).

After incubation, the plate was washed five times with 50 μL/well of washing buffer and 50 μL of the secondary antibody was added to each well (secondary antibody conjugated to horseradish peroxidase with buffered saline containing surfactant and stabilizers). Since the substrate is photosensitive, the plate was incubated at room temperature for 30 min in the dark. The plate was washed five times with washing buffer, 50 μL substrate was added (urea peroxide and tetramethylbenzidine), and the plate was incubated at room temperature for 30 min. The plate was protected from light during this incubation. Finally, 50 μL of stop solution (0.5 M H_2SO_4) was added to each well (blue colour changed to yellow), and OD was measured at 450 nm on a microplate reader (Bio-Rad Model 680 Microplate Reader). Antibody quantification in serum samples was calculated by the cut-off value. The cut-off value supplied by the manufacturer was determined by the following formula:

$$\frac{NC1 + NC2 + NC3}{3} + 0.1$$

where NC is the optical density (OD) value of negative controls and 0.1 is a factor determined by the manufacturer. Thus, samples with ODs lower than the cut-off value were considered negative, and samples with ODs values higher than the cut-off value were considered positive.

3.6. Identification of higher reactive *Plasmodium falciparum* proteins fractions

The Western blotting technique or protein immunoblot consists of the specific immunodetection of protein fractions in samples of cell lysates, tissues or pathogens. This technique can be summarized in five steps: (i) protein extraction and quantification, (ii) polyacrylamide gel electrophoresis (PAGE), (iii) protein transfer, (iv) immunodetection of protein fractions and (v) developing (Towbin *et al*., 1979, Burnette., 1981).

Electrophoresis is a separation method based on the differences of electrophoretic mobility when a uniform electric field is applied, allowing the separation of different molecules electrically charged in a solution.

Commonly, the migration of a molecule depends on two factors, its size and charge, which translates into different electrophoretic mobilities in the separation gel.
In this study, we used the polyacrylamide gel electrophoresis technique (sodium-dodecyl sulphate–polyacrylamide gel electrophoresis, SDS-PAGE), which allows the visualization of the different proteins, separated according to their molecular mass.
The proteins present in the *P. falciparum* extract were separated by SDS-PAGE, protocol outlined in Figure 14. A 12% polyacrylamide gel was used whose characteristics are described in Table 7.

Table 7. . Composition of stacking and resolving gels

Components	Running Gel (12% polyacrylamide) (mL)	Resolving Gel (4% polyacrylamide) (mL)
Deionized water	4.1	6.1
Acrylamide Solution*	3.3	1.3
Tris-HCl 0, 5M pH 6.8	-	2.5
Tris-HCl 1, 5M pH 8.8	2.5	-
Sodium dodecyl sulfate (SDS) 10% (w/v)	0.1	0.1
Ammonia persulfate 30% (w/w)	100	100
Tetramethylethylenediamine TEMED	10	10

*Acrylamide solution - 30% (w/v) acrylamide + 0.8% (w/v) bis-acrylamide

3.6.1. *Identification of Plasmodium falciparum protein fractions by immunoblot*

SDS-PAGE separated the proteins present in the *P. falciparum* extract, and a 12% polyacrylamide gel was used. Initially, *P. falciparum* extract was diluted (1:2) in a sample buffer [water, 0.5 M Tris- HCl pH 6.8, glycerol, 10% SDS, bromophenol blue 0.5%, β-mercaptoethanol]. The antigenic solution was heated at 97 °C for 5 min to aid in protein denaturation by breaking electrical interactions. After gel polymerization, 10 µL of a molecular mass marker (Hyper PAGE Pertained Protein Marker, Bioline, UK) and 60 µL of diluted extract (1:2) were applied. Then, the gel was submerged in a running buffer [250 M Tris, 1.92 M glycerin, 1% SDS (w/v], water) and subjected to a constant electric current of 80 V. After the samples entered the running gel, the voltage was increased to 120 V.

To determine the ideal amount of protein to be placed per well to obtain bands with a proper resolution, different amounts (2.8 μg, 5.7 μg, 10.5 μg and 14.2 μg) of total *P. falciparum* extract were added to each well. To visualize the protein fractions, the gel was stained with coomassie bright blue, and the ImageJ software was used to determine the molecular mass. Thus, the protein fractions, separated during SDS-PAGE, were transferred to a nitrocellulose membrane (GE Healthcare, UK) on which the antigen-antibody complexes were established. The electro-transfer of *P. falciparum* proteins to the membrane was done using the Semi-Dry system (BIO-RAD, USA) at 18 V for 1 h. Thus, the membrane was washed twice with washing solution [0.05% (v/v) Tween-20 in PBS] for 10 min each, at room temperature, on an orbital shaker. The membrane was blocked with a blocking solution [3% (w/v) powdered milk in PBS] for 1 h at room temperature in orbital stirring. The membrane was washed two times with washing solution for 10 min each, at room temperature, with orbital shaking and then with PBS. The membrane was cut into strips, and each strip was placed on a plate and incubated with 10 μL of primary antibody diluted 1/100 (v/v) into antibody buffer (1% blocking solution in washing buffer), for 1 h at room temperature with orbital stirring. A pool of the human serum samples without a clinical history of malaria was used as negative control. Again, the membrane was washed with washing solution two times for 10 min each at room temperature with orbital shaking, and once with PBS. After washing, 1 μL of the secondary antibody HRP-conjugated (anti-human IgG antibody, AbD Serotec, Bio-Rad) diluted 1:10000 (v/v) in antibody buffer, was added to each strip and the strips were incubated for 1 h at room temperature with orbital stirring. The strips were washed five times with washing solution for 10 min each at room temperature with orbital shaking, and once with PBS. The strips were incubated in the development of the solution, consisting DAB tablets [(3,3'-Diaminobenzidine) (Sigma-Aldrich)] diluted in deionized water, at room temperature until the colour development was observed (~10 min). Then, the reaction was stopped with deionized water, and the strips were air-dried.

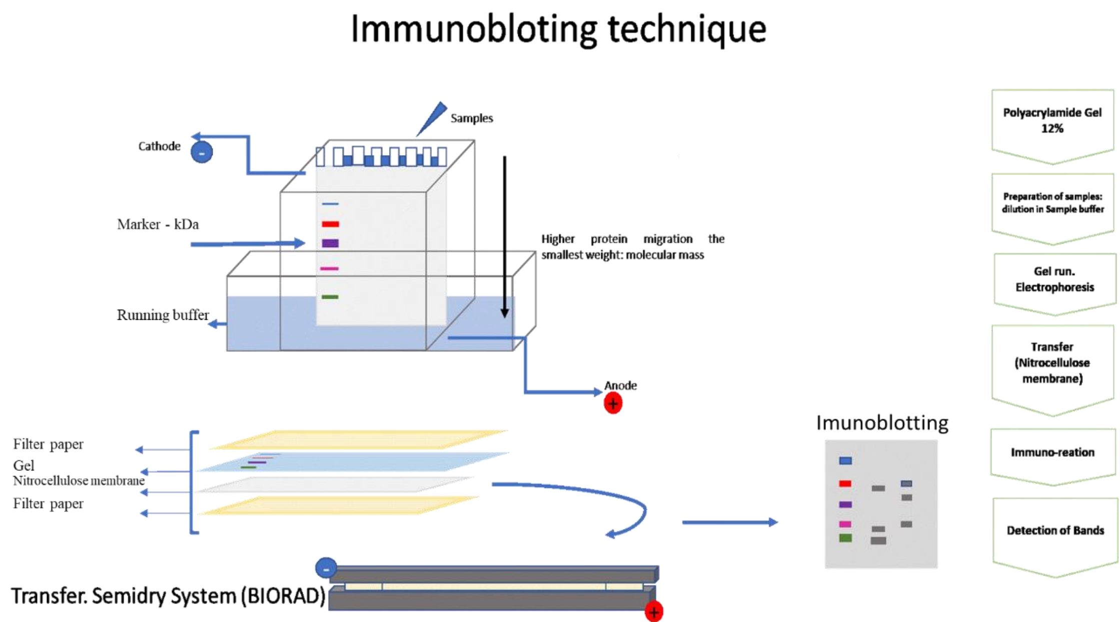

Figure 14. Western blot. Schematic representation of the immunoblotting assay.

Table 8. List of primary antibodies (Serum samples) used in immunoblotting assays.

Identification	Characteristics
P1	Pool of high reactive sera
P2	Pool of positive samples for IgG antibodies
P3	IgM positive samples and low IgG antibody
P4	IgG positive samples
P5	Pool of positive samples IgG antibodies
R1 -R7	High reactive sera
1-24	Low reactive sera
NC	Pool of serum from individuals without a clinical history of malaria. - Negative control

Each pool (P1, P2, P3, P4 and P5) is constituted by different reactive serum for IgG antibodies.

3.7. *In silico* analysis of *Plasmodium falciparum* antigens

To predict immunogenic epitopes of *P. falciparum,* an *in silico* methodology was used, schematized in the figure 15. Based on spectrometry data previously obtained by our group and described by Costa *et al*., (2013), the prediction of linear B-cell epitopes was carried out using BepiPred, BcePred, BCPred and Epitopia Software's (Tong, Tan and Ranganathan, 2006; Emanuelsson *et al.*, 2007; Soria-Guerra *et al.*, 2015).
To perform *in silico* annotation of protein functional regions, the following criteria were raised: (i) propose the secondary protein structure, (ii) propose the subcellular localisation of proteins, and (iii) to predict exposed epitopes.

In the current study, the following *P. falciparum* proteins were analysed:

- Elongation factor - 1 α (EF- 1α)
- Protein disulfide isomerase (PDI)
- Phosphoglycerate kinase (PGK)
- 78 kDa glucose-regulated protein homologue (GRP- 78)
- Rhoptry- associated protein 2 (RAP-2)
- Rhoptry- associated protein 3 (RAP-3)

3.7.1. Sequence Data: Retrieved sequences from the UniProt database

To predict possible antigenic properties and the secondary structure of *P. falciparum* proteins by using bioinformatic tools, the entire protein sequence was downloaded from the UniProt database (UniProt Consortium) and analysed. The annotation of functional sites in the selected *P. falciparum* proteins was performed.
Together with protein sequence, the sequence of another similar high protein was also obtained. Then, the analysis of the two sequences was made by a multiple sequence alignment program (Clustal Omega database, EMBL-EBI), identified the subcellular location of *P. falciparum* protein, predicted the signal peptide, and identified the transmembrane domain. Results were saved in Notepad program and displayed in SeaView multiplatform.

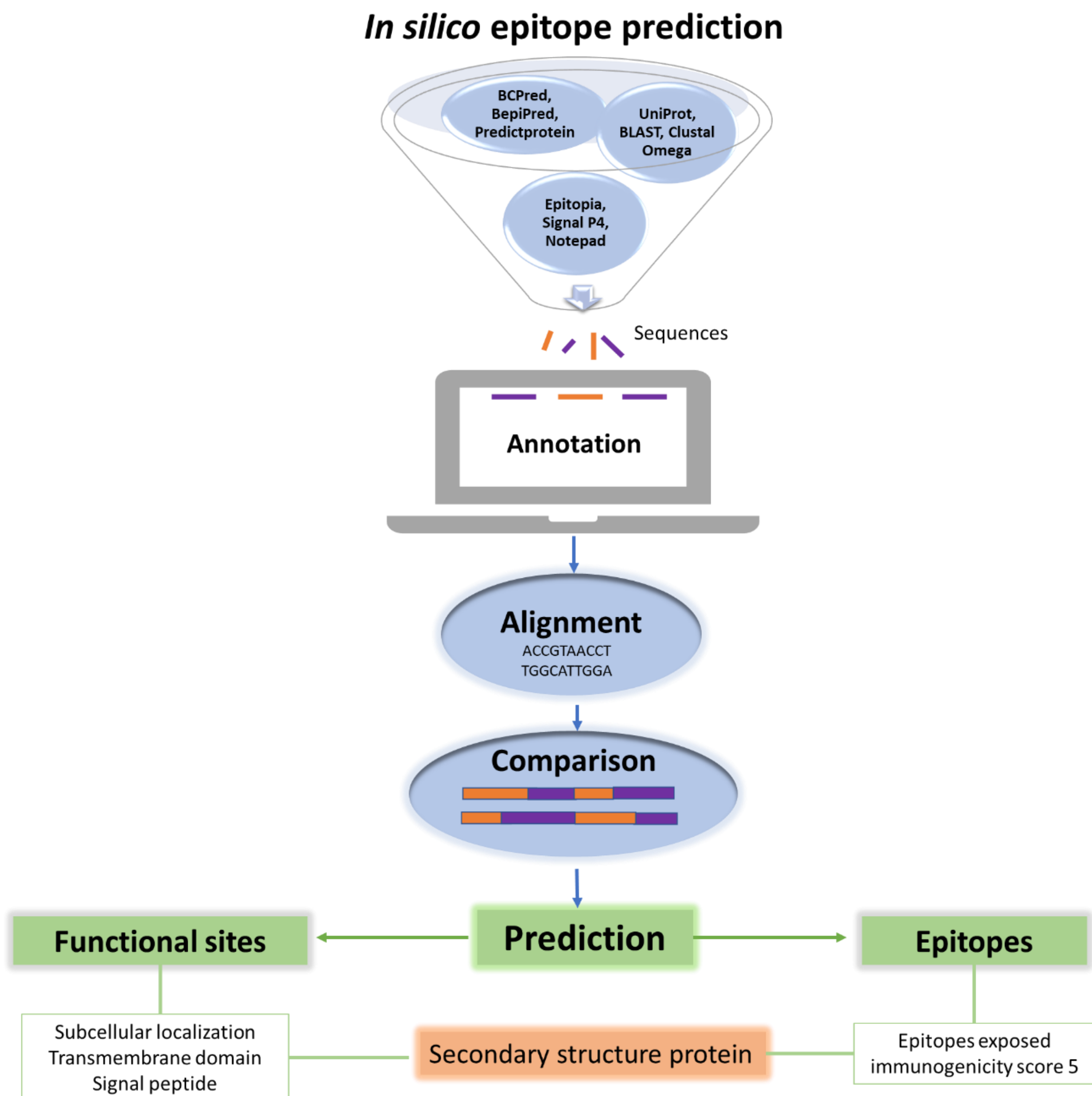

Figure 15. *In silico* epitope prediction. Schematic representation of the computational methodology used for *in silico* prediction of *P. falciparum* epitopes.

3.7.2. Epitopes prediction

Identification of antigenic epitopes is of interest of biomedical research since it can have a potential impact in the development of new diagnosis and the design of prophylactic and therapeutic vaccines. Linear epitopes and conformational epitopes are associated with humoral immune responses. Signal P prediction is one of the best methods used to epitopes prediction and most cited in the literature (Emanuelsson *et al.*, 2007; Soria-Guerra *et al.*, 2015).

To predict linear B-cell epitopes in *P. falciparum* proteins, the BcePred tool, which is based on physicochemical properties such as hydrophilicity, flexibility and polarity. In this work was predicted epitopes with more than 8 eight amino acids and set to a 75% specificity. After identifying the best-predicted epitopes based on score, each of these epitopes was examined for predicted antigenicity, using the Epitopia software with the following scale:

1 = low;

2, 3 and 4 = average;

5 = high.

Figure 16 shows an image representative of the immunogenicity scale of Epitopia software used for epitopes prediction. Lastly, the potential conservation within each of these epitopes was investigated, using the BLAST tool at PlasmoDB. All epitopes presenting scores above 0.5 were included in the final list.

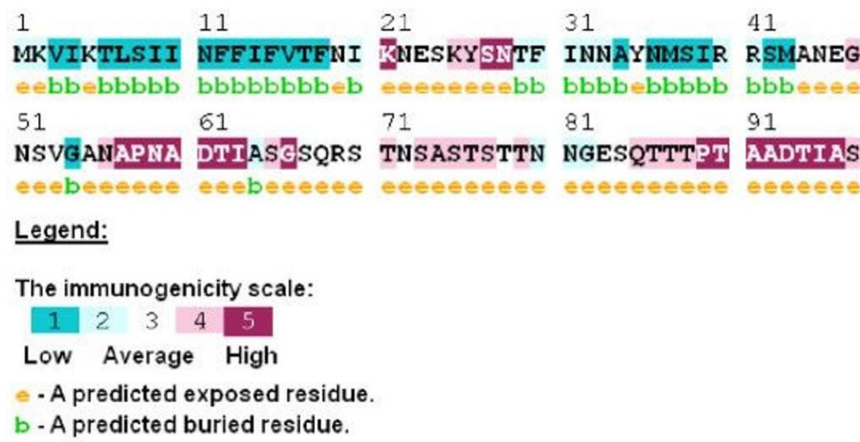

Figure 16. Schematic representation of scale immunogenicity established for identified epitopes. Image obtained from Epitopia program**.** The immunogenicity scale is represented by number and colour: 1(blue) = low, 2, 3 and 4 (light blue, white and pink) = medium, 5 (purple) = high. The residue exposure is represented by letters and colour: **e** (yellow) = a predicted exposed residue and **b** (green) = a predicted buried residue. Regions that have a score > 0.5 and pass the filtering criteria were predicted as disordered binding. All epitopes with a score of 5 and with an exposed (e) amino acid sequence were included in the results. Finally, a secondary protein structure was proposed, identifying the functional sites, the transmembrane domain, peptide signal, and immunogenic epitopes.

3.8. Statistical Analysis

The following Statistics analyses were performed using Prism 6.0 for Windows (GraphPad Software, Inc., San Diego, USA), Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA) and MedCalc. Ink (Statistic Software MedCalc, Ostend, Belgium). The analysis of the data allowed to evaluate the reliability of the ELISA developed, using statistical measures such as sensitivity, specificity, negative and positive predictive values, Youden index (*J*) and Receiver Operator Curves (ROC). The correlation between the two ELISAs was tested by the Bland–Altman test and Pearson correlation test. The Fishers exact test and chi-square tests ($\chi 2$) were used to evaluate the associations among continuous and categorical variables, Kruskal-Wallis test, was used when comparing more than two groups. The Student T test was applied to determine the difference between the distributions of values of the different antibodies used; and normalized transformations were performed on raw data before testing by one-way analysis of variance (ANOVA). Statistical tests were applied with a confidence level of 95%.

4. Results

4. Results

In the context of human malaria, the mechanisms of immunity and protection during infections caused by *Plasmodium* sp. represent a major challenge and priority for research. Studies of humoral and cellular immunity mechanisms seem to be of fundamental importance to understand the parasite-host interaction and may contribute to the identification of potential serological markers, an essential step in the development of new laboratory diagnostic tools and new alternative for vaccine development studies. In this work we want to identify immunogenic *P. falciparum* antigens, to identify serological reactivity against *P. falciparum* infection. In this retrospectively experimental study, sera from 436 sera from individual were analysed through *in-house* ELISA for detection of isotypes (total, IgM, IgG and IgG subtypes 1- 4) during human malaria. In this work, 419 sera from individuals with a clinical history of malaria were tested. As a negative control, a group of 17 sera from healthy Portuguese individuals, who had never been in malaria-endemic area, were used.

4.1. Characterisation of serum samples used to identify immunogenic *Plasmodium falciparum* antigens

The demographic characterisation of the population used in the current study, represented in table 9, was performed by analysing the information extracted from questionnaires (Annex B) and organised in a database. The 419 sera samples were socio-demographically and serologically categorised. All serum donors gave their consent to the interview and answered an inquiry for the collection of socio-demographic data, including age (<20, [20-40[, [40-60[, [60-80[, >80 years), gender (Male, Female), and origin (most are adults who were in different endemic regions of Africa, including Angola, Guinea, Mozambique, Sao Tome and Principe, Cape Verde, Congo, Tanzania, Guinea-Bissau, Nigeria and Morocco; some sera were from individuals of the American continent, including Brazil, Ecuador, Mexico and Haiti, and some sera from Asia, including India and Indonesia). The majority (64%) of the sera used in the current study were from males. Sera were collected from individuals with ages ranging from 6 to 87 years, and the average age was 43.5 years (SD - 13.9 years). The group of adults aged between 21 and 40 years included the highest number of sera.

Sera obtained from 419 individuals with a clinical history of malaria and residing in areas where malaria transmission occurs were considered naturally exposed to *Plasmodium* sp. infections. Thus, 92% of sera were from individuals living on the African continent, and 6.6% were from South America and 1.4% from Asia continent.

Table 9. Demographic characteristics of serum donors. N, absolute number; CI, the 95% confidence interval.

Population		%	N. 419	CI 95%
Gender	Male	64	268	
	Female	36	151	
Age (years)	< 20	2	8	10 - 18
	[20-40[	47	197	30 - 34
	[40-60[	39	163	49 - 52
	[60-80[	11	47	62 - 70
	>80	1	4	
Africa	Angola	53.5	224	
	Cape Verde	3.6	15	
	Guinea – Bissau	6.2	26	
	Morocco	1.0	4	
	Congo	2.6	11	
	Equatorial Guinea	5.5	23	
	Tanzania	0.2	1	
	Mozambique	12.4	52	
	Nigeria	1.0	4	
	São Tomé e Principe	6.0	25	
America	Mexico	0.2	1	
	Ecuador	0.7	3	
	Brazil	5.5	23	
	Haiti	0.2	1	
Asia	Indonesia	0.2	1	
	India	1.2	5	

4.2. *Plasmodium falciparum* total extract is recognised by human immunoglobulins

In this work, we want to identify some protection mechanisms against *P. falciparum* infection. To characterise the reactivity of *P. falciparum* total extract, a total of 436 samples were analysed by *in-house* ELISA and OD index (OD value normalised to the cut-off) determined. The samples, that presented reactivity to total antibodies anti-*P. falciparum* (n=270), were selected for isotypes detection and correlation analysis. The deeper understanding of the role of *P. falciparum* antigens in inducing a humoral immune response during *Plasmodium* infection is crucial for the rational development and deployment of malaria control tools.

4.2.1. Naturally acquired anti-*Plasmodium falciparum* antibodies are predominately IgG, IgG1 and IgG3

Antibody isotype and subclass influence the ability to humoral immune response. Therefore, we characterized naturally acquired antibodies to *P. falciparum* antigens, for possible contribution in acquired immunity. We measured antibody levels by ELISA and converted OD/cut-off, among a selection of malaria-exposed individuals. In this study, naturally acquired anti-*P. falciparum* antibodies are predominately IgG, IgG1 and IgG3. The antigenic activity of total extract of *P. falciparum* was evaluated and presented in figure 17, where it is shown that most of the sera collected from individuals with a clinical history of malaria (64%, N=270), reacted positively with *P. falciparum* antigen. These 270 sera serologically positive for total antibodies against *P. falciparum*, a 72% (N=197) were reactive for total IgG antibodies and a 38% (N=101) were reactive for IgM antibodies anti-*P. falciparum*.
Subtypes IgG1 (43%, n=84) and IgG3 (64%, n= 126) recognised more *P. falciparum* antigens in comparison with IgG2 17% (N=33), and IgG4 19% (N=37), respectively (Fig 17).

Statistical comparisons (Kruskal Wallis) made among the reactivity values (Optical density OD) found for the different isotypes, suggest that IgG isotype recognised better *P. falciparum* antigens than IgM isotype. For IgM antibodies, the maximum value was around 5 OD/cut-off, and for IgG antibodies the maximum value was 16 OD/cut-off.

The comparative analysis between the reactivity of IgG subclass showed statistical differences between IgG1 and IgG4 ($p = 0.03$), IgG2 and IgG4 ($p = 0.021$), and IgG3 and IgG4 ($p = 0.01$). It showed that IgG1 and IgG3 isotypes recognised majority *P. falciparum* antigens than IgG2 and IgG4 subtypes (Fig 18a,18b). The IgM and IgG outliers correspond to sera from the African continent, mainly from the following countries: Angola, Congo, Mozambique, Guinea-Bissau, and São Tomé and Principe. All these donors were male with ages ranging from 45 to 62 years, with an average of 55 years.

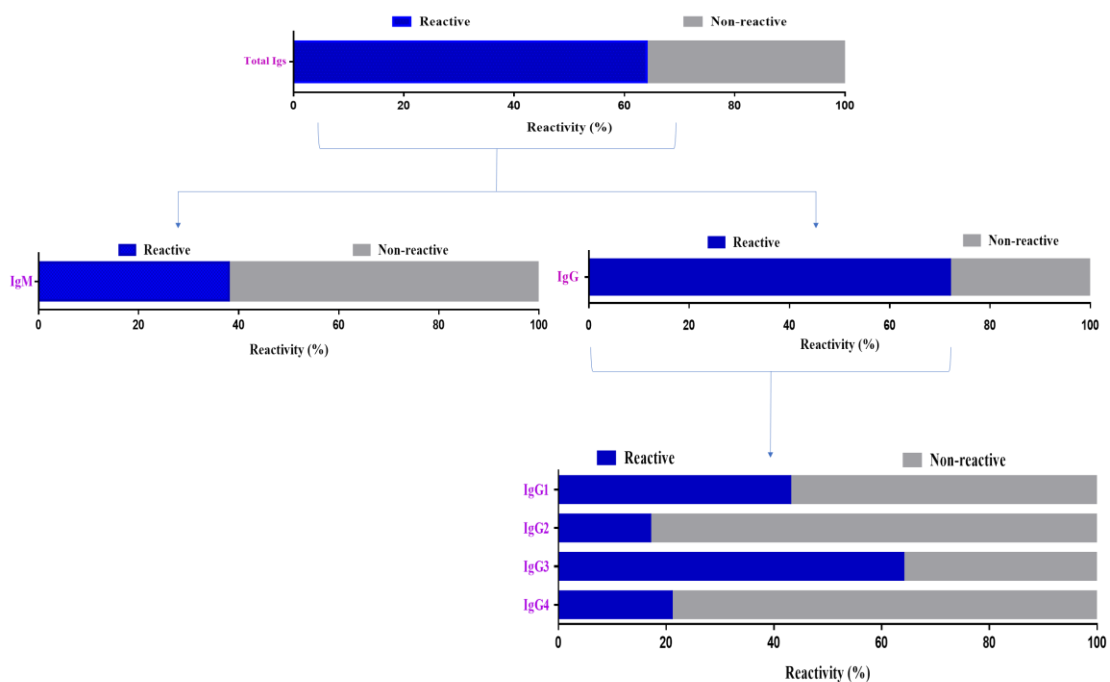

Figure 17. Seroprevalence to total extract of *Plasmodium falciparum*. Reactivity of total extract to total immunoglobulins (total antibodies), IgM, IgG and IgG subtypes (IgG1, IgG2, IgG3 and IgG4) antibodies were evaluated by *in-house* ELISA, using sera of individuals exposed to *Plasmodium* sp. (N=419). The percentages of sera that react with *P. falciparum* antigens are represented in blue bars and non-reactive samples in grey colour.

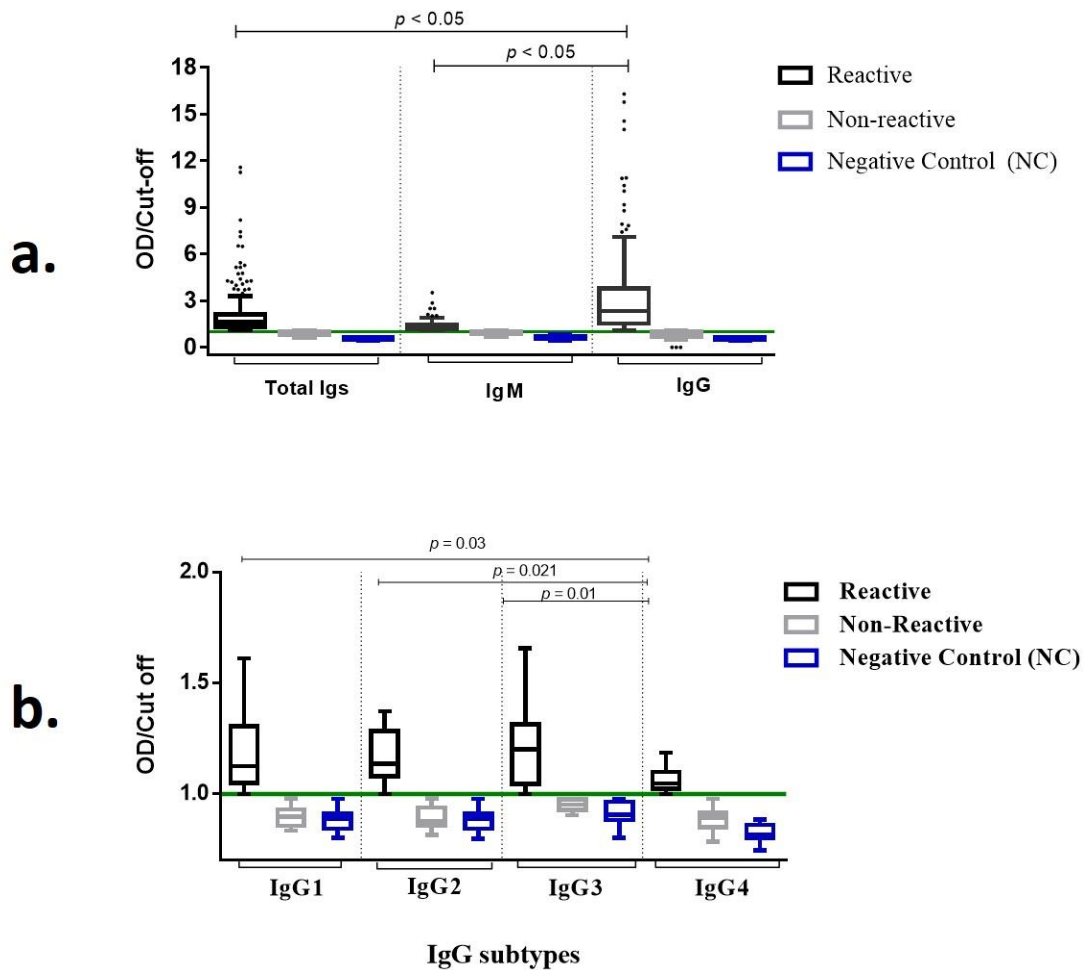

Figure 18. Serological response against *Plasmodium falciparum*. Individual with a clinical history the malaria (N= 419) and individual non-exposed to the parasite (negative control, NC (N = 17) were tested for ELISA. (**18a**) Reactivity for Total Ig, IgM and IgG antibodies anti-*P. falciparum*. (**18b**) Reactivity for IgG subclasses (IgG1, IgG2, IgG3, and IgG4) anti-*P. falciparum*. Results were analysed using the Kruskal Wallis test, and are represented by medians, and interquartile ranges and percentage of positive samples**.** The green line represents the cut-off value that was considered to classify individuals as positive (> 1.0) and negative (<1.0). The OD index (OD normalised by the cut-off) was calculated for each serum, p<0.05.

In this study, results of the serological reactivity detected against total extract of *P. falciparum* are shown in figure 19; in the samples analysed, *Plasmodium* infection seems to induce the production of IgM antibodies in the highest amount of sera (p<0.05) when compared with total Igs (Figure 19a). However, most of the sera samples were positive for IgG when compared with total Igs (p=<0.05, Figure 19b) and IgM (p=<0.05, Figure 19c).

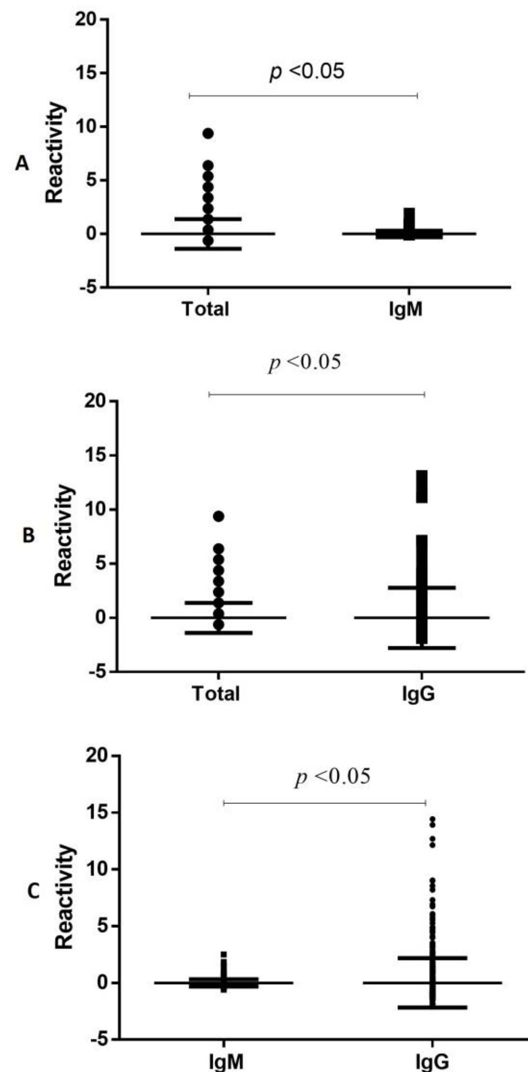

Figure 19. Comparison of serological reactivity against *Plasmodium falciparum*. The student t-test was used to determine the differences among continuous and categorical variables of the OD/cut-off values of total Igs total, IgM and IgG antibodies anti-*P. falciparum*. (**a**) Differences between total Igs and IgM. (**b**) Differences between total Igs and IgG. (**c**) differences between IgM and IgG. Results are represented by aligned dot plot, medians and interquartile ranges**.** Dots indicate outlier values.

4.3. Seroprevalence of isotypes against *Plasmodium falciparum* antigens according to demographic characteristics

To identify the contributions of the epidemiological data on the distribution observed in humoral response to *P. falciparum*, we associated the epidemiological data with the frequency at antibodies response in a heatmap, represented in Figure 20. Two hundred seventy samples, which presented reactivity to total anti-*P. falciparum* antibodies were categorised socio-demographically and used in these analyses. We found higher IgM and IgG antibody prevalence in the individual with age between 20 to 39 years old, and in individuals with less than 20 years. To understand the profile of humoral response against *P. falciparum* and the relationship to epidemiological data, we used a clustered bidimensional heatmap.

The IgM responses were heterogeneous; IgG response was more reactive in sera from Africa and individual with ages between 40-60 years compared to IgM response; nevertheless, IgG1 and IgG3 responses for *P. falciparum* antigens were heterogeneous and not matched with high OD values for sera from Africa, compared with ser from America and from Asia. We also observed a mixed profile of isotype individuals with ages less than 60 years responded to *P. falciparum* antigens but not in individuals with 60 or more years.

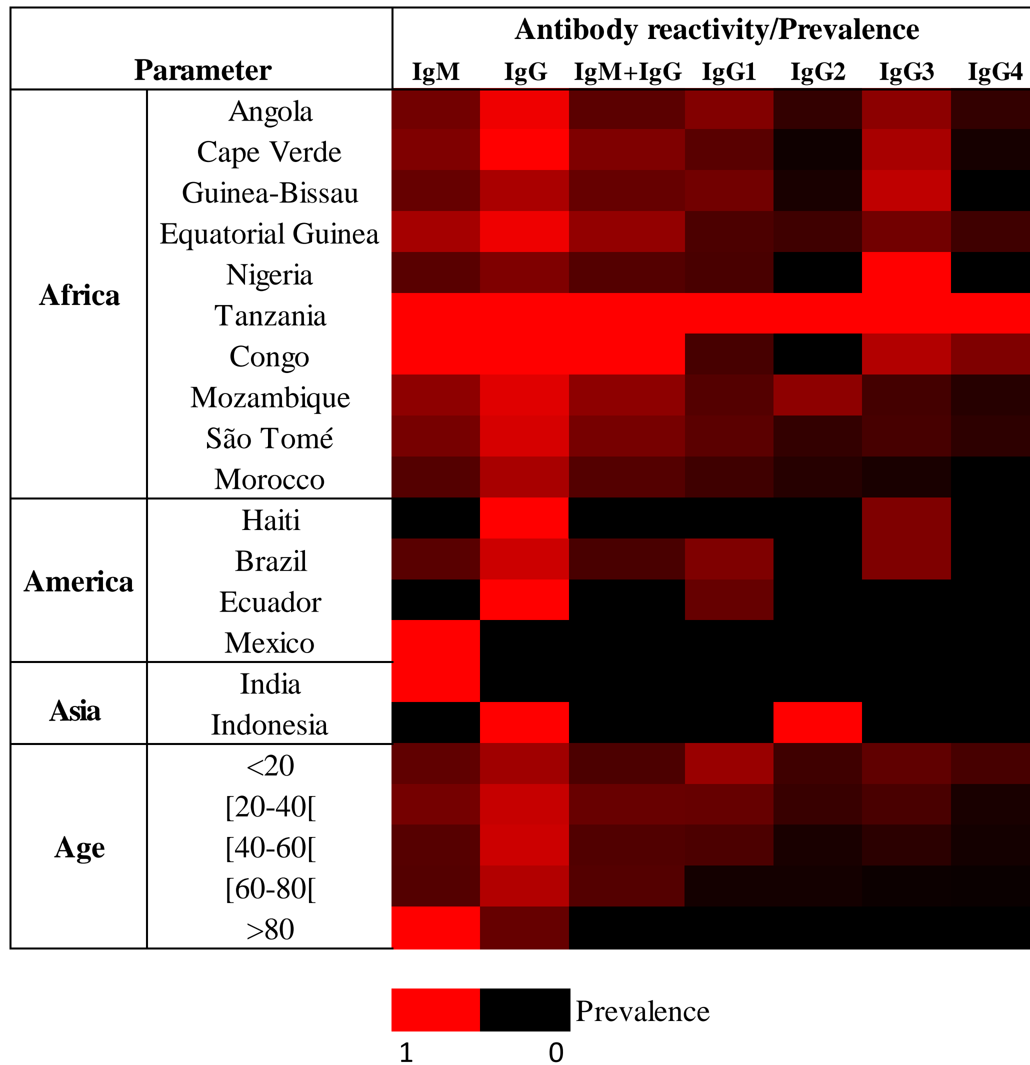

Figure 20. Distribution of antibodies response against *Plasmodium falciparum* antigens according to socio-demographic characteristics of the participants. Clustered heatmap. Each cell represents the isotypes value studied by *in-house* ELISA. The vertical cluster shows the isotypes (IgM, IgG, IgM+IgG, IgG1, IgG2, IgG3, IgG4) along the horizontal axis, and epidemiological data (origin, age). The red colour in the cells indicates high Igs values and the black colour indicates low.

4.4. High antibody responses against *P. falciparum* antigens in sera from African

The antigenic reactivity of *P. falciparum* antigen was evaluated considering the geographical area of the sera, assessing parasite role in the maintenance of humoral immune response, figure 21 shows these findings. IgM antibody showed highest level response in sera from Angola and Tanzania (Fig 21A), and IgG antibody showed highest level response in sera from Angola, Mozambique Tanzania, and Guinea-Bissau (Fig 21B).

Thus, it seems that the antigen, used in this study, can differentiate among zones of major and minor endemicity. In this study, the relationship among malaria-endemic area and the capacity of IgG antibodies to react with *P. falciparum* antigens suggest that IgG antibodies present in sera of individuals from Angola, Mozambique, Tanzania and Guinea- Bissau were capable of recognised total *P. falciparum* antigens.

Only IgM antibodies were detected in sera from individuals who mentioned having been in Mexico and India, which may suggest that these individuals had a brief exposure to the parasite and did not develop IgG antibody. All sera from Tanzania and Congo presented joint serological reactivity of IgM and IgG.

However, the comparative analysis shown in figure 22, suggests that IgG antibodies detected in African sera recognised more *P. falciparum* antigen than IgM antibody ($p= 0.03$), detected in the same population and that IgG antibody response of American sera ($p= 0.05$). IgG1 antibodies of African sera recognised significantly more *P. falciparum* antigen than IgG2 ($p= 0.03$) and IgG4 ($p= 0.04$) antibodies in the same population.

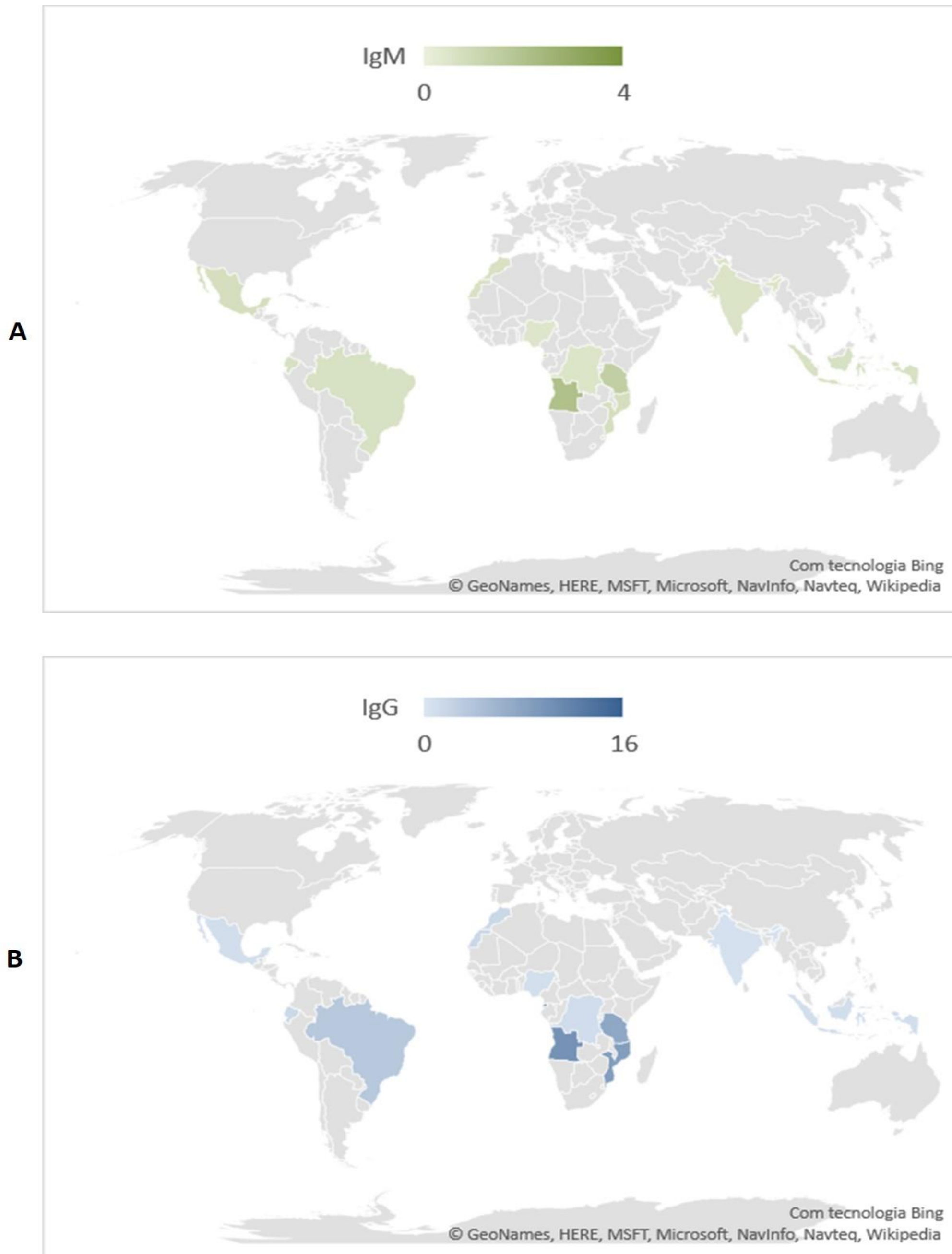

Figure 21. Geographical distribution of IgM and IgG antibody responses against *Plasmodium falciparum* antigens. (**A**) **IgM** antibody response was detected in sera of individuals exposed to *Plasmodium* sp. (N=270) by *in-house* ELISA; the results are shown by geographical distribution according to OD/cut-off values (Minimum 0 – Maximum 3.51). (**B**) **IgG** antibody response was detected in sera of individuals exposed to *Plasmodium* sp. by *in-house* ELISA, using sera of individuals exposed to *Plasmodium* sp. (N=270); the results are shown by geographical distribution, according to OD/cut-off values detected (Minimum 0 – Maximum 16.2).

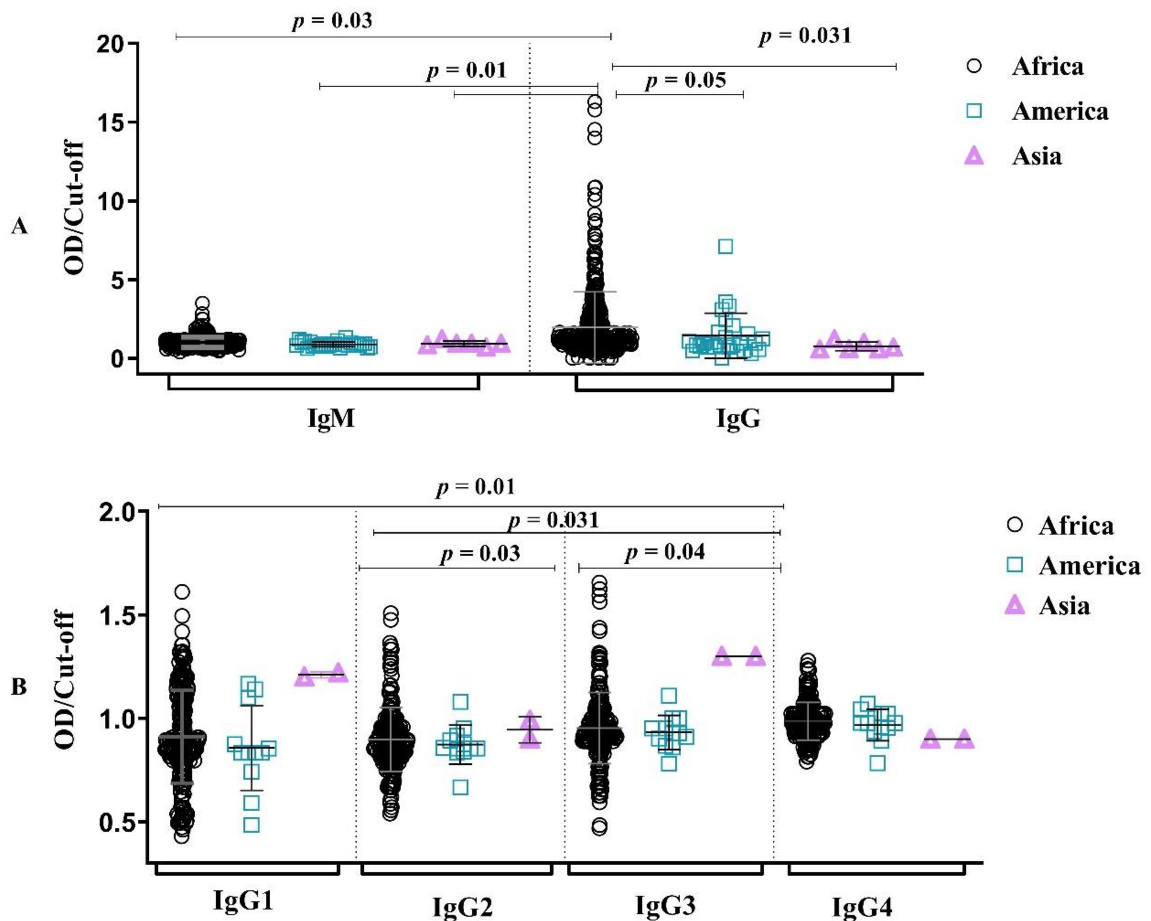

Figure 22. **Antigenic reactivity of *Plasmodium falciparum* antigens according to origin area of the sera**. (**A**) Geographical distribution of *P. falciparum* antigen reactivity to IgM and IgG antibodies and (**B**) Geographical distribution of *P. falciparum* antigen reactivity to IgG1, IgG2, IgG3 and IgG4 antibodies, of sera from individuals exposed to *Plasmodium* sp. (N=270). Results are represented by scattering dot plot, median and interquartile range. The Kruskal–Wallis rank test was used for statistic comparisons.

4.5. Correlation between IgM and IgG anti-*Plasmodium falciparum* antibodies according to malaria-area origin of the sera

To verify if IgM and IgG antibodies would have the same capacity to detect *P. falciparum* antigens, a linear regression analysis was performed, shown in figure 23; the results are shown according to origin area of the sera. Pearson correlation analysis showed a significant positive correlation between IgM and IgG antibody detection by *in-house* ELISA in sera from Africa (r= 0.6521, y= 0.06668*X+0.8598, p<0.05) (Fig23A) and from America (r=0.5442, y= 0.05008*X+0.82119, *p*<0.05) (fig 23B) sera, indicating that *P. falciparum* total extract has an equal capacity to detect IgM and IgG antibodies. But it was not possible to find a good correlation in sera from Asia Pearson r = 0.6917, y =0.5777*X+0.5022, p>0.05).

4.6. Relationship between human isotype levels and the gender of the participants

To evaluate the relationship among antibody's levels and participant gender, the Student t-test was used to determine the differences among continuous and categorical variables of the OD/cut-off values of IgG and IgM antibodies anti-*P. falciparum.* The graphical results of this analysis are shown in figure 24. As expected, there were no statistical differences to IgM (p = 0.548) and IgG (p = 0.741) in all samples tested (Fig 24A and 24B), and in African sera (Fig 24C).

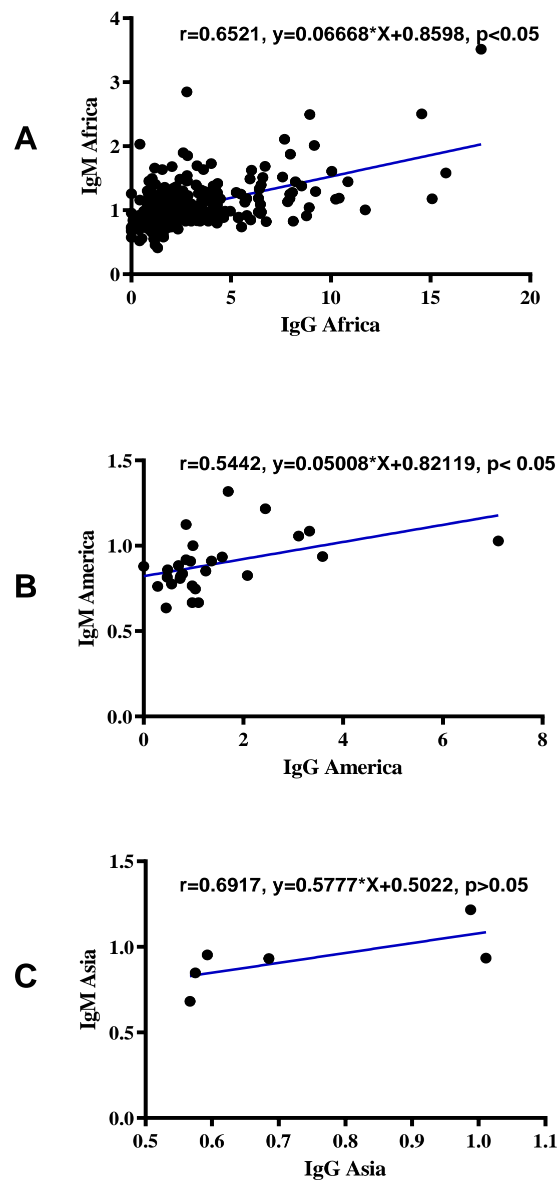

Figure 23. Correlation among IgM and IgG antibody responses according malaria-endemic area of the sera. Correlation coefficients were determined by Pearson correlation method. We show data for sera samples as a dot plots (OD index for IgM and IgG antibodies evaluated by *in-house* ELISA). **(A)** Correlation between IgM and IgG from Africa (Pearson r =0.6521, y = 0.06668*X+0.8598, p<0.05)**. (B)** Correlation between IgM and IgG from America (Pearson r=0.5442, y= 0.05008*X+0.82119, p<0.05) and **(C)** Correlation between IgM and IgG from Asia (Pearson r = 0.6917, y =0.5777*X+0.5022, p>0.05). p-value <0.05 indicates a statistically significant correlation.

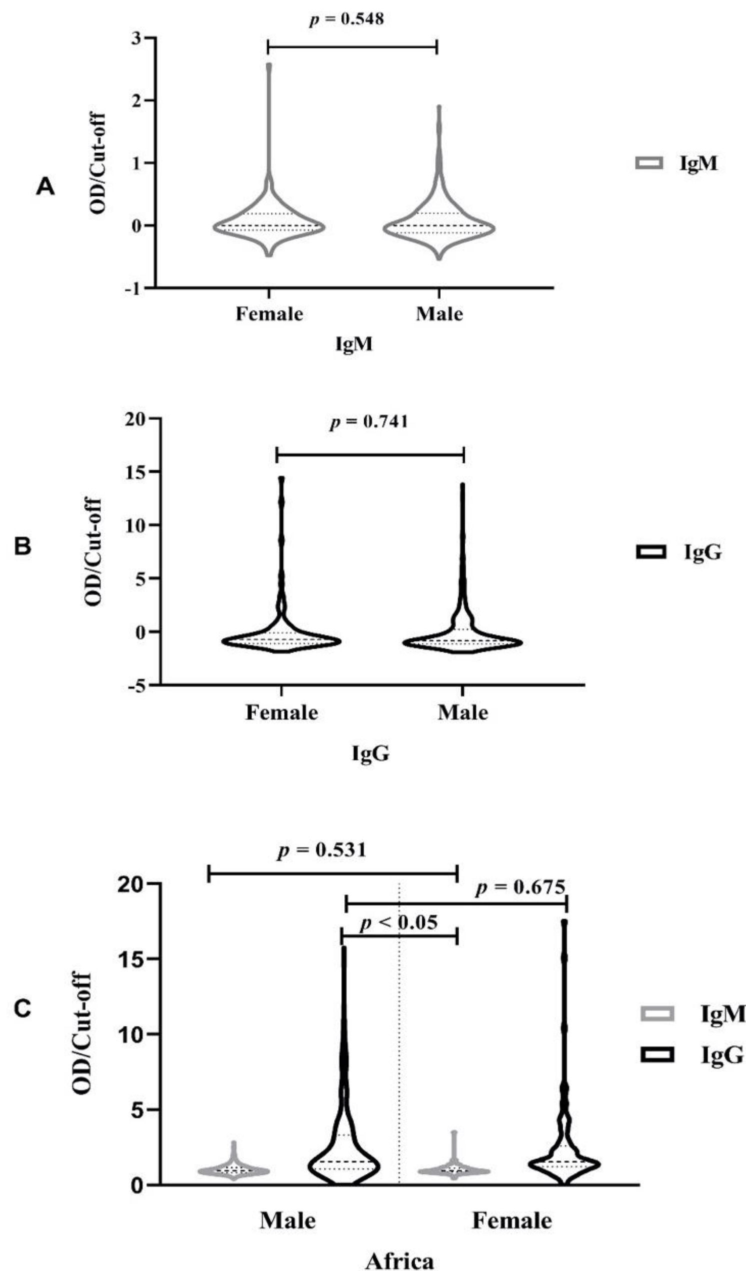

Figure 24. Comparison between serological response against *P. falciparum* and participant gender. **(A)** IgM reactivity index versus gender of all sera. (**B**) IgG reactivity index versus gender of all sera. **(C)** IgM and IgG reactivity index versus gender of the participants from Africa. All samples were evaluated by *in-house* ELISA. Results are represented by violin plot, median and interquartile range. The student t-test was used to determine the differences among continuous and categorical variables of the OD/cut-off values of IgG and IgM anti-*P. falciparum* antibodies.

4.7. Relationship between human isotype levels and age of the participants

In this study, the antigenicity of *P. falciparum* total extract was analysed according to the age of participants, and graphical results are shown in figure 25. No statistic differences were found concerning to the reactivity of the IgM and IgG antibodies, and to the five age groups established (Figure 25A). Curiously, IgG subtypes in the individual older than 80 years did not detect *P. falciparum* antigens (Figure 25B).

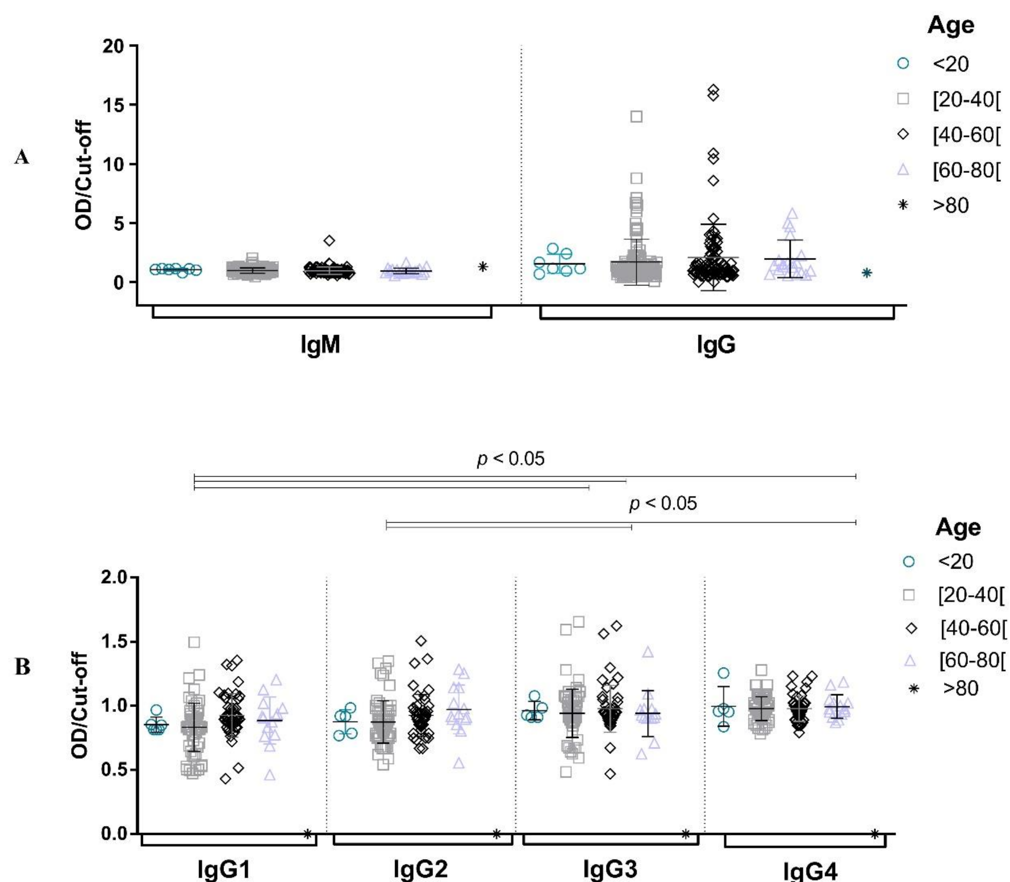

Figure 25. Relationship between anti-*Plasmodium falciparum* antibodies and age (years old) of the participants. (**A**) IgM and IgG antibody response and age. (**B**) IgG subclass IgG1, IgG2, IgG3, and IgG4 and age. All samples were evaluated by *in-house* ELISA, using sera of individuals exposed to *Plasmodium* sp. (N=270). Results were analysed by Kruskal Wallis test and represented by scattering dot, median and interquartile range.

4.8. *In-house* ELISA validation - Concordance analysis of two ELISAs used for detection of antibodies anti-*Plasmodium falciparum*

The capacity of anti-*P. falciparum* antibodies of to react with *P. falciparum* antigens was evaluated by an *in-house* ELISA and compared with results obtained from a commercial ELISA, which incorporates recombinant antigens of *P. vivax*, *P. falciparum*, *P. malariae*, and *P. ovale*. The degree of agreement of two ELISAs, suggesting the possibility of having other important antigens involved in the serological response during human malaria, indicates that total antigens are more reactive by ELISA that recombinant proteins. Using commercial ELISA, increased antibodies reactivity was observed in serum samples, that reached maximum values of about 24 OD/cut-off, whereas with *in-house* ELISA the same sample achieved maximum OD/cut-off values around 14 (Figure 26). Serological reactivity of total antibodies against *P. falciparum* was of 53% for commercial ELISA and 60% for *in-house* ELISA (Table 10). A paired analysis of samples fulfilling acceptance criteria for both methods showed a significant difference (p = <0.05; t student test) (Figure 27).

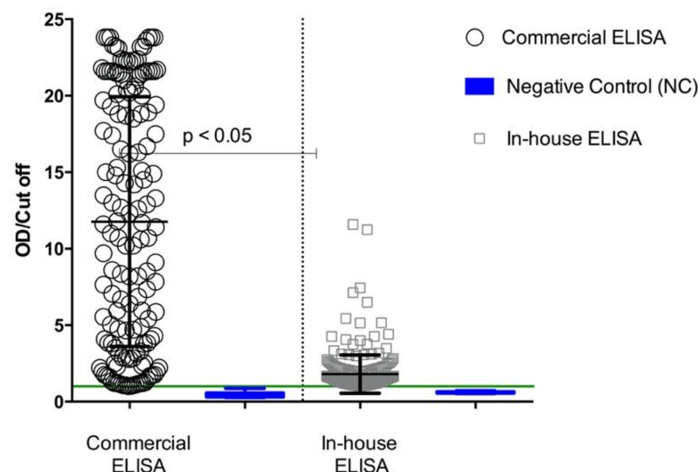

Figure 26. Serological reactivity of *in-house* and commercial ELISAs. Serum samples were evaluated by commercial ELISA that incorporates recombinant antigens of *P. vivax*, *P. falciparum*, *P. malariae* and *P. ovale*; and by *in-house* ELISA (*P. falciparum* total extract). Sera of individuals exposed to *Plasmodium* sp. (n=365) and non-exposed to the parasite (negative control, NC n=17) were evaluated in parallel by the two ELISAs.

The results were analysed by Student t-test and represented by scatter dot plot, medians and interquartile ranges. Green line represents cut-off.

Sera from individuals non-exposed to the parasite (control group n= 17) were identified as non-reactive by *in-house* and commercial ELISAs, indicating 100% of concordance. However, sera from individuals exposed to *Plasmodium* sp. (n= 365) presented 67% (n= 242) of concordance. The agreement between both ELISAs for sera of individuals exposed to *Plasmodium* sp. was 40% (n= 144) of positive sera, and 27% (n=98) of negative sera samples, being the disagreement of 33% (Table 10).

Table 10. Comparative analysis and agreement between commercial and *in-house* ELISAs.

Results	Reactive		Non-reactive		Negative control	
	n	%	n	%	n	%
Commercial ELISA	192	53	173	47	17	100
***In-house* ELISA**	219	60	146	40	17	100
Both ELISAs	144	40	98	27		

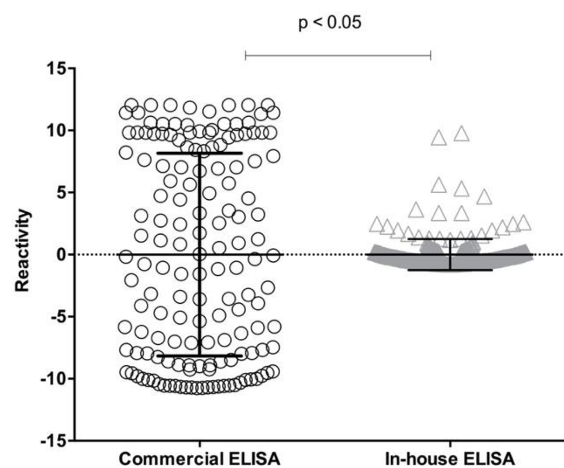

Figure 27. Comparison of serological reactivity between two ELISAs. Serological reactivity of total extract of *P. falciparum* to total anti-*P. falciparum* antibodies was evaluated by commercial and *in-house* ELISAs using sera from individuals exposed to *Plasmodium* sp. (n=365). Results were analysed by Student t-test and are represented by whisker boxes, medians and interquartile ranges, with a representation of the statistical value ($p < 0.05$).

Pearson correlation analysis shows a significant positive correlation of *in-house* ELISA versus commercial ELISA (Pearson r=0.45; p=0.02; y=0,07179*X+1.186). Examination through Bland-Altman analysis shows a good agreement across the range of samples tested, with a bias of less than 5% between both ELISA readings (R square; Rs=0.885; p=0.01; y=1.773*X–2.207) (Figure 28).

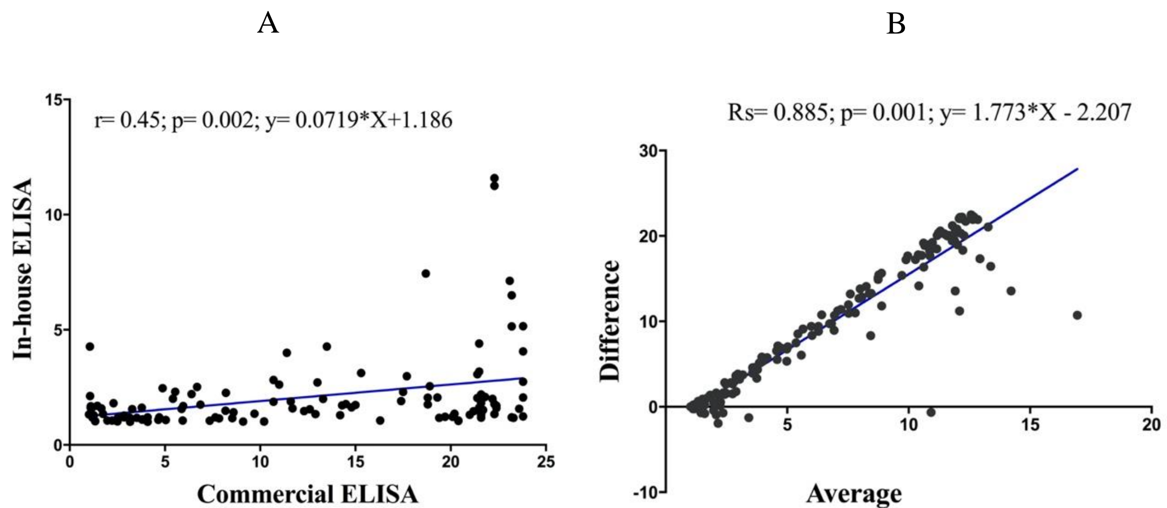

Figure 28. Correlation analysis between commercial and *in-house* ELISAs. Pearson correlation plots evaluated the correlation between commercial and *in-house* ELISAs (a) and Bland Altman plots (b). Blue line represents the linear regression of data. Pearson correlation analysis shows a significant positive correlation of *in-house* versus commercial ELISAs (Pearson r=0.45; p=0.002; y=0.07179*X+1.186). Bland Altman plots show significant correlation between both ELISAs (Rs=0.885; p=0.001; y =1.773*X – 2.207). *p*-value <0.05 indicates a statistically significant correlation.

The quality of *in-house* and commercial ELISAs was expressed through the ROC curve. The area under of the ROC curve measured the accuracy. An area of 1 represents a perfect test; an area of 0.5 represents a worthless test. A rough guide to classify the accuracy of a diagnostic test is the traditional academic point system: 90-100 = excellent, 80-90 = good, 70-80 = fair, 60-70 = poor, 50-60 = fail.

The area under the ROC curve to the *in-house* ELISA was of 89% (95% IC= 0.8464 - 0.9267, SD =0.0204), and 71% to commercial ELISA (95% IC= 0.663 - 0.757, SD =0.0267) (Figure 29).

Antigen performance was evaluated by the following parameters: area under the curve (Table 11), diagnosis parameters showing sensitivity (75%), specificity (57%), positive predictive value (VPP= 66%), and negative predictive value (NPV=68%), shown in table 12.

The formulas used to obtain these variables were:

Sensitivity: PV/ (PV + FN)

Specificity: NV/ (NV + FP)

Positive Predictive Value: PV/ (PV + FP)

Negative Predictive Value: NV/ (NV + FN)

Where PV represents positive values, NV negative values, FN false negative values, and FP false positive values.

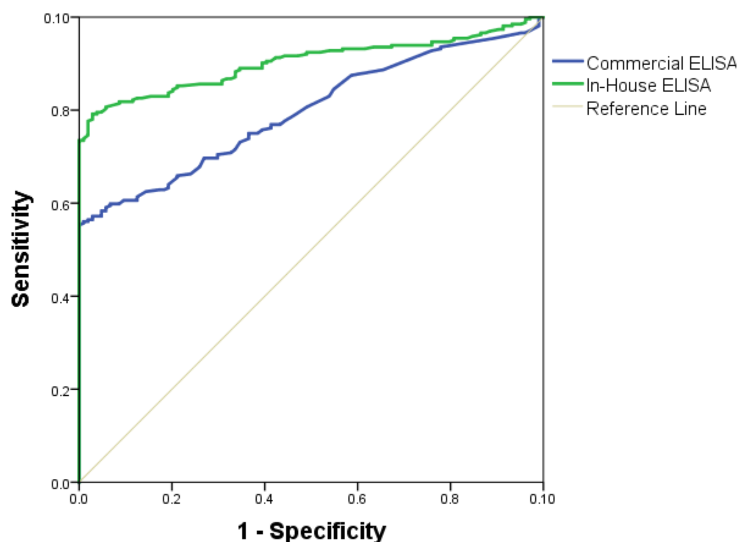

Figure 29. ELISA ROC curves. The Absorbance of sera samples obtained by *in-house* and commercial ELISAs was plotted and ROC curves generated. Blue line represents the sensitivity to commercial ELISA, green line represents the sensitivity of *in-house* ELISA, and black line represents the specificity. The area under the curve was determined with a 95% confidence interval.

Table 11. Parameters of Area Under Curve (AUC) - ROC curves of commercial ELISA and *in–house* ELISAs.

Test	AUC	S	95% CI	*p*-value
ELISA commercial	0.712	0.0267	0.663 to 0.757	<0.0001
ELISA *in-house*	0.891	0.0203	0.876 to 0.937	<0.0001

Abbreviations: AUC (Area under the curve); CI= 95% (Confidence interval); *p*-value (Significance level); S (Sensitivity).

Table 12. Serological reactivity parameters used for the determination of anti-*Plasmodium falciparum* antibodies by *in–house* ELISA.

Parameters	Value	CI 95%
Sensitivity	75%	(0.6826-0.8095)
Specificity	57%	(0.4891-0.6415)
Positive Predictive Value (PPV)	66%	(0.5906-0.7201)
Negative Predictive Value (NPV)	68%	(0.5887-0.7467)
Youden index J	0.873	
AUC	0.886	

Abbreviations: AUC (Area Under the Curve); CI = 95% (Confidence Interval).

In this work, we also compared the methodological conditions of the two ELISAs, in order to persist or detect any significant variation that existed between both protocols, which could be affecting the procedure. For this analysis, we relied on theoretical findings on the optimization of clinical trials.

The main difference found between the two ELISAs was the incubation temperature. For *in-house* ELISA, the antigen (*P. falciparum* total extract) was incubated at 4ºC overnight, unlike commercial ELISA, where the incubation was at 37ºC for 60 min.

Other methodological differences between the procedures used in the current study are shown in table 13.

Table 13. Comparison of the protocols used *in-house* ELISA and commercial ELISA.

Variables of the technique	Commercial ELISA	*In-house* ELISA
Antigen	Recombinant antigen: *P. falciparum, P. vivax, P. malariae, P. ovale*	Crude extract of *P. falciparum* 3D7
Antigen amount	50 uL (1:10 conjugate buffer)	100 ng/10 ml (bicarbonate buffer)
Antigen coating temperature	37°C/60 min	4°C overnigth
Amount of serum sample/ dilution	50 µL Undiluted	1: 200 (0.5 µL)
Incubation primary antibody	30 min/ TA	1 h/TA
Incubation Secondary antibody	30 min/TA	1 h/TA
Enzyme	HRP	HRP
STOP Solution	Sulfuric Acid	Sulfuric Acid
Microplate reader	model 680, Biorad, USA	model 680, Biorad, USA
Absorbance reading	450 nm	405

4.9. *Plasmodium falciparum* antigenic protein fraction complexes with malaria IgG antibodies

It is well established that B-cells and antibodies play a crucial role in malaria immunity. Natural immunity, acquired by individuals living in malaria-endemic areas, is slow to develop and depends largely on the acquisition of a repertoire of specific protective antibodies directed against the antigens of the parasite blood-stage. Protein profile and molecular mass identification (kDa) of *P. falciparum* antigenic fractions were evaluated by electrophoresis. Polyacrylamide gel stained with Coomassie blue was used to select the protein concentration, allowing better gel resolution and better visualization of *P. falciparum* protein fractions on a nitrocellulose membrane. Thus, 14 µg of *P. falciparum*

total extract was applied to each well. Figure 30 shows the electrophoretic profile of the total extract of *P. falciparum* used in this study.

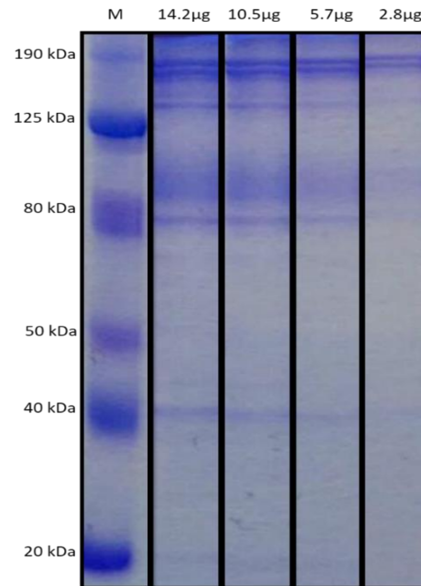

Figure 30. **Electrophoretic profile of *Plasmodium falciparum* total extract**. Electrophoresis of parasite antigen was performed in 12% polyacrylamide gel stained with Coomassie brilliant blue. M: molecular mass marker. Antigen dilutions between 14.2 to 2.87 µg/well were applied to the gel.

To identify the protein fractions of *P. falciparum* antigen that formed immuno-complexes with IgG antibodies against *P. falciparum,* it was performed an immunoblot assay (Western blotting) using a pool of negative control sera (NC), pools of serum samples with high reactivity (5 > OD/cut-off < 16) and pools with high levels of IgG antibodies, and serum samples with low reactivity (1.1 > OD/cut-off < 4.9) to IgG antibodies detected by *in-house* ELISA. Immunoblot analysis revealed a total of approximately 15 antigenic bands with relative masses recognised by sera, which can be seen in figure 31. The antigenic bands were observed between 20 and 190 kDa. Sera from healthy individuals (NC) did not reveal any bands of human sera: high reactive serum samples are represented in figure 31a, and low reactive serum samples are represented in figure 31b. *P. falciparum* protein fractions were categorized as high molecular mass (bands with more than 80 kDa), median molecular mass (bands between 40 and 80 kDa) and low molecular mass (bands with less than 40 kDa).

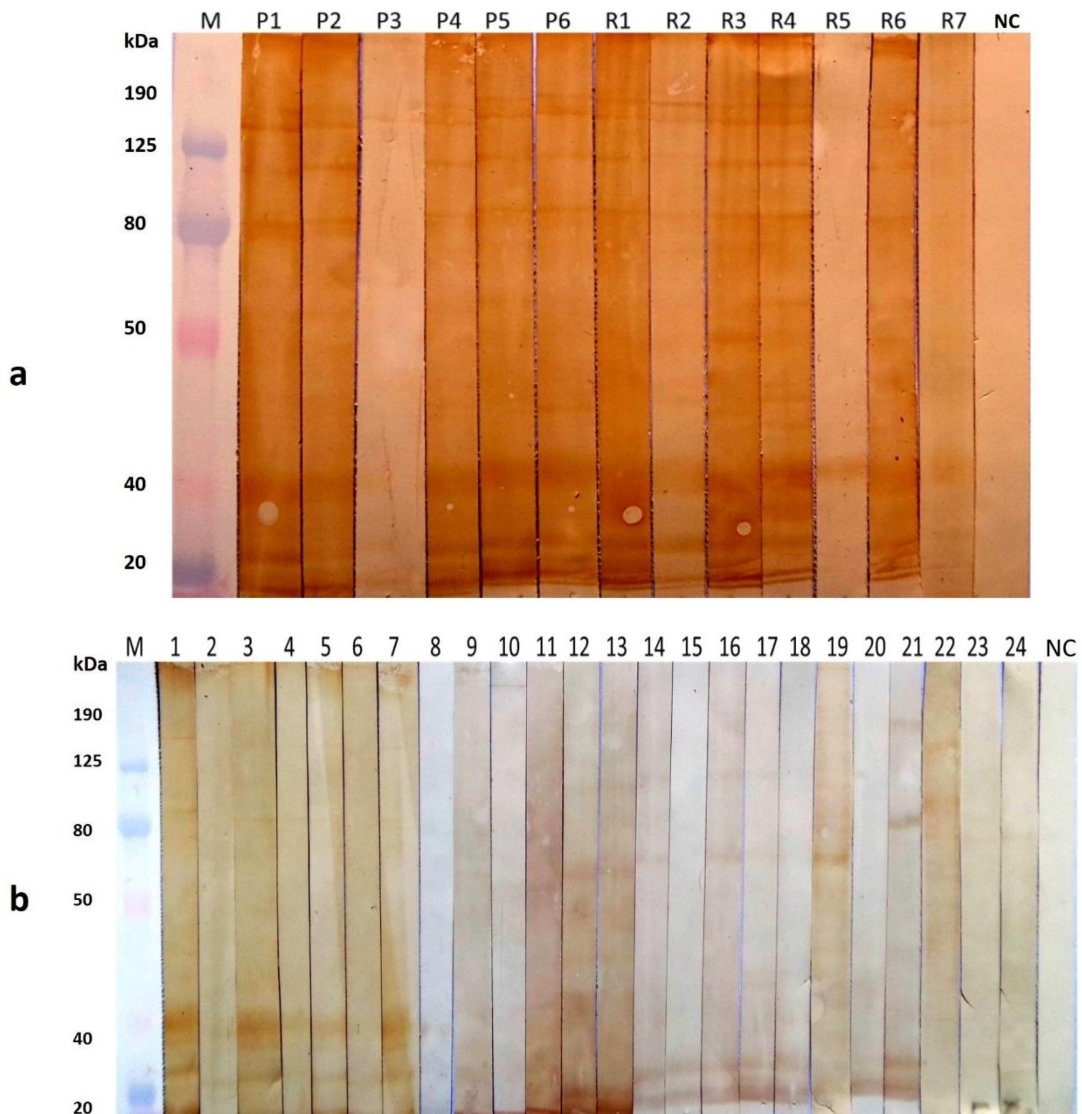

Figure 31. *Plasmodium falciparum* antigen fractions bind to malaria IgG antibodies. *P. falciparum* protein fractions, separated by SDS-PAGE, were transferred to a membrane and incubated with human sera: high reactive serum samples - the strips P1 to R7. (**a**); low reactive serum samples- the strips 1 to 24 (**b**). Each strip contained approximately 14 µg of *P. falciparum* 3D7 extract; M, molecular mass marker (kDa); NC, negative control.

4.9.1. Immunogenic protein fractions of *Plasmodium falciparum* total extract

Immunoprotein studies are crucial for more accurate identification of antigens that can be useful for the development of diagnostic assays and design of prophylactic tools. The antigenicity protein fraction of total extract *P. falciparum* was evaluated by Image *J* software. The Heatmap, in figure 32, shows the antigenicity results of the protein fractions of total *P. falciparum* extract, expressed as a percentage of reactivity when in contact with sera from individuals with malaria clinical history, who had serological reactivity for IgG type antibodies. Protein fraction with a molecular mass of approximately 40 kDa was the most reactive and recognised for a large number of the sera tested. Protein fractions, with a molecular mass between 40 and 80 kDa, were better recognised by both sera groups; protein fractions with a molecular mass greater than 80 kDa, had a heterogeneous recognition for both groups of sera and protein fractions, with a molecular mass inferior to 40 kDa, were faintly recognised by the group of low reactive sera for IgG antibodies.

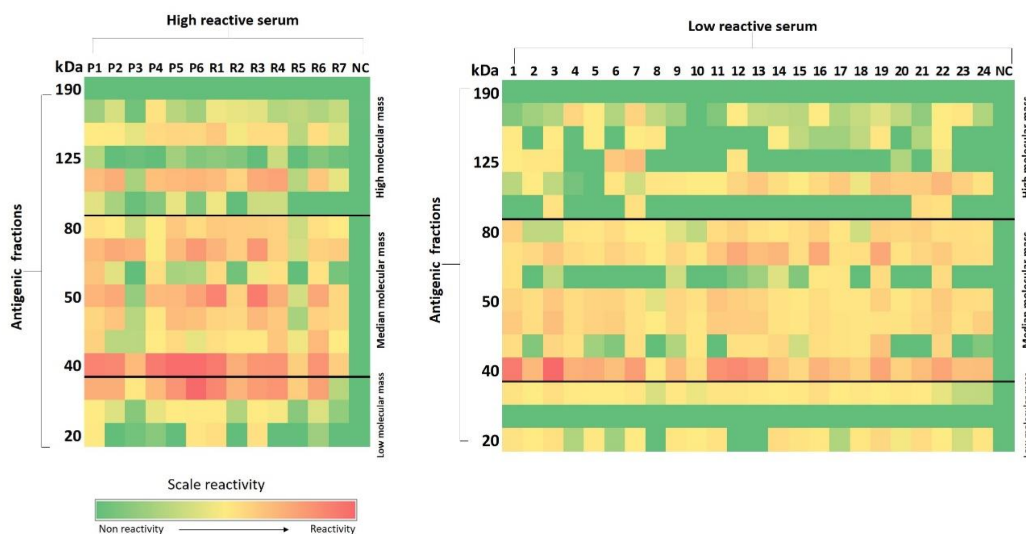

Figure 32. **Antigenicity of *Plasmodium falciparum* extract**. The intensity of the heatmap signal of the *P. falciparum* protein fractions, capable of binding to antimalaria IgG antibodies, was identified by immunoblot. P1 to R7 reactive sera; 1 – 24 indicate low reactive sera. Results represent antigenic reactivity of *P. falciparum* total extract against IgG antibodies and are represented by a standard dual-colour heatmap. Green colour, no-reactivity; red colour, greater reactivity.

4.10. *In silico* prediction of immunogenic epitopes of *Plasmodium falciparum* 3D7 proteins

In this work, it is proposed a new *in silico* methodology that allowed the consensual prediction of *P. falciparum* epitopes. It is expected that the predicted epitopes can be used for the development of new serological assays able to diagnosis *Plasmodium* infection and prophylactic tools, like a vaccine to prevent and avoid malaria expansion.

P. falciparum proteins with antigenic reactivity were previously evaluated by mass spectrometry, and the amino acid sequence was obtained by our research group (Costa *et al.*, 2013). The following sequences were used for *in silico* analysis: (i) Elongation factor-1α (EF-1α), (ii) Protein disulphide-isomerase (PDI), (iii) Phosphoglycerate kinase (PGK), (iv) 78 kDa glucose-regulated protein homolog (GRP-78), (v) Rhoptry-associated protein 2 (RAP-2) and (vi) Rhoptry-associated protein 3 (RAP-3).

4.10.1. Identification of consensual epitopes.

There are specific sequences that indicate the export of proteins. To be exported, the specific peptide sequence must be recognised through the signal peptide, which after being exported, is removed by proteases.

Using BCPred to epitope prediction, 29 epitopes with a specificity above 0.75, score 5 of immunogenicity, and evidencing exposure of amino acid sequence, were identified in this study, which is described in table 14. (see Annex C).

- Five epitopes of EF-1α protein, localised in the cytoplasm, without peptide signal, evidenced the following amino acid sequences: LGGIDRRTIEKFEKESAEMGKG, YSEDRYEEIKKEVKDYLKKVGYQADKVDF, TMEPPKRPYDKPLR, DSKIDKRSGKVVEENPKAIKSGD, SVEKKEPGAVTAKAPAKK);

- Five epitopes of PDI protein, localised in the endoplasmatic reticulum and with peptide signal, presented the following sequences: MNRKYFSSLFLFLISFVFESFVRS, epitopes amino acid sequences: KNKINYGGGRT, TDEKKVEYDEKTP, SNEGRYLLKNPKESLLNHNA, AGKIEKSLKSEPIPEDDKNAP, NKHATNTPISIDGVPEFEDGTSEEL;
- Five epitopes of PGK protein localised in the cytoplasm, without peptide signal, presented the following amino acid sequence: LGNKLSISDLKDIKNKK, IENGIIKDTNR, PDGLRNEKYT, EEGKGVDANGNKVK, NNANTKFVTDEEG);
- Five epitopes of RAP-2 protein localised in plastids, with peptide signal presented following amino acid sequences: MGLKFYVLVFLILCLKNVVKG, EFSKLYPESNS, SEREHLVIKKNPFLRVLNKASTTTHA, HTPYKDYLGDFNKYTEISVL, VNNVISKNKTL, DNKSEYYGTPDDL;
- Five epitopes of RAP-3 protein localised in Plastids, with peptide signal present the following amino acid sequences; MIRKFLISLFLIFLCLNNVVIG, KALIDIDTKDL, EKDASHIVKRTDFLQDILEKADLNNH, EINFFRTFQLEGKPHIPDDQ, GNYNISKNRTR, KNGKEEFFGTPDD;
- Four epitopes of GRP-78 protein localised in the endoplasmatic reticulum, without peptide signal, presented the following amino acid sequence: FFNGKEPNRGINPDE, DKGTGKSRGITITNDKGRLSKEQ, ATVEDKDKLADKIEKEDKNT, QPGGPSPQPSGDEDVDSDEL.

These molecular characteristics suggest that *P. falciparum* PDI, RAP-2 and RAP-3 could be immunogenic proteins with a vital role in host immunity against *P. falciparum* infection. With these characteristics, it is possible that these proteins can be used in a new diagnostic assay or exhibit a protective potential that can be used for the development of a malaria vaccine.

4.10.2. Proposal of the secondary structure of *P. falciparum* proteins

In order to understand which of the analysed proteins have the best immunoreactivity to a *P. falciparum* humoral immune response, with the protein sequence data a figure was projected for each protein, identifying immunogenic sites of interest (epitopes, transmembrane domain, functional sites), which is described in figure 33.

EF-1α protein evidenced a genetic sequence consisting of 443 amino acids, and five epitopes in the following positions (Figure 33a):

- 31-52 (LGGIDRRTIEKFEKESAEMGKG);
- 159-187 (YSEDRYEEIKKEVKDYLKKVGYQADKVDF);
- 220-233 (TMEPPKRPYDKPLR);
- 363-384 (DSKIDKRSGKVVEENPKAIKSGD);
- 426-443 (SVEKKEPGAVTAKAPAKK).

GRP-78 protein exhibited a genetic sequence consisting of 279 amino acids, and four epitopes in the following positions (Figure 33b):

- 138-160 (FFNGKEPNRGINPDE);
- 173-181 (DKGTGKSRGITITNDKGRLSKEQ);
- 197-216 (ATVEDKDKLADKIEKEDKNT);
- 260-279 (QPGGPSPQPSGDEDVDSDEL).

PDI protein showed a genetic sequence consisting of 483 amino acids, a transmembrane domain in the position 6-23, and five epitopes in the following positions (Figure 30c):

- 114-125 (KNKINYGGGRT);
- 205-217(TDEKKVEYDEKTP);
- 306-325 (SNEGRYLLKNPKESLLNHNA);
- 335-358 (AGKIEKSLKSEPIPEDDKNAP);

- 460-483 (NKHATNTPISIDGVPEFEDGTSEEL).

PGK protein evidenced a genetic sequence consisting of 416 amino acids, and five epitopes in following positions (Figure 33d):

- 2-18 (LGNKLSISDLKDIKNKK);
- 29–39 (IENGIIKDTNR);
- 67–76 (PDGLRNEKYT);
- 127-140 (EEGKGVDANGNKVK);
- 293-305 (NNANTKFVTDEEG).

RAP-2 protein showed a genetic sequence consisting of 398 amino acids, a transmembrane domain in the position 2-18, and five epitopes in the following positions (Figure 33e):

- 27-37 (EFSKLYPESNS);
- 154-179 (SEREHLVIKKNPFLRVLNKASTTTHA);
- 194-213 (HTPYKDYLGDFNKYTEISVL);
- 243-253 (VNNVISKNKTL);
- 280-292 (DNKSEYYGTPDDL).

RAP-3 protein evidenced a genetic sequence consisting of 399 amino acids, a transmembrane domain in the position 4-23, and five epitopes in the following positions (Figure 33f):

- 27-37 (KALIDIDTKDL);
- 154-179 (EKDASHIVKRTDFLQDILEKADLNNH);
- 194-213 (EINFFRTFQLEGKPHIPDDQ);
- 243-253 (GNYNISKNRTR);
- 280-292 (KNGKEEFFGTPDD).

Table 14. *In silico* analysis of *Plasmodium falciparum* proteins. Signal peptide; E, exposed epitopes; and SE, functional sites were analysed in Elongation factor-1α (EF-1α), Protein disulfide-isomerase (PDI), Phosphoglycerate kinase (PGK), 78 kDa glucose-regulated protein homolog (GRP-78), Rhoptry-associated protein 2 (RAP-2) and Rhoptry-associated protein 3 (RAP-3).

Protein	Subcellular Localization	Signal peptide	Sequence signal peptide	Exposed Epitopes (E)	Position E	Functional sites (SF)	Function SF	Position	Sequence SF
				LGGIDRRTIEKFEKESAEMGKG	31 - 52	GTP		14 - 21	GHVDSGKS
				YSEDRYEEIKKEVKDYLKKVGYQADKVDE	159 - 187	GTP	GTP Ligation	91 - 95	DAPGH
Elongation factor- 1 alpha (EF-1α)	Cytoplasm	No		TMEPPKRPYDKPLR	220 - 233	GTP		153 - 156	NKMD
				DSKIDKRSGKVVEENPKAIKSGD	363 - 384				
				SVEKKEPGAVTAKAPAKK	426 - 443				
				KNKINYGGGRT	114 - 125			1 - 24	
				TDEKKVEYDEKTP	205 - 217				
Protein disulfide isomerase (PDI)	Endoplasmatic reticulum	Yes Position (1-24)	MNRKYFSSLFLFLISFVFESFVRS	SNEGRYLLKNPKESLLNHNA	306 - 325	Thioredoxin	Signal Peptide	25 - 483	
				AGKIEKSLKSEPIPEDDKNAP	335 - 358				
				NKHATNTPISIDGVPEFEDGTSEEL	460 - 483				
				LGNKLSISDLKDIKNKK	2 -18	Substrate	Glycosylation	39	R
				IENGIIKDTNR	29 - 39	Substrate	Glycosylation	122	R
				PDGLRNEKYT	67 - 76	Substrate	Glycosylation	176	R
Phosphoglycerate kinase (PGK)	Cytoplasm	No		EEGKGVDANGNKVK	127 - 140	ATP	Phosphorylation	215	K
				NNANTKFVTDEEG	293 - 305	ATP	Phosphorylation	312	G
						ATP	Phosphorylation	343	E
						ATP	Phosphorylation	374	D
				FFNGKEPNRGINPDE	138 - 160				
Glucose-regulated protein	Endoplasmatic reticulum	No		DKGTGKSRGITITNDKGRLSKEQ	173 - 181	Heat shock protein	Undetermined	1 - 279	
				ATVEDKDKLADKIEKEDKNT	197 - 216				
				QPGGPSPQPSGDEDVDSDEL	260 - 279				
				EFSKLYPESNS	27-37			1 - 21	
				SEREHLVIKKNPFLRVLNKASTTTHA	154-179			22 - 348	
Rhoptry-associated protein 2 (RAP-2)	Plastids	Yes Position (1-24)	MGLKFYVLVFLILCLKNVVKG	HTPYKDYLGDFNKYTEISVL	194-213	_____	Signal peptide		
				VNNVISKNKTL	243-253				
				DNKSEYYGTPDDL	280-292				
				KALIDIDTKDL	27-37			1 - 22	
				EKDASHIVKRTDFLQDILEKADLNNH	154-179			23 - 400	
Rhoptry-associated protein 3 (RAP-3)	Plastids	Yes Position (1-22)	MIRKFLISLFLIFLCLNNVVIG	EINFFRTFQLEGKPHIPDDQ	194-213	_____	Signal peptide		
				GNYNISKNRTR	243-253				
				KNGKEEFFGTPDD	280-292				

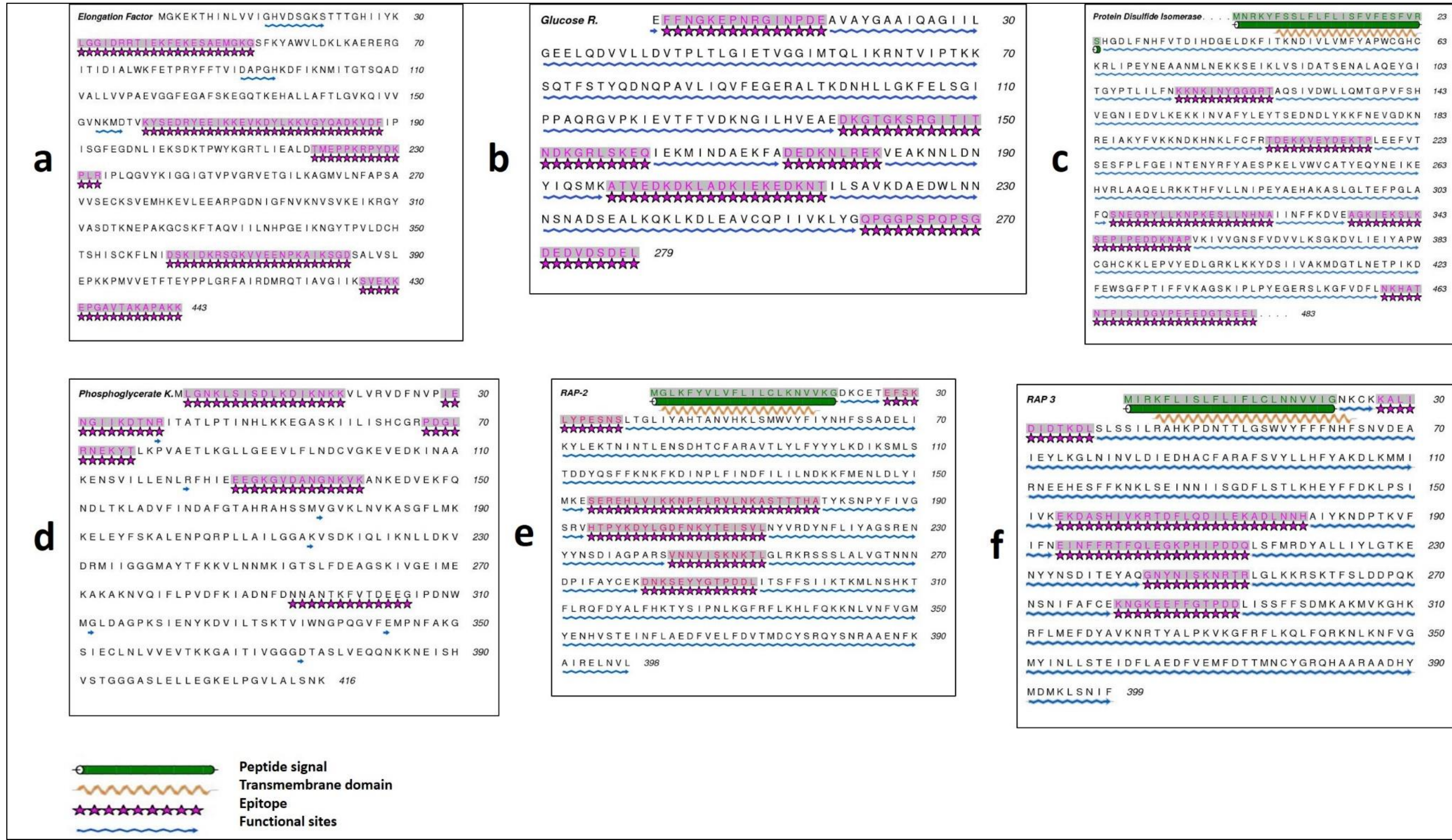

Figure 33. Schematic representation of the genetic constitution of *Plasmodium falciparum* proteins, including functional sites, peptide signal, transmembrane domain and epitopes predictions scores. Elongation factor protein (**a**), Glucose-regulate protein (**b**), Disulfide Isomerase protein (**c**), Phosphoglycerate Kinase protein (**d**), RAP-2 protein (**e**), RAP-3 protein (**f**). The region corresponding to the amino acid sequence was selected using BepiPred and Aline programs. The Green cylinder, peptide signal; yellow spiral, transmembrane domain; pink star, exposed epitopes and; blue line, functional sites.

5. Discussion

5. Discussion

For each stage of the parasite, the mechanism that promotes a protective response to the disease is specific and complex. The development of protective immunity can be slow and only is evidenced after the individual has been exposed over the years to repeated infections (Bull and Marsh, 2002).

The present study aimed to identify immunogenic *P. falciparum* antigens, to characterise the mechanism of an early humoral immune response of malaria, by serologic and antigenic reactivity during *P. falciparum* infection. The profile isotypes measured indicated its importance for parasite control. In the present work, a group of 419 sera from individual naturally exposed to *P. falciparum* infections from a malaria-endemic area (Africa, America and Asia) were analysed.

Recently, many studies have been developed to identify the parasite target protein of *Plasmodium* sp., to understand the exact mechanism by which protection is achieved, and the reason for such protection only to be acquired after repeated exposures over a long time (Crompton *et al*., 2014). The role of antibodies in protective immunity against *P. falciparum* infections has been studied, the passive transfer of anti-*P. falciparum* antibodies contributed to the reduction of parasite density and protection against infection (Grant *et al.*, 2015; Dobbs and Dent, 2016). Antibodies are associated with the control of parasitemia levels by directly inhibiting the invasion of merozoites (Crompton *et al.*, 2014).

Human immunity is an essential factor in determining the outcome of infection, especially among adults in areas of moderate or intense malaria transmission. Partial immunity is developed over years of exposure to malaria, and while it never provides complete protection, it does reduce the risk that malaria infection will cause severe disease. The natural immunity acquired in malaria is complex and poorly understood, varying according to age, endemicity, stage, parasitic species, and specific genetic markers (Artavanis-Tsakonas, Tongren and Riley, 2003).

In areas where malaria is endemic, individuals repeatedly exposed to the infected mosquito gradually acquire relative clinical immunity. This immunity would inhibit the invasion and parasite multiplication (Mombo *et al.*, 2003). In the current study it was showed that antibodies type IgM, IgG and IgG subclass (1,2,3,4) recognise *P. falciparum* antigens, and particularly showed a predominant response of IgG and subclass IgG1,

IgG3, and lower response of IgM, IgG2 and IgG4 antibodies, agreeing with recent studies (Saavedra-Langer *et al.*, 2018; Ubillos *et al.*, 2018). On the other hand, the fact that *Plasmodium* antigens less recognise some antibodies can be a consequence of antibody kinetics (Kurtovic *et al.*, 2018).
During the humoral immune response, IgM is first produced and generated by B lymphocytes in the absence of apparent stimulation by specific antigens. Then IgM progressively disappears or decreases, and IgG is generated (Boes, 2000). There is a probability that most sera are from late infections, and therefore have mainly IgG; and therefore, only few of these sera showed IgM.

In the present study, it was verified that cytophilic antibodies IgG1 and IgG3 recognised manly *P. falciparum* antigens, compared with non-cytophilic antibodies IgG2 and IgG4. Cytophilic antibodies tend to bind to cells, promoting phagocytosis and stimulating cytotoxicity. Cytophilic antibodies, promote phagocytosis and cytotoxicity processes mediated by antibody-dependent cells (ADCC). Unlike the IgG2 and IgG4 subclasses, which are non-cytophilic antibodies, which compete for the same epitopes as the antibodies cytophilic and, thus, blocking their protective activity (Aucan *et al.*, 2000). It has been proposed that the primary role of the humoral immune response directed against merozoite antigens is to act upon the inhibition of merozoite invasion (Beeson *et al.*, 2016), it has also been thought that they can intervene in the opsonisation and phagocytosis of merozoites by monocytes (Osier *et al.*, 2014). Experimental and standardisation tests have been carried out to measure the level of invasion and / or inhibition of antibody growth (Rodríguez-Galán *et al.*, 2017).

Several studies have shown that the humoral immune response against *P. falciparum* in people from endemic malaria areas demonstrated a higher predominance of IgG, IgG1 and IgG3 specific antibodies, and was strongly associated with clinical protection against *P. falciparum* infection (Roussilhon *et al.*, 2007; Wang *et al.*, 2016). Also, other studies have shown that, in malaria-endemic areas, the predominance of cytophilic antibodies is associated with low parasitemia (Shi *et al.*, 1996) and low risk of malaria (Polley *et al.*, 2006).

Cytophilic antibodies IgG1 and IgG3 play a crutial role in merozoite opsonisation and merozoite lysis, being associated with parasite inactivation and, therefore, with malaria protection, as indicated elsewhere (Polley *et al.*, 2006; Tongren *et al.*, 2006; Nebie *et al.*, 2008).

Other studies showed similar results and concluded that IgG3 anti-*P. falciparum* is associated with malaria humoral immunity (Weaver *et al.*, 2016). In addition, MSP3 experimental trials showed that IgG3 antibodies must be strongly associated with clinical protection against *P. falciparum* (Roussilhon *et al.*, 2007; Wang *et al.*, 2016). However, another study performed by Aucan *et al*., (Aucan *et al.*, 2000) reported that antigens *P. falciparum* RESA and MSP2 stimulated mainly the production of antibodies IgG2 and IgG4. The higher reactivity of IgG, IgG1 and IgG3 found in the current study suggest that the antigen used in the serological tests presents an good performance in detecting the antibodies predominate in individuals that developed immunity against malaria (Bouharoun-Tayoun and Druilhe, 1992).

In this study antibodies present in sera from individual with clinical history of malaria recognised *P. falciparum* antigens in both men and women; in the characterisation of the patients, it was demonstrated that there was a more significant antibody response in men than in women; however, a direct relationship between gender and the humoral immune response against malaria has not yet been described. It was observed that the highest immune response presented in both genders was by IgG antibodies; however, both men and women did not show significant statistical differences among types of antibodies. It is also shown, in the literature, that malaria is not a disease related to sex (Reuben, 1993; Parks and Bryan, 2001).

Concerning gender distribution, our results revealed an increase in the distribution of male participants, which might suggest that the male population has more antibodies probably because they are more likely to come into contact with the parasite when exposed to the mosquito bite, possibly due to the type of activities they perform; for example, in some locations on the African continent, men are the ones who regularly work in labour camps until late at night (Dlamini *et al.*, 2017), men go more easily to soccer fields and nightspots where, unfortunately, they are exposed to the *Anopheles* mosquito bites; while women would be more protected because they work in domestic work, regularly stay at home and rarely go out at night (Parks and Bryan, 2001).

In some African communities where there are low levels of education, women have a little more knowledge about malaria as they attend prenatal care in health centers where they receive health education that improves their understanding of the disease's etiology (Reuben, 1993; Parks and Bryan, 2001; Bbosa and Ehlers, 2017); this condition makes women more aware and not easily exposed to the mosquito.

It can also be related to the use of the bed-net, illustrating the variability of the relationship between the use of bed-net and malaria incidence, a study in southern Ethiopia, where the use of bed-nets was frequent, showed that the prevalence of malaria was also high, since men and women, who stay outside the house while socialising or working at night, did not receive any protection from the bed-net mosquito nets (Debo and Kassa, 2016). Other study carried out in Portugal on imported malaria, shown similar results (Askling *et al.*, 2012; Portugal-Calisto *et al.*, 2016). Another study performed in Lundu district, Sarawak, Malaysia, men were seven times more likely to be infected with malaria than women (Jusoh and Shah, 2007).

In this study, IgG antibodies had response against *P. falciparum* total extract at all ages, showing a steady increase in serological reactivity in young adults (20-40 years) and a decrease in young and seniors, this change is probably explained by the fact that protective immunity in malaria is relatively low in the first years of life and in seniors , the senile failure of the immune system would explain this decrease in serological detectability, probably due to a parasite density-reducing effect of naturally acquired immunity (NAI) (Bretscher *et al.*, 2015). However, in Africa, where malaria is endemic, all individuals, regardless of the age, have some degree of immunity (Artavanis-Tsakonas, Tongren and Riley, 2003).

A study performed in children from Mozambique reported that age, the number of previous malaria episodes, current infection, and the neighborhood of residence, were the main factors that influenced serological reactivity against *P. falciparum* antigens (Dobaño *et al.*, 2012). In this study, the highest serological reactivity against *P. falciparum* was in adults between 21 - 40 years, and majority in male individuals, these findings are consistent with the European data on imported malaria concerning malaria distribution by age group, when the age groups of 15 - 24 and 25 - 44 years were who presented most serological reactivity against malaria parasite (*Introduction to the Annual Epidemiological Report*, 2017) [ECDC, 2017]. IgG subclass was not detected in the

senior individual population (older than 80 years), which might be because that these subtypes tend to disappear over time. However, IgM and IgG reactivity levels remain, probably because acquired malaria immunity in endemic area is acquired over the years, probably due to continued parasite exposure (Artavanis-Tsakonas, Tongren and Riley, 2003).

In this study, higher serological reactivity was showed in sera from Africa; the results presented here agree with other studies carried out with samples of hyperendemic areas of malaria, where it was demonstrated that *P. falciparum* antigens in human blood are recognised more frequently by antibodies such as IgG, IgG1, and IgG3 (Roussilhon *et al.*, 2007; Wang *et al.*, 2016; Cherif *et al.*, 2017). These findings are probably related to the complexity and variability of *P. falciparum* antigens. For the knowledge of malaria, it is important to study of the relationship between the antibodies-mediated immune response and the degree of protection in an individual.

A recent study revealed that IgG1 and IgG3 antibodies recognised different *P. falciparum* antigens, but IgG1 showed better responses to *P. falciparum* antigens (HBsAg, LSA-1, MSP-5, P41, RH1, RH2, PTRAMP, RH 4.2, RH 4.9 and SSP2) and IgG3 antibodies better recognised *P. falciparum* antigens (MSP-2, MSP-1 Block 2 and Kh4). Moreover, despite the complexity of the serum used, rich in several types of immunoglobulins due to the constant exposure to the parasite, our antigen showed good reactivity for IgG total, IgG1 and IgG3 anti-*P. falciparum* antibodies. However, in another study, the incidence and response of anti-*P. falciparum* antibodies against *P. falciparum* antigens were also evaluated, where it was evidenced that the association between antibodies - antigens and malaria-endemic area are not always consistent (Fowkes *et al.*, 2010), these immune responses may depend on parasitic antigens or collective characteristics of the malaria-endemic area (Hviid, 2005). It is of great importance to know that anti-*P. falciparum* antibodies present in human sera from individual with clinical history of malaria recognised *P. falciparum* antigens suggesting that these extracts contained some proteins that could be used as diagnosis targets or as a vaccine candidate for malaria.

A study carried out in Senegal to understand humoral immune response to *P. falciparum* infection used an ELISA that include only merozoite antigens (MSP1p19, Pf13-DBL1a), and obtained good seroprevalence responses (Vigan-Womas *et al.*, 2011; Perraut *et al.*, 2014). In this study, a total extract of *P. falciparum* 3D7 was used as antigen in *in-house* ELISA, this antigen was composed by proteins extracted from merozoite, gametocyte, and trophozoite forms; possibly this characteristic contributes to better induction of antibodies response.

A concordance analysis between two ELISA was made. This analysis was done to detect whether proteins or protein fractions, present in the antigen, have could be recognised by anti-*P. falciparum* antibodies, and to see if *P. falciparum* total extract is more or low reactive than the recombinant proteins of commercial ELISA. The results suggest that commercial ELISA, which is constituted by recombinant proteins, has a higher cut-off but identifies a lower number of positive sera. On the other hand, the *in-house* ELISA has a lower cut-off, but it identifies a greater number of positive sera. These findings probably suggest that *P. falciparum* total extract contains a diverse protein from different parasite forms capable of to be recognised by a greater number of antimalarial antibodies. In another study, a comparative analysis was performed using two types of antigens (PfAMA-1 and PfMSP1) alone and combined, concluding that the use of several antigens in the diagnostic test presented greater serological reactivity (84-87%), than when a single antigen is used (Rouhani *et al.*, 2015).

This study indicates that *in-house* ELISA and commercial ELISA are suitable for detecting anti-*P. falciparum* antibodies, since they show a high level of correlation and concordance, which allows the clinical use of any them. However, the variability observed between the ELISA protocols and between the number of reactive samples suggests that the antigen used *in-house* ELISA is more complex, probably containing a greater number of immunogenic proteins capable of to induce a higher response of antibodies against *P. falciparum*. In addition, the concentration of *P. falciparum* antigens used for ELISA plate adsorption is an essential step in the process of optimizing an immunoassay for the determination of antimalarial antibodies.

In this study, serological reactivity for antimalarial antibodies was evaluated by ELISA using different concentrations of the total extract of *P. falciparum* (from 10 to 100 ng/ well). Here, the *P. falciparum* extract concentration, considered appropriate for the ELISA, was 100 ng/ well, since the best serological reactivity (absorbance) was obtained at this concentration. On the other hand, it is known that excess antigen adsorbed to the ELISA plate may cause an "antigen stacking" phenomenon, resulting in the elimination of antigen-antibody complex during the washing steps of the assay. Therefore, in this study, the total concentration of *P. falciparum* antigens of 100 ng / well was considered. *In-house* ELISA is also a technique that could minimize cost difficulties, as it uses reagents prepared *in house* and it is an easy technique to execute that could be closer to be a rapid test or point-of-care tests (POCT), becoming a useful diagnostic for malaria.

When trying to evaluate complex antigens, it is also important to evaluate the assay conditions (incubation and temperature), several antibody responses in sera from different geographic areas, serve to define the best conditions where results with good sensitivity would be obtained. In this study, dilution of conjugated antibodies was used according to the manufacturer's standards. Serum samples were diluted according to the availability of biological material, optimizing a 1/200 (v/v) dilution of all tested samples. In addition, increasing the incubation temperature from 5 to 37°C decreases the affinity of the antigen-antibody complexes by decreasing the stability of the coupling complex (Lipschultz *et al.*, 2002; Voets, 2017).

In *in-house* ELISA, antigens were incubated at 4°C overnight, unlike the commercial ELISA antigen, which was incubated at 37°C for 2 hours. Studies carried out by Lipschultz *et al.* (2002) and Reverberi *et al.* (2007) concluded that the incubation temperature influences the antigen-antibody affinity, as it decreases the affinity of antigen-antibody complexes by declining the stability of the coupling complex (Lipschultz *et al.*, 2002; Reverberi and Reverberi, 2007). Therefore, we suggest that the temperature variations could influence the antigenic reactivity and affect the performance of serological assays. In another study, it was shown that in ELISA, the samples incubation at 4°C overnight gave better performance for the measurement of antigen reactivity, since the antibody is strongly bound to the antigen and there is evidence of increased serological reactivity (Moreno-Perez *et al.*, 2011; Ubillos *et al.*, 2018).

Moreno *et al* (2011) found that soluble antigen rhoptry neck protein 1 (PvRON1) of *P. vivax* performed at 4ºC, presented better antigenicity than when performed at 37°C (Moreno-perez *et al.*, 2011) . Previous studies concluded that the incubation temperature influences the reversible antigen-antibody kinetics by changing the constant association/ dissociation equilibrium (Reverberi and Reverberi, 2007), which can affect the sensitivity of the assay (Tijssen, 1985).

The use of specific and sensitive antigens in the execution of serological diagnostic tests can interfere with the changes or the impact of malaria transmission. Other authors have been interested in evaluating the specific antibody response against *Plasmodium* sp. involved in the innate and humoral immune response (Rouhani *et al.*, 2015). It is important to remember that many antigens, present in the pre-erythrocyte and erythrocyte stages, play an essential role in protecting against malaria (Perraut *et al.*, 2014; Drew *et al.*, 2016; Mclean *et al.*, 2016). Several studies have been carried out on the functionality of *P. falciparum* antigens in the diagnosis, and it has been found, for example, that MRCAg1 chimeric antigen, a potential marker for the detection of malaria, that containing 8 epitopes of *P. falciparum* (Wilson *et al.*, 2011; Offeddu *et al.*, 2012; Yao *et al.*, 2016).

In addition, some studies have been conducted to understand the antibody response against malaria infection using ELISA (Toure-Balde *et al.*, 2009; Perraut *et al.*, 2014), suggesting that ELISA is a good method for evaluating the humoral immune response. However, for rapid and efficient diagnosis of human malaria through immunoassays, it is necessary to develop faster, more specific and more sensitive tests. This analysis is vital because endemic malaria communities would indirectly benefit from having more extensive information on the antigenic reactivity of *P. falciparum* that could be used in the development of new immunoassay. The results obtained in ELISA assays are consistent with the reactivity shown in immunoblotting. The immunoblotting analysis in this study, revealed high reactivity of protein fractions of median molecular mass (40-80 kDa).

Similar results were found in studies of immunoblotting transfer of patients with malaria; these studies reported that *P. falciparum* protein fractions of 100, 75, 50 and 45 kDa, which are probably components of the merozoite coat, presented antigenic reactivity (Crosnier *et al.*, 2013).
Likewise, another study demonstrated that *P. falciparum* proteins fractions of 33-35 kDa exhibited high specificity and antigenicity (Leoratti *et al.*, 2008), there is controversy among studies regarding the immunogenicity of parasite protein fractions which may be related to the *Plasmodium falciparum* strain used or the serum samples used.

Numerous antigens on the surface of merozoites (e.g., Merozoite 1, 2 and 3 surface proteins) and erythrocyte invasive ligands (e.g., erythrocyte binding antigens, invasion ligands PfRh and apical membrane antigen 1) have been identified as crucial targets for the acquisition of immunity and as candidates for vaccines (Roussilhon *et al.*, 2007; Fowkes *et al.*, 2010; Richards *et al.*, 2013; Cibulskis *et al.*, 2016).

Previous studies identified protein fractions with a molecular mass similar to the bands detected in this study; some of these proteins reported in the literature are shown in Table 15. However, these findings do not limit the possibility that our antigen may contain immunogenic proteins that could be diagnostic targets or candidates for a vaccine for malaria. Previous studies evidenced the discovery of serological biomarkers that can, more accurately, identify the existence of recent exposure to *P. falciparum,* not only for a single community but also for several communities of malaria-endemic areas (Cai *et al.*, 2007; Helb *et al.*, 2015).

Previous studies suggest that *Plasmodium* immunodominant epitopes can provide a useful tool for the identification of antigens that can be used as vital targets for vaccine candidates or for malaria diagnosis (Longley *et al.*, 2015). Since the prediction of *P. falciparum* epitopes by computational methodology has been highly useful in finding immunogenic epitopes (Panda and Mahapatra, 2017), in this study, it was performed the *in silico* analysis of the *P. falciparum* proteins EF-1α, GRP-78, PDI, PGK, RAP-2 and RAP-3, which were previously selected for their capacity to react with anti-*P. falciparum* antibodies from individual with a clinical history of malaria.

Table 15. Some *P. falciparum* protein with similar molecular mass finding in this study and reported in the literature.

Estimated molecular mass (kDa)	Putative correspondence	Host immune response	Role	Reference
158	RH4 protein (of 160 kDa)	Antibodies from individuals exposed to malaria show reactivity against RH4.	Involved in the parasite connection to erythrocyte surface.	(Triglia *et al.*, 2009)
150	AARP2 protein (of 150 kDa)	Protein 2 rich in asparagine and aspartate is accumulates in trophozoites and schizonts.	Involved in invasion processes.	(Barale *et al.*, 1997)
125	SERP protein (of 126 kDa)	Involved in humoral response.	Inhibition *P. falciparum* growth *in vitro*.	(Banic *et al.*, 1998; Soe *et al.*, 2002)
97	Protein of 96 kDa	Humoral immune response.	Is recognised for antibodies that inhibiting the reinvasion.	(Jouin *et al.*, 1987)
75	Protein of 75 kDa	Humoral immune response.	Stimulate IgG antibodies production.	(Richman and Reese, 1988)
50	Protein of 51 kDa	Antibodies react with a 51 kilodalton merozoite surface antigen.	Inhibit parasite growth *in vitro.*	(Epping *et al.*, 1988)
47	MSP1 fragment protein of 47kDa	Involved in parasite inoculation.	Necessary for invasion of merozoite into of the erythrocyte.	(Yamauchi *et al.*, 2007)
45	Protein of 45 kDa	Humoral immune response: Epitope elicits antibodies that react with the native antigen.	Inhibits the growth of the asexual blood-stages of the parasite *in vitro*.	(Ramasamy, Jones and Lord, 1990)
42	MSP142 protein of 47kDa	Involves a highly site-specific proteolytic activity, during merozoite release and erythrocyte invasion.	Necessary for invasion of the merozoite into erythrocyte.	(Blackman, Whittle and Holder, 1991; Lyon *et al.*, 2008)
20	MSP133 and MSP119	Induce an antibody response.	Inhibit erythrocyte invasion.	(Guevara Patiño *et al.*, 1997)

The list of immunogenic epitopes of *P. falciparum* proposed in the present study does not match the 386 epitopes of *P. falciparum* and *P. vivax* listed in two epitope databases available on the web (http://www.imtech.res.in/raghava/bcipep and http://www.immuneepitope.org), suggesting that the proposed epitopes, that are not reported in any of these databases, may be new antigenic epitopes of *P. falciparum* proteins, which have not yet been experimentally studied.

The *in silico* methodology used here to identify putative epitopes that can be useful immunogenic biomarkers for malaria diagnosis or as possible vaccine candidates for malaria, showed that RAP-2, RAP-3 and PDI presented better immunogenic characteristics, suggesting that they can be crucial targets to malaria diagnosis, but it is not possible to guarantee which one is the most antigenic, without experimental evidence.

Previous studies have predicted epitopes of *P. falciparum* proteins that might be useful to produce a vaccine against malaria, as is the case of RAP-2 (Dodoo *et al.*, 2002; Damfo *et al.*, 2017). RAP-2 is considered an essential protein in the erythrocyte invasion by merozoites. This protein is transferred from the parasite to the surface of the uninfected erythrocyte during the invasion (Layez *et al.*, 2005; Awah *et al.*, 2009). RAP-3 is a 45 kDa polypeptide that shares 68% of similarity with RAP-2 protein (Baldi *et al.*, 2002), suggesting that they probably share similar important characteristics in immunity acquiring malaria immunity. This protein has an essential role in erythrocyte invasion during *Plasmodium* infection (García *et al.*, 2010), although the mechanism by which RAP-3 binds to the host cell is still not clear (Sterkers *et al.*, 2007).

PDI, a protein located in the endoplasmic reticulum, which appears to have non-negligible participation in the biology of *P. falciparum,* can be a potential antimalarial target (Mouray *et al.*, 2007). PGK is a protein key in the glycolytic pathway and catalysis a stage of phosphorylation that leads to ATP production (Smith, Chattopadhyay and Pal, 2011), since this protein has control over ATP production, experimental studies suggested that PGK inhibition could block glycolysis (Pal *et al.*, 2004). GRP-78 is a molecular chaperone present in the lumen of the endoplasmic reticulum, which maintains endoplasmic reticulum homeostasis (Lee *et al.*, 2014). A study suggested that GRP-78 could be a pharmacological target (Chen *et al.*, 2018). EF-1α plays an essential role in the synthesis, morphology and cellular expression of *Plasmodium;* GRP-78 has two genes

that encode EF-1α (eef1aa and eef1ab), experimental studies have shown that the lack of one these genes can affect the proliferation of parasite blood stages (Janse *et al.*, 2003), this aspect is important because it can contribute to infection control. Overall, the results suggest that the interaction between *P. falciparum* antigens and antibodies are important for maintaining the humoral immune response, as it is suggested that in areas of malaria transmission, protective immunity from malaria can be obtained through the production of the appropriate antibodies.

6. Conclusion

6. Conclusion

The results suggest that *in-house* ELISA is a good technique to detect anti-*P. falciparum* antibodies. Antibodies present in sera of individuals with a clinical history of malaria tested in this work, suggest to be capable to detect *P. falciparum* antigens; we found higher response of IgG isotype, and cytophilic antibodies IgG1 and IgG3 compared to non-cytophilic antibodies, suggesting that there may be a beneficial factor for the host during the pathogenesis and immunity of the infection, since previous studies have shown that these antibodies have protective activity in the *Plasmodium* infection, which would be beneficial for the host. The results also suggest that *P. falciparum* total extract contains protein or protein fractions with immunogenic activity, which could be used as diagnostic in context of human malaria. This analysis is important because malaria-endemic communities would benefit from having more extensive information on the antigenic reactivity of *P. falciparum* that could be used in the developing of new immunoassays.

Reactivity results were analysed in association with epidemiological characteristics (age, gender and origin). The most prevalence anti-*P. falciparum* antibodies were found in individuals from Africa, a major malaria-endemic area; these results can suggest that exposure to the parasite may have influence in the humoral response in malaria infection. However, results showed that, even in human serum from regions with less malaria transmission (America continent) when compared with African continent, a certain degree of immunity could be developed naturally, since in this study it was evidenced that antibodies against *P. falciparum* recognised *P. falciparum* antigens regardless of the origin, but with the greatest contribution of individual from the African continent. These results, though only suggestions towards the identification of proteins that reacted with IgG antibodies specific for *P. falciparum*, can prove new knowledge in the development of new protein targets as vaccine candidates. Additionally, these findings can contribute to a better understanding of the protection role of antibodies in communities exposed to *Plasmodium* sp.

6.1. Final Remarks

- There is natural antigenicity against *P. falciparum* total extract in the population studied.
- *In-house* ELISA is a good technique to diagnose *P. falciparum* infection in Africa.

- Total IgG, IgG1 and IgG3 antibodies are more reactive against *P. falciparum* in sera from individual with clinical history of malaria.
- Sera from individuals of African continent suggest are more reactive against *P. falciparum* total extract.
- Protein fractions of *P. falciparum* with an approximate molecular mass of 25, 35, 40, 45, 50, 75, 80, 100 and 150 kDa were more reactive.
- 29 immunogenic epitopes were predicted from six proteins of *P. falciparum* total extract:

- Five epitopes from PDI [114-125 (KNKINYGGGRT); 205-217(TDEKKVEYDEKTP); 306-325 (SNEGRYLLKNPKESLLNHNA); 335-358 (AGKIEKSLKSEPIPEDDKNAP); (NKHATNTPISIDGVPEFEDGTSEEL)].

- Five epitopes from EF-1α [(31-52 (LGGIDRRTIEKFEKESAEMGKG); 159-187 (YSEDRYEEIKKEVKDYLKKVGYQADKVDF); 220-233 (TMEPPKRPYDKPLR); 363-384 (DSKIDKRSGKVVEENPKAIKSGD); 426-443 (SVEKKEPGAVTAKAPAKK)].

- Five epitopes from PGK (2-18 (LGNKLSISDLKDIKNKK); 29–39 (IENGIIKDTNR); 67–76 (PDGLRNEKYT); 127-140 (EEGKGVDANGNKVK); 293-305 (NNANTKFVTDEEG)],

- Four epitopes from Grp [138-160 (FFNGKEPNRGINPDE); 173-181 (DKGTGKSRGITITNDKGRLSKEQ); 197-216 (ATVEDKDKLADKIEKEDKNT); 260-279 (QPGGPSPQPSGDEDVDSDEL)].

- Five epitopes from RAP-2 [27-37 (EFSKLYPESNS); 154-179 (SEREHLVIKKNPFLRVLNKASTTTHA); 194-213

(HTPYKDYLGDFNKYTEISVL); 243-253 (VNNVISKNKTL); 280-292 (DNKSEYYGTPDDL)].

- Five epitopes from RAP-3 [-37 (KALIDIDTKDL); 154-179 (EKDASHIVKRTDFLQDILEKADLNNH); 194-213 (EINFFRTFQLEGKPHIPDDQ); 243-253 (GNYNISKNRTR); 280-292 (KNGKEEFFGTPDD)].

- *P. falciparum* total extract suggest has protein fractions with immunogenic characteristics, which could be useful in the development of a new serological diagnosis for malaria.

In addition, these findings can contribute to a better understanding of the role of *P. falciparum* antigens in malaria immunity and can provide new insights into the development of malaria control strategies, especially diagnosis and vaccination.

6.2. Future perspectives

Further *in silico* studies will help us understand and know the viability of *P. falciparum* antigens involved in the protection, diagnosis, treatment, and control of malaria, and to evaluate the protective role of different isotypes, especially against other antigens targets with high use in new serological markers and/or vaccine designs.

Although there are numerous studies on malaria protection and immunity, there are still many immunogenic proteins that have not yet been characterized. Therefore, we detected molecular characteristics which we can attribute immunogenicity to proteins that might be critical targets in the development of new serological diagnostic tests or as a candidate vaccine for malaria.

The epitopes predicted in this study could be validated experimentally through the synthesis of a chimeric protein. The potential of this chimeric protein can be evaluated or used in the development of a new serological assay for malaria (ELISA, immunochromatography).

7. References

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8. Annex

8.1. Annex A.

Parecer 4-2012-PN do CEIHMT sobre a utilização de amostras de soros disponíveis no IHMT

O Inv. Doutor Marcelo Sousa Silva apresentou um pedido de parecer sobre a utilização de amostras de soros proveniente de 500 indivíduos e disponíveis no Laboratório Central da UEI Clínica Tropical. O CEIHMT analisou o pedido e observou que:

- A proposta de utilização por fins de investigação oferece garantia de confidencialidade ainda que não descreva o processo de passagem das informações dos doentes para os investigadores com detalhes. Desta forma não oferece risco para os participantes.

- A investigação proposta pode vir a produzir um melhor entendimento da imunopatogenia da malária e da sua relação com a clínica. Para isto será eventualmente necessário obter dados da história do doente que permitiram localizar no tempo e caracterizar o(s) episódio(s) de malária.

O pedido de parecer apresentado oferece uma solução para a ausência de Consentimento Informado aquando da obtenção de sangue que é aceitável pelo CEIHMT. O CEIHMT recomenda no entanto que em caso de armazenamento para fins de investigação de biodados no futuro o Consentimento Informado do indivíduo dador seja obtido.

Com base dessas observações, o CEIHMT dá um parecer favorável e recomenda que mais adiante sejam descritos os procedimentos que garantem o anonimato.

Gilles Dussault

Presidente

Lisboa, 22 de Fevereiro 2012

1

Annex

UNIDADE DE ENSINO E INVESTIGAÇÃO
CLÍNICA DAS DOENÇAS TROPICAIS

Exmo. Senhor Presidente do
Comité de Ética do IHMT
Prof. Doutor Gilles Dussault

Ref.: Pedido de apreciação de um protocolo de estudo retrospectivo intitulado *"Caracterização de anticorpos anti-Plasmodium em pacientes com malária importada".*

Na qualidade de Investigadores deste Instituto, vimos por este meio consultar este Comité de Ética (CEIHMT) relativamente à apreciação de um protocolo de estudo retrospectivo, protocolo este intitulado *"Caracterização de anticorpos anti-Plasmodium em pacientes com malária importada".* Os motivos que justificam tal pedido de apreciação estão apresentados abaixo.

O Laboratório Central do IHMT rotineiramente recebe amostras de sangue de pacientes com suspeita de malária. As amostras de sangue são utilizadas para os testes de diagnósticos convencionais da malária, nomeadamente: (i) os testes parasitológicos - microscopia direta, (ii) teste rápido, e algumas vezes o (iii) teste molecular (PCR). Neste contexto, durante os anos de 2006 a 2011, todas as amostras de sangue, utilizadas para o diagnóstico da malária, foram processadas para fins de diagnóstico e posteriormente armazenadas a uma temperatura de -20ºC. Este procedimento de armazenamento foi anteriormente autorizado pelo Investigador Doutor Luís Távora Tavira, na altura, responsável pelo Laboratório Central do IHMT e atualmente também participante deste estudo.

Nesta data, temos um banco de amostras de soros (aqui denominado de *"soroteca"*) provenientes do diagnóstico laboratorial da malária de aproximadamente 500 indivíduos. Este material encontra-se sob a nossa responsabilidade e nas dependências do atual Laboratório Central da UEI Clínica Tropical. Esta soroteca poderá representar uma valiosa ferramenta de investigação no âmbito do diagnóstico serológico da malária, e também no âmbito da caracterização de mediadores inflamatórios na imunopatogenia da malária aguda.

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UNIDADE DE ENSINO E INVESTIGAÇÃO
CLÍNICA DAS DOENÇAS TROPICAIS

Neste sentido, viemos por este meio solicitar autorização a este CEIHMT para realizar uma linha de investigação exploratória, utilizando esta soroteca como um instrumento de estudo.

Se aprovado por este CEIHMT, os principais objetivos desta linha de investigação, utilizando a soroteca de indivíduos submetidos ao diagnóstico de malária, serão:

1. Determinar, por imuno-ensaio (ELISA), os níveis de anticorpos totais IgG anti-*Plasmodium sp* e anticorpos espécie-específicos anti-*Plasmodium falciparum* em todos os indivíduos representados na soroteca (período de obtenção das amostras compreendido entre os anos de 2006 a 2011);
2. Utilizar os soros com reatividade positiva para malária como reagentes imunoquímicos para a pesquisa de potenciais candidatos antigênicos apresentados na infecção aguda por *Plasmodium falciparum* utilizando as técnicas de *Western Blotting* e proteómica;
3. Definir os subtipos (IgG1, IgG3, IgG4) e subclasses (IgM e IgG) dos anticorpos anti-*Plasmodium falciparum* como possíveis marcadores dos processos inflamatórios na fase aguda da malária humana;
4. Utilizar os dados hematológicos de cada amostra biológica, tais como (i) hematócrito, (ii) hemoglobina, (iii) contagem de eritrócitos, (iv) contagem de leucócitos; para encontrar possíveis correlações com os diferentes níveis de anticorpos anti-*Plasmodium*, e assim contribuir para a definição de alguns dos parâmetros da imunopatogenia da infecção.

Caso este protocolo de estudo seja aprovado pelo CEIHMT, a equipa de Investigadores envolvida neste projecto compromete-se a:

1. Não utilizar os dados pessoais de identificação dos pacientes em nenhum momento do estudo;
2. Não utilizar as amostras biológicas para outros fins não especificados neste protocolo

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de estudo;

3. Não emprestar ou doar as amostras, ou parte delas, a outros investigadores ou outras instituições de investigação;
4. Os resultados deste estudo não trará nenhuma consequência ao diagnóstico ou decisão clínica e terapêutica dos indivíduos, representados aqui pelas as amostras de sangue;
5. As amostras biológicas serão processadas laboratorialmente durante o período mínimo de um ano e máximo de dois anos.

Investigador Responsável:

Doutor Marcelo Sousa Silva

Investigadores envolvidos no projeto:

Doutor Luís Távora Tavira

Doutor Jorge Atouguia

Estudantes envolvidos no projeto:

Karina Pires de Sousa – Bioquímica e estudante de mestrado em Ciências Biomédicas - IHMT/UNL.

Vanessa Martins – Bióloga e estudante de mestrado em Saúde Tropica – IHMT/UNL.

Anaxore Casimiro – Medico e estudante de mestrado em Saúde Tropical – IHMT/UNL.

Rita Costa – Bioquímica, mestre e candidata a doutoramento em Ciências Biomédicas – IHMT/UNL.

Assim, vimos por este meio solicitar apreciação por parte deste CEIHMT.

Com os nossos melhores cumprimentos,

Marcelo Sousa Silva	Jorge Atouguia
Investigador Responsável	Investigador Participante

Lisboa, 22 de Fevereiro de 2012.

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8.2. Annex B.

110 ANOS . 1902-2012
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MEDICINA TROPICAL
UNIVERSIDADE NOVA DE LISBOA

Número de identificação do participante
(a preencher pelo Técnico)

IMUNIDADE EM MALÁRIA:

CONTRIBUIÇÃO PARA O ESTUDO DE ANTICORPOS EM INDIVÍDUOS COM ESTADIA EM ZONA ENDÉMICA

QUESTIONÁRIO

Género __ Masculino __ Feminino

Data de Nascimento: ______-______-________
(Dia) (Mês) (Ano)

Em que país nasceu?______________________ **Após o nascimento, quantos anos viveu nesse país?**_________

Para responder ao questionário considere como país/zona com risco de malária (ou paludismo) os países que constam da tabela de países no fim do questionário.

Obrigado pela sua colaboração.

1. Em que ano esteve pela primeira vez num país com malária (ou paludismo)? ________________________
2. Em que ano esteve pela última vez num país com malária (ou paludismo)? ________________________
3. Quantas vezes esteve em países com malária (ou paludismo)?____________________________________
4. **Alguma vez na vida teve malária (ou paludismo)?**

	Sim
	Não **(passe para a pergunta 7)**
	Não sabe/Não se recorda

(coloque um X no quadrado da resposta)

5. Quantas vezes teve malária (ou paludismo) durante toda a sua vida?______________________________
6. Em que ano ou há quanto tempo teve o último episódio de malária (ou paludismo)? _________________

7. Indique na tabela abaixo os países e regiões com malária (ou paludismo) onde esteve, mesmo que não tenha tido a doença. Responda às perguntas com um X no espaço de resposta. Cada coluna corresponde a uma estadia.

	EXEMPLO:	Descrição da 1ª vez que esteve num país onde existe malária	Descrição da última vez que esteve num país onde existe malária
País e Região onde esteve	Angola, Cuanza Sul		
Ano de Entrada no país	1983		
Ano de Saída do país	1984		
Duração da Estadia	__ menos de 6 meses X 6 meses - 1 ano __ 1 - 5 anos __ 5 - 10 anos __ 10 - 15 anos __ 15 - 20 anos __ mais de 20 anos	__ menos de 6 meses __ 6 meses - 1 ano __ 1 - 5 anos __ 5 - 10 anos __ 10 - 15 anos __ 15 - 20 anos __ mais de 20 anos	__ menos de 6 meses __ 6 meses - 1 ano __ 1 - 5 anos __ 5 - 10 anos __ 10 - 15 anos __ 15 - 20 anos __ mais de 20 anos
Motivo da Estadia	__ Nascimento X Residência/Trabalho __ Serviço Militar __ Visita/Férias __ Estudos __ Voluntariado __ Outro. Qual?______	__ Nascimento __ Residência/Trabalho __ Serviço Militar __ Visita/Férias __ Estudos __ Voluntariado __ Outro. Qual?______	__ Nascimento __ Residência/Trabalho __ Serviço Militar __ Visita/Férias __ Estudos __ Voluntariado __ Outro. Qual?______
Teve malária (ou paludismo)?	X Sim __ Não __ Não sabe/Não se recorda	__ Sim __ Não __ Não sabe/Não se recorda	__ Sim __ Não __ Não sabe/Não se recorda
Quem lhe disse que teve ou não teve malária (ou paludismo)?	__ Médico __ Curandeiro __ Amigos/Familiares/Vizinhos X Eu próprio sabia __Outro. Quem?______ __ Não fiquei doente __ Não sabe/Não responde	__ Médico __ Curandeiro __ Amigos/Familiares/Vizinhos __ Eu próprio sabia __Outro. Quem?______ __ Não fiquei doente __ Não sabe/Não responde	__ Médico __ Curandeiro __ Amigos/Familiares/Vizinhos __ Eu próprio sabia __Outro. Quem?______ __ Não fiquei doente __ Não sabe/Não responde
Fez análises de sangue?	X Sim __ Não __ Não sabe/Não se recorda	__ Sim __ Não __ Não sabe/Não se recorda	__ Sim __ Não __ Não sabe/Não se recorda

Se teve malária (ou paludismo) relacionada com outra viagem, por favor preencha a tabela da página seguinte.

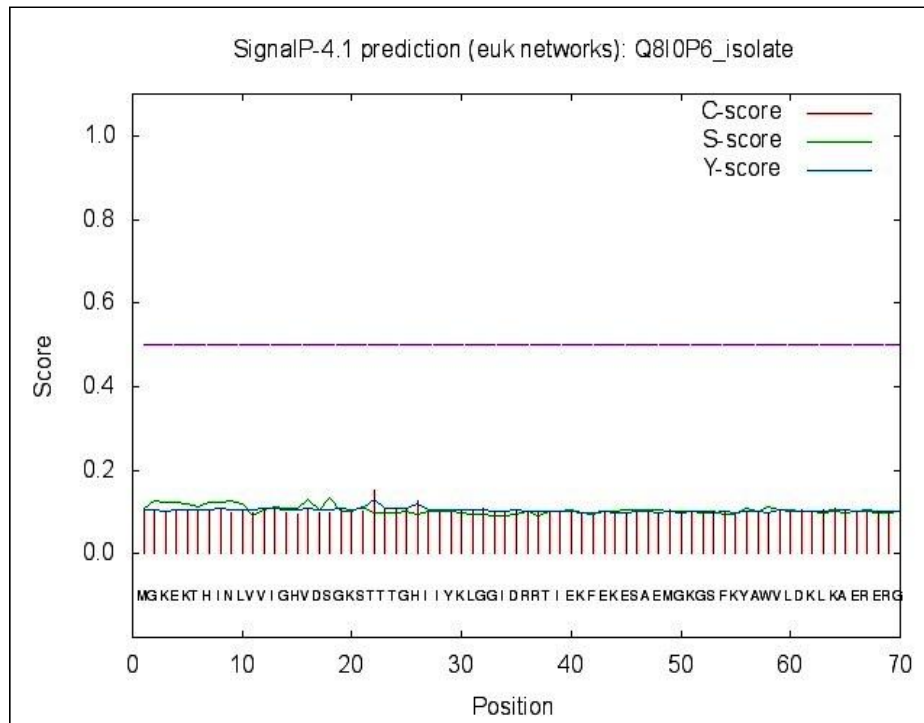
8. Se teve malária (ou paludismo) relacionada com outra viagem, por favor preencha a tabela da página seguinte.

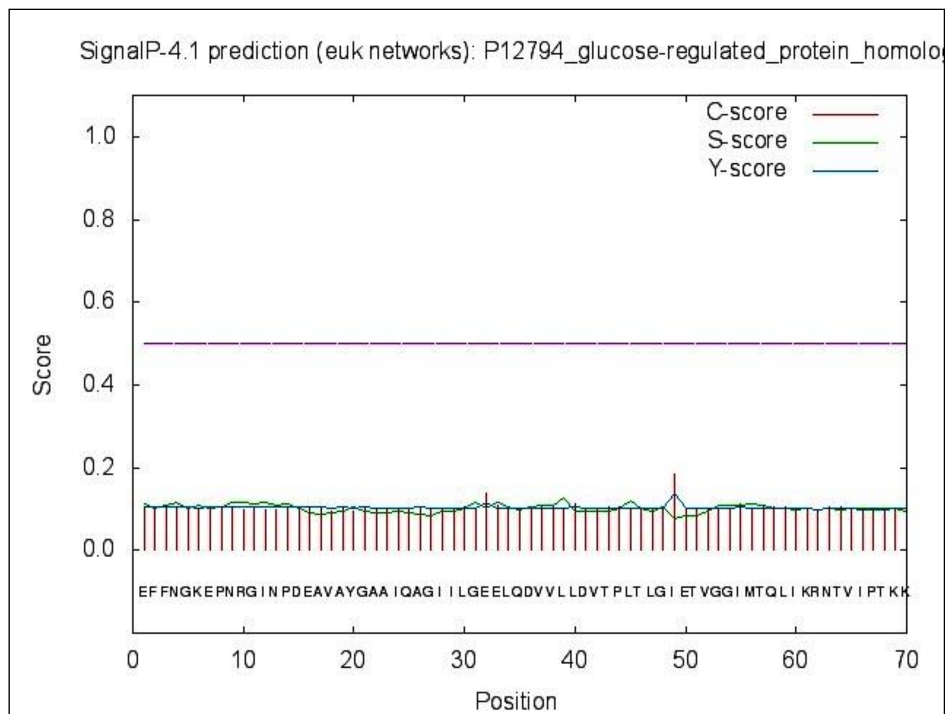
	Outra viagem em que teve malária (ou paludismo)	Outra viagem em que teve malária (ou paludismo)	Outra viagem em que teve malária (ou paludismo)
País e Região onde esteve			
Ano de Entrada no país			
Ano de Saída do país			
Duração da Estadia	__ menos de 6 meses __ 6 meses - 1 ano __ 1 - 5 anos __ 5 - 10 anos __ 10 - 15 anos __ 15 - 20 anos __ mais de 20 anos	__ menos de 6 meses __ 6 meses - 1 ano __ 1 - 5 anos __ 5 - 10 anos __ 10 - 15 anos __ 15 - 20 anos __ mais de 20 anos	__ menos de 6 meses __ 6 meses - 1 ano __ 1 - 5 anos __ 5 - 10 anos __ 10 - 15 anos __ 15 - 20 anos __ mais de 20 anos
Motivo da Estadia	__ Nascimento __ Residência/Trabalho __ Serviço Militar __ Visita/Férias __ Estudos __ Voluntariado __ Outro. Qual?__________	__ Nascimento __ Residência/Trabalho __ Serviço Militar __ Visita/Férias __ Estudos __ Voluntariado __ Outro. Qual?__________	__ Nascimento __ Residência/Trabalho __ Serviço Militar __ Visita/Férias __ Estudos __ Voluntariado __ Outro. Qual?__________
Quem lhe disse que teve malária (ou paludismo)?	__ Médico __ Curandeiro __ Amigos/Familiares/Vizinhos __ Eu próprio sabia __Outro. Quem?__________ __ Não sabe/Não responde	__ Médico __ Curandeiro __ Amigos/Familiares/Vizinhos __ Eu próprio sabia __Outro. Quem?__________ __ Não sabe/Não responde	__ Médico __ Curandeiro __ Amigos/Familiares/Vizinhos __ Eu próprio sabia __Outro. Quem?__________ __ Não sabe/Não responde
Fez análises de sangue?	__ Sim __ Não __ Não sabe/Não se recorda	__ Sim __ Não __ Não sabe/Não se recorda	__ Sim __ Não __ Não sabe/Não se recorda

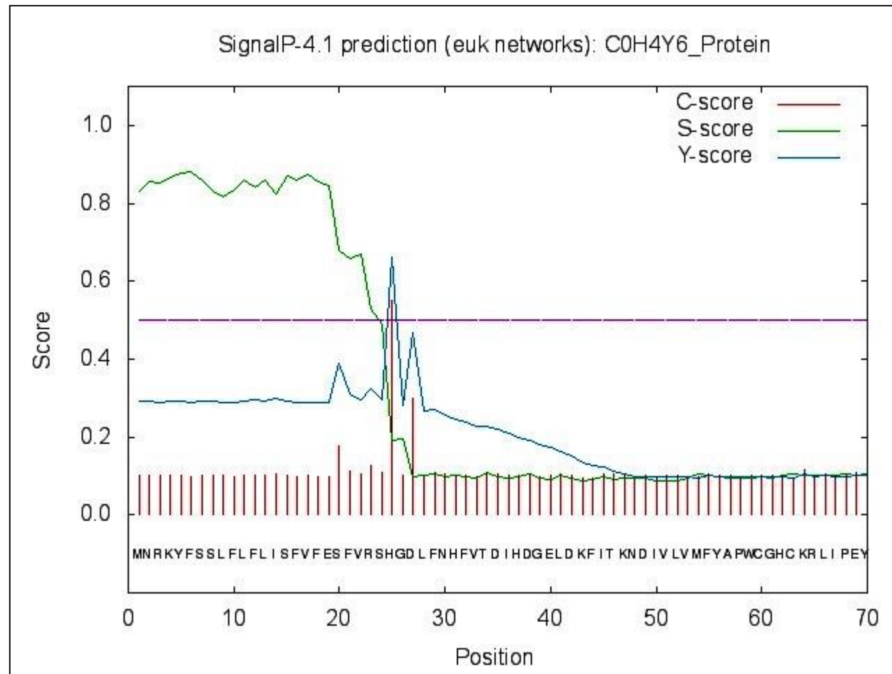
NOTA: SE TEVE MAIS EPISÓDIOS DE MALÁRIA PEÇA NOVO QUESTIONÁRIO E PREENCHA APENAS OS ESPAÇOS NO QUADRO.

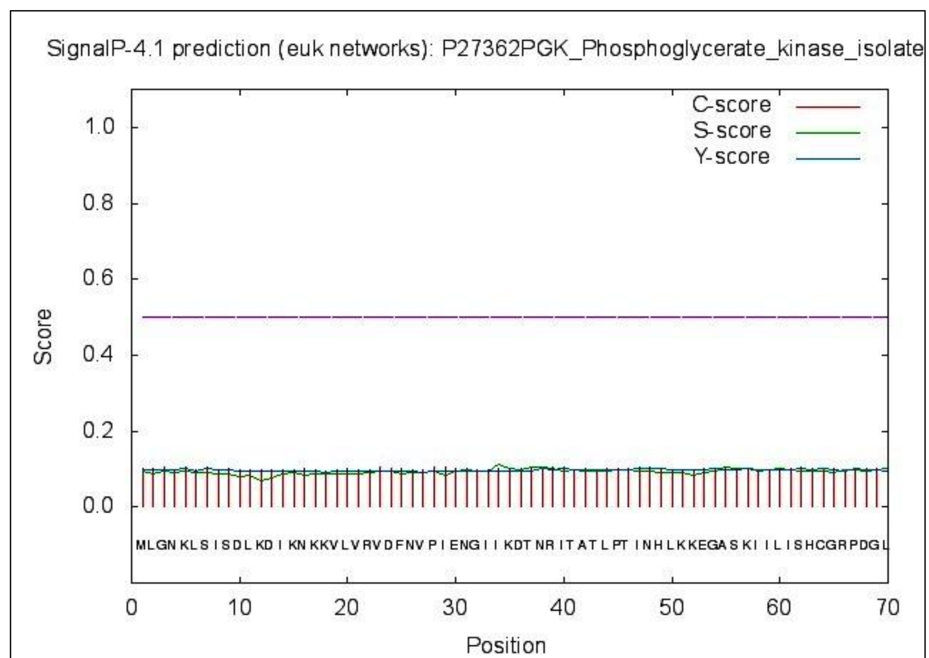
8.3. Annex C.

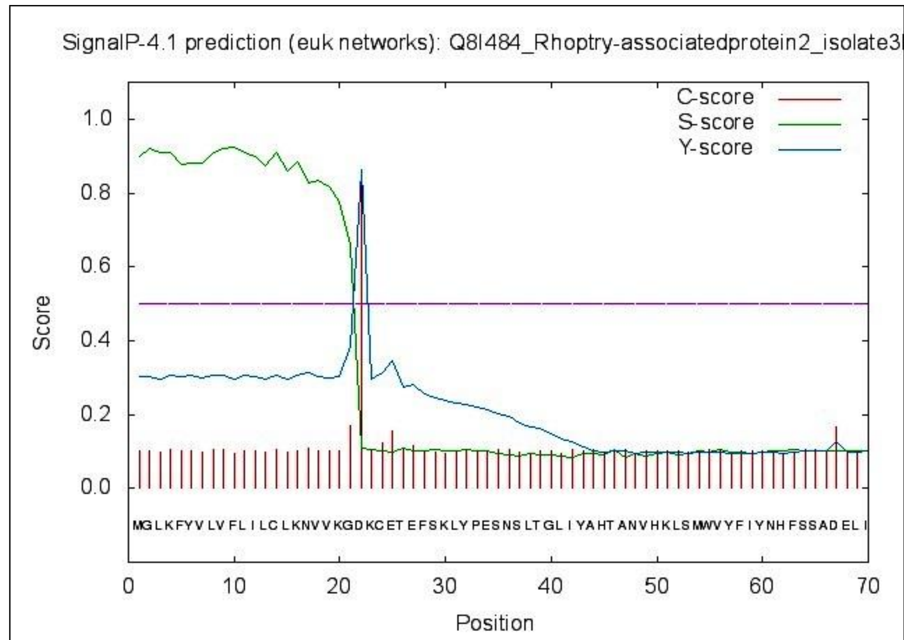
Signal P. Prediction Results

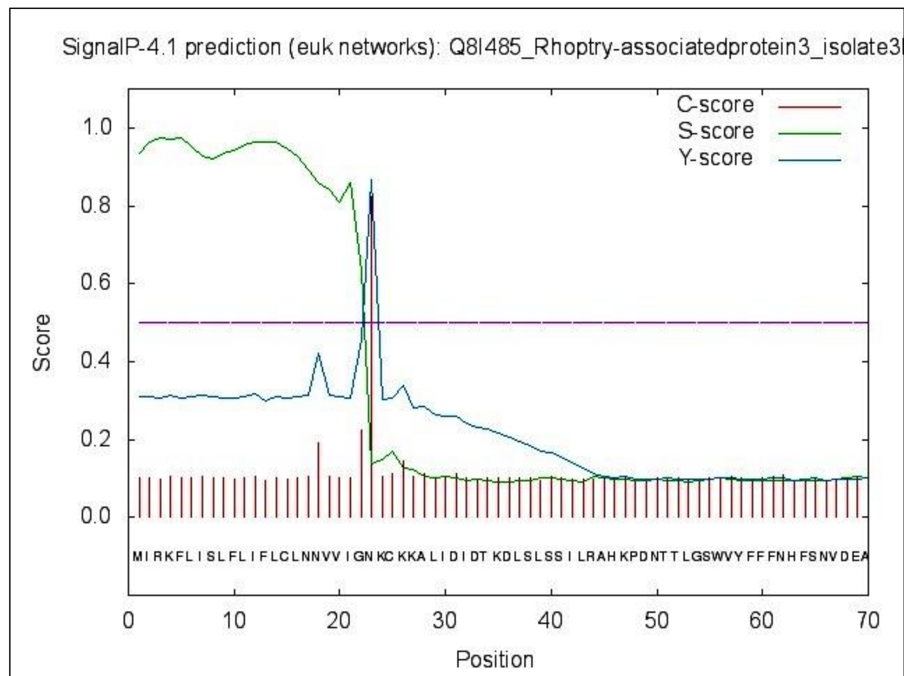





SignalP-4.1 prediction (euk networks): C0H4Y6_Protein
C-score
S-score
Y-score
Score
1.0
0.8
0.6
0.4
0.2
0.0
MNRKYFSSLFLFLISFVFESFVRSHGDLFNHFVTDIHDGELDKFITKNDIVLVMFYAPWCGHCKRLIPEY
0
10
20
30
40
50
60
70
Position


SignalP-4.1 prediction (euk networks): P27362PGK_Phosphoglycerate_kinase_isolate
C-score
S-score
Y-score
Score
1.0
0.8
0.6
0.4
0.2
0.0
MLGNKLSISDLKDIKNKKVLVRVDFNVPIENGIIKDTNRITATLPTINHLKKEGASKIILISHCGRPDGL
0
10
20
30
40
50
60
70
Position


SignalP-4.1 prediction (euk networks): Q8I484_Rhoptry-associatedprotein2_isolate3
C-score
S-score
Y-score
Score
Position
MGLKFYVLVFLILCLKNVVKGDKCETEFSKLYPESNSLTGLIYAHTANVHKLSMWVYFIYNHFSSADELI


SignalP-4.1 prediction (euk networks): Q8I485_Rhoptry-associatedprotein3_isolate3
C-score
S-score
Y-score
Score
Position
MIRKFLISLFLIFLCLNNVVIGNKCKKALIDIDTKDLSLSSILRAHKPDNTTLGSWVYFFFNHFSNVDEA

Article

Comparative Analysis of the Serological Reactivity of Individuals with Clinical History of Malaria using Two Different ELISA Tests

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Abstract: Early diagnosis of malaria reduces disease, prevents deaths, and contributes to decreased malaria transmission. The use of specific and sensitive antigens in the execution of serological diagnostics may have an impact on the transmission of the disease. However, many individuals cannot be easily diagnosed by serological tests due to low levels of antibodies in the serum. Using two different Enzyme-Linked Immunosorbent Assay (ELISA) tests (a commercial and an in-house ELISA), a total of 365 serum samples from individuals with a clinical history of malaria were analyzed. From the serum samples analyzed, 192 (53%) samples from the commercial ELISA and 219 (60%) samples from the in-house ELISA presented positive serological reactivity to malaria. The concordance of the samples tested (n = 365) between both ELISAs was of 67% (n = 242), and with the negative control was 100% (n = 17). We demonstrated that the in-house ELISA showed high antigenic reactivity to *Plasmodium falciparum* antigens when compared with the commercial ELISA. The degree of concordance of both ELISAs suggested the possibility of existence of other *P. falciparum* antigens present in the crude extract of *P. falciparum* that are important in the serological response during malaria infection.

Keywords: *Plasmodium falciparum*; malaria; ELISA; serological diagnosis; serological markers

1. Introduction

Early diagnosis and treatment of human malaria reduces disease, prevents deaths, and contributes to reduced transmission [1]. Detection of antibody response against malaria antigens is often done through Enzyme-Linked Immunosorbent Assay (ELISA) [2]; many of these diagnostic assays are designed with a single antigen or with recombinant antigens of different stages of the parasites (tissue infection or blood), where merozoite surface proteins can be detected, and have demonstrated good sensitivity and specificity [3]. However, many individuals cannot be easily diagnosed due to the low levels of antibodies present in their blood. In this context, the use of ELISA as a tool for laboratory diagnosis of malaria has some limitations, namely: (i) low antibody titres presented by individuals in the acute phase of infection; (ii) many *Plasmodium* sp. are involved in human malaria; (iii) different evolutionary forms of *Plasmodium* spp. during infection and life cycle; and (iv) different