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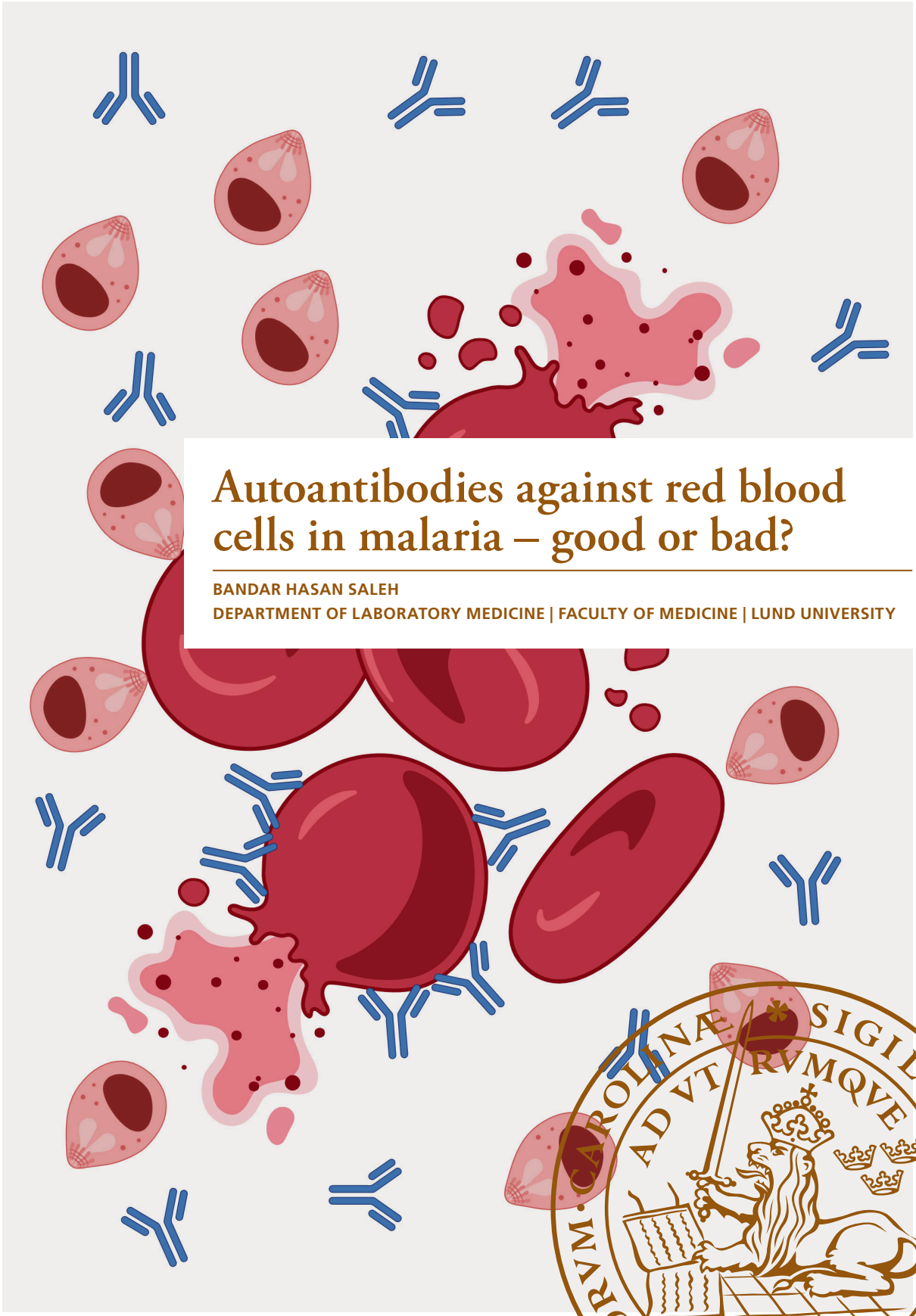

Autoantibodies against red blood cells in malaria – good or bad?

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Bandar Saleh

LUND
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Abstract:

Malaria is a life-threatening disease today occuring mostly in tropical regions and it is transmitted through the bite of female Anopheles mosquitoes. Many species of Plasmodium infect humans where P. falciparum is the most fatal. Red blood cells (RBCs) are the main target where the parasite replicates. A better understanding of how naturally immune individuals control infections could facilitate future vaccine studies. This thesis investigates the ambiguous role of autoantibodies against RBCs in malaria development. We have investigated healthy adults living in a malaria endemic area in Uganda and found that more than half had antibodies against RBCs. Specific antibodies directed against RBC surface antigens, incriminated in merozoite invasion such as glycophorins A, B and C, as well as JMH (SEMA7A), were detected, and potentially these antibodies could be beneficial. Other antibodies had partial specificity against the blood group antigens Rh, Diego, and Cromer. 15% were DAT-positive and 28% were parasite positive (by PCR), and these had higher anti-parasite extract IgG levels and more inhibition in growth/invasion assays, however there was no correlation with presence of antibodies against RBCs. We also measured different inflammatory markers and found some Ugandans to have low haptoglobin levels and more than half had low orosomucoid levels. There was no correlation with PCR-positivity or anti-RBCs or anti-parasite antibodies, and in general there were very few signs of inflammation in spite of presence of parasites.

In contrast to the above possibly beneficial autoantibodies, anti-Phosphatidylserine (PS) antibodies have been linked to anemia, a common complication in pregnancy. The levels of anti-PS IgG antibodies were investigated in pregnant Ugandan women. Anti-PS inversely correlated with packed cell volume (PCV) but not with anti-VAR2CSA IgG or parasitemia, indicating a multifactorial modulation. Anti-PS levels were lowest in multigravidae which may explain the better control of anaemia in these individuals. During the first year of life, levels of anti-PS gradually increase and show several correlations with atypical P. falciparum-specific B-cells, implicating that further investigations are needed to conclude the functional aspects of these antibodies and which part they play in development of immunity against malaria.

In conclusion, our results showed that specific autoantibodies against RBCs are very common in malaria endemic areas. A clear presence of antibodies against RBCs in parallel with high levels of IgG and almost no signs of inflammation was observed in healthy adults living in such areas, even though many were carrying parasites, indicating that these Ugandan adults have reached a state of immunity where they can harbor parasites without the need for any major inflammatory response. We speculate that antibodies against different RBC surface antigens which are also receptors for merozoite invasion might be beneficial for long term protection, while other antibodies such as those against PS could be more harmful.

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To my precious family

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Bandar Hasan Saleh, Allan Lugaajju, Jill R. Storry, Kristina E.M. Persson (2023). *Autoantibodies against red blood cell antigens are common in a malaria endemic area*. Microbes and Infection, 25 (3), 105060. doi.org/10.1016/j.micinf.2022.105060

Paper II

Adebimpe Fasanya, Nurat Mohammed, **Bandar Hasan Saleh**, Muyideen Kolapo Tijani, Alexandra Teleka, Maria del Pilar Quintana, Lars Hviid and Kristina E. M. Persson (2023). Anti-phosphatidylserine antibody levels are low in multigravid pregnant women in a malaria-endemic area in Nigeria, and do not correlate with anti-VAR2CSA antibodies. Frontiers in cellular and infection microbiology, 13, 1130186. doi.org/10.3389/fcimb.2023.1130186

Paper III

Bandar Hasan Saleh, Allan Lugaajju, Muyideen Kolapo Tijani, Lena Danielsson, Ulrika Morris, Jill R. Storry, and Kristina EM Persson. *An immuno-inflammatory profiling of asymptomatic individuals in a malaria endemic area in Uganda.* (Manuscript).

Paper IV

Muyideen Kolapo Tijani, **Bandar Hasan Saleh,** Allan Luggaajju, Lena Danielsson, Kristina E M Persson. *Acquisition of anti-phosphatidylserine IgM and IgG antibodies by infants and their mothers over time in Uganda*. (Manuscript).

Author's contribution to the papers

Paper I

I have performed all the laboratory work and methodology of this paper. I did the laboratory assays such as Direct antiglobulin test (DAT), antibody screening by gel-indirect antiglobulin test (gel- IAT), antibody identification test of common and rare phenotypes, Lewis inhibition assay, ELISA for Anti-*P. falciparum* schizont extract IgG. I wrote this paper myself and got some comments and suggestions from the other authors and reacted accordingly.

Paper II

My share in the laboratory methodology of this paper was the assaying of anti-PS antibodies by Enzyme Linked Immunosorbent Assay (ELISA). I wrote part of this paper and got some comments and suggestions from the other authors and reacted accordingly.

Paper III

I have performed several laboratory techniques in this paper such as ELISA for Anti-*P. falciparum* schizont extract IgG antibody, plasma protein analysis, ELISA for IgG directed against RBC/RBC antigens, DNA Extraction, Polymerase chain reaction (PCR) and Gel electrophoreses analysis, and I shared the work related to growth/invasion inhibition assay. I wrote this paper myself and got some comments and suggestions from the other authors and reacted accordingly.

Paper IV

My share in the laboratory methodology of this paper was the assaying of anti-PS IgG and IgM antibodies by Enzyme Linked Immunosorbent Assay (ELISA). I wrote part of this paper and got some comments and suggestions from suggestions from the other authors and reacted accordingly.

Abbreviations

AMA1	Apical Membrane Antigen 1
APCs	Antigen-Presenting Cells
CRP	C-reactive protein
DAT	Direct Antiglobulin Test
EBL-1	erythrocyte-binding ligand-1
ELISA	Enzyme-linked immunosorbent assay
GPA	Glycophorin A
GPB	Glycophorin B
GPC	Glycophorin C
GPD	Glycophorin D
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
iRBCs	infected Red Blood Cells
IFN-γ	Interferon-γ
IL-1	Interleukin-1
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IPTp	Intermittent preventive treatment in pregnancy
ITNs	Insecticide-treated bed nets
JMH	John Milton Hagen
PCV	Packed Cell Volume
PADH	Post-Artesunate Delayed Haemolysis

PBMC	Peripheral blood mononuclear cells
P. cynomolgi	*Plasmodium cynomolgi*
P. falciparum	*Plasmodium falciparum*
P. ovale	*Plasmodium ovale*
P. malariae	*Plasmodium malariae*
P. knowlesi	*Plasmodium knowlesi*
P. vivax	*Plasmodium vivax*
PvDBP	*Plasmodium vivax Duffy binding protein*
PS	Phosphatidylserine
RBC	Red Blood Cell
Rh	Reticulocyte-Binding Homologue
RIFIN	Repetitive Interspersed Family
RHAG	Rh Associated Glycoprotein
STEVOR	Subtelomeric Variant Open Reading frame
RT	Room Temperature
TNFα	Tumor necrosis factor alpha
TLRs	Toll-like receptors
VCAM-1	Vascular cell adhesion molecule-1
WHO	World Health Organization
WMR	World Malaria Report

1 Introduction

1.1 Red Blood Cells (RBCs)

Red blood cells, also known as erythrocytes, play a crucial role in the circulatory system and are the most numerous types of cells in the human body. Their main tasks involve carrying oxygen from the lungs to the body's tissues and then transporting carbon dioxide from the tissues back to the lungs. This vital exchange is made possible by hemoglobin, a specialized protein in RBCs, which has the ability to bind both oxygen and carbon dioxide efficiently.

RBCs are distinctive in their structure. They are shaped like biconcave discs, which increases their surface area for oxygen exchange and allows them to deform as they navigate through narrow capillaries. This shape is maintained by a cytoskeletal structure composed of proteins like spectrin and ankyrin. Unlike most other cells, RBCs lack a nucleus and most organelles, including mitochondria. This absence provides additional space within the cell for hemoglobin, thereby maximizing the cell's capacity for oxygen transport.

Erythropoiesis, the production of RBCs, occurs in the bone marrow. This process is tightly regulated by the hormone erythropoietin, produced primarily in the kidneys in response to low oxygen levels. New RBCs enter the bloodstream as reticulocytes, a slightly immature form, and mature within a day or two.

The average lifespan of an RBC is about 120 days. As they age, they undergo changes in their membrane and become more prone to hemolysis. Old or damaged RBCs are typically removed from circulation by phagocytic cells in the spleen and liver. The components of these cells, such as iron from hemoglobin, are recycled to form new blood cells, while bilirubin, a byproduct of hemoglobin breakdown, is excreted in bile.

Further understanding of RBCs is crucial in addressing various hematological disorders, such as anemia and polycythemia, and in managing conditions requiring blood transfusions.

Surface Antigens on RBCs and Blood Groups

The surface of RBCs is adorned with a variety of proteins and glycoproteins, many of which can serve as antigens. These antigens can be recognized by the immune system and are key in determining blood compatibility during transfusions. The most widely recognized and clinically significant of these are the ABO and RhD blood group systems.

ABO Blood Group System:

- **A Antigen**: People with type A blood have A antigens on the surface of their RBCs and anti-B antibodies in their plasma.
- **B Antigen**: Those with type B blood have B antigens and anti-A antibodies.
- **AB Antigens**: Individuals with "AB" blood group have both A and B antigens and lack both types of antibodies. Therefore, those individuals can serve as "universal recipients" during any blood transfusion process accepting blood from any other major blood group (A, B or O).
- **No A/B Antigens**: Individuals with "O" blood group have neither A nor B antigens but have both anti-A and anti-B antibodies. Consequently, they can donate RBCs to anyone with the other major blood groups (A, B or AB), during blood transfusion. Therefore, they are considered "universal donors". Blood group O is indeed more common in certain regions of Africa, and it's believed to be connected to resistance against malaria. Sickle-cell anemia, caused by a mutation in the hemoglobin subunit beta (HBB) gene, results in abnormal hemoglobin molecules that lead to the characteristic sickle shape of RBCs. While individuals with sickle-cell trait (one copy of the gene) may suffer from the disease, those with two copies (sickle-cell disease) face severe health challenges. However, carriers of the sickle-cell trait have a survival advantage in regions where malaria is endemic. Malaria parasites are less likely to survive and reproduce in the RBCs of individuals with sickle-cell trait, as the deformed cells are less hospitable for the parasites (Allison, 1954). Studies have suggested that individuals with blood group O may also have a degree of protection against severe malaria. It's theorized that the absence of these antigens may interfere with the parasite's ability to attach to and invade RBCs, reducing the severity of malaria infection (Modiano et al., 2001; Rowe, Opi, & Williams, 2009).

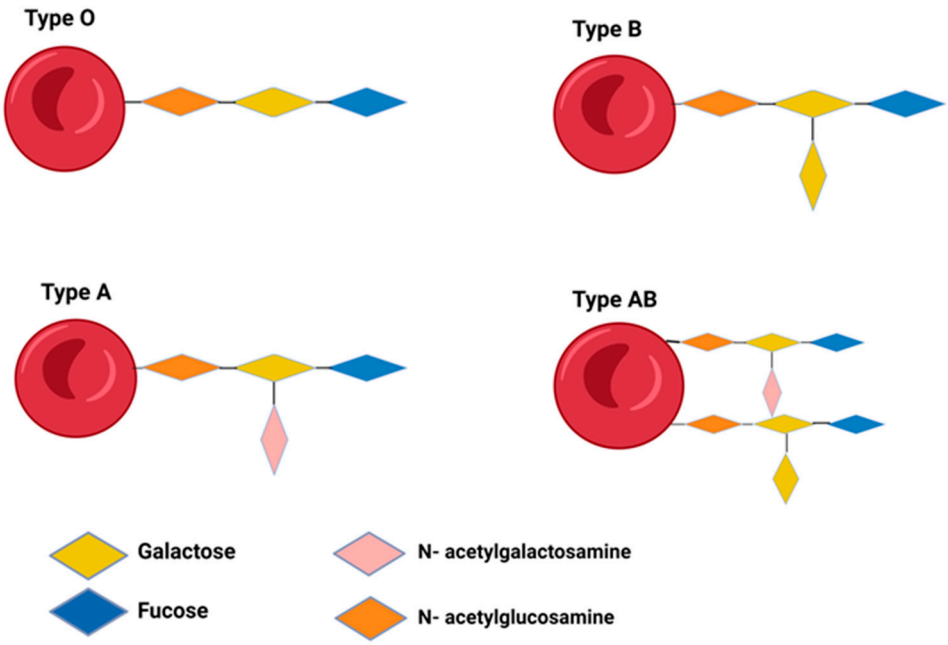

Figure 1. A, B, AB and O blood group. Created with Biorender.

RhD Blood Group System:

- The RhD antigen is either present (+) or absent (-) on the surface of RBCs. Those who possess the antigen are termed "Rh positive" (e.g., A+, B+), while those who lack the antigen are "Rh negative" (e.g., A-, B-). The presence or absence of this antigen adds another layer of compatibility that must be considered during blood transfusions.

Beyond the ABO and Rh systems, there are numerous other blood group systems and antigens (e.g., Kell, Duffy, Kidd, Lewis, MNS, P1PK, Lutheran, Diego, Yt, Xg, Scianna, Dombrock, Colton, Landsteiner-Wiener, Chidi/Rodgers, H, Kx, Gerbich, Cromer, Knops, Indian, Ok, Raph, JMH, I, Globoside, Gill, Rh-associated glycoprotein, Forssman, JR, LAN), but the ABO and RhD systems are the most significant in terms of transfusion medicine (Reid, Lomas-Francis, & Olsson, 2012).

In summary, the surface antigens present on RBCs play a crucial role in determining blood compatibility and ensuring safe blood transfusions.

1.2 Burden of Malaria

Malaria is the predominant parasitic tropical/subtropical and fatal infectious disease (World Health Organization, 2023). The global health issue of malaria, predominantly due to the *Plasmodium falciparum* (*P. falciparum*) parasite, represents a significant challenge across the world. This particular strain of malaria is noted for its severity and widespread impact on public health, affecting numerous countries and communities. The presence and prevalence of *P. falciparum* have made malaria not just a regional concern but a matter of international importance, demanding attention and resources for effective management and control. Over one-third of the current human population lives in regions, mainly in sub-Saharan Africa, where there is a risk of contracting malaria, and where almost 70% of malaria cases exist and 90% of deaths occur **(Figure 2).** According to the World Health Organization's (WHO) malaria report for 2023, there were approximately 249 million malaria cases globally and approximately 608,000 deaths. The highest burden of mortality was observed predominantly in children under the age of five and pregnant women (World Health Organization, 2023). The highest mortality rates due to malaria have been recorded in the lowest gross national income (GNI) countries (World Health Organization, 2023). Besides the high mortality of the disease, the overall burden of malaria is huge. It is among the top diseases causing economic loss, calculated by disability-adjusted life years (DALYs) (Y. Wang et al., 2022). The huge costs for prevention, control and treatment of malaria in the endemic low-income countries has aggravated the levels of poverty in such regions (Malaney, Spielman, & Sachs, 2004). Population migration to high malaria infection regions, agricultural transitions, and long-term climate change are potential hazard agents that could have an adverse effect on the emerging malaria patterns in such communities (Sachs & Malaney, 2002).

Globally, Uganda ranks 3rd highest in terms of the number of malaria cases with approximately +597 000 cases and 7th highest in terms of the number of malaria deaths (World Health Organization, 2023). The impact of malaria on the economy of Uganda is further compounded by the loss of workdays resulting from sickness, the reduction in productivity, the decrease in school attendance and the poor performance of schools, and the decline in foreign direct investment (World Health Organization, 2023)

The report on malaria in Nigeria for the year 2022, published by the WHO African Region, provides a comprehensive overview of the malaria situation in the country. It highlights Nigeria's status as having the highest global malaria burden, with an estimated 194,000 deaths in 2021, accounting for nearly 27% of the global malaria burden. Additionally, WHO reported that Nigeria showed around +1.3 million cases

(World Health Organization, 2023). The report details the nationwide efforts to combat this, including the distribution of insecticide-treated nets, implementation of malaria prevention programs, and the challenges faced, such as funding dependencies and the need for increased domestic support. The document emphasizes the significant progress made in reducing malaria incidence and mortality rates over the years, while also pointing out the areas requiring further attention to sustain and advance these gains (World Health Organization, 2023).

The enforcement of control programmes including prompt treatment of infected patients, prophylactic chemotherapy to people at-risk, as well as vector control using Insecticide-treated bed nets (ITNs) and indoors application of insecticides can led to reductions in morbidity and mortality due to malaria. Measures implemented for vector control has expanded over the last decade in Sub-Saharan Africa. However, it is important to note that malaria is a complex and dynamic disease, and its prevalence can vary over time and across different regions. Several factors can contribute to an increase in malaria cases, such as:

1. **Climate Change:** Changes in temperature and rainfall patterns can alter mosquito breeding and malaria transmission, potentially expanding the geographical range of malaria and increasing the number of at-risk populations.
2. **Drug Resistance:** The emergence of drug-resistant strains of the malaria parasite poses significant challenges and can lead to treatment failures and increased malaria cases.
3. **Inadequate Healthcare Infrastructure:** Limited access to healthcare facilities, diagnostic tools, and effective treatment can hinder malaria control and prevention, potentially leading to increased malaria transmission.
4. **Population Movements:** Human migration can contribute to the spread of malaria as infected individuals moving to non-endemic areas can introduce new cases and potentially trigger localized outbreaks.
5. **Socioeconomic Factors:** Poverty, lack of education, and poor living conditions can create environments conducive to malaria transmission, limiting access to preventive measures and proper healthcare, resulting in increased malaria cases.
6. **Malaria Service Disruptions during Covid-19:** The disruption of malaria services during the pandemic has added to the regions' malaria burden due to decreased access to necessary preventative and treatment services.
7. **Urbanization and Land Use Changes:** Changes in land use and increased urbanization can create new breeding grounds for mosquitoes, potentially altering transmission dynamics and increasing the risk of malaria.

8. **Lack of Awareness and Education:** Insufficient knowledge and awareness about malaria prevention and treatment among populations can contribute to the spread of the disease.
9. **Inadequate Funding and Resources:** Limited funding and resources can hinder the implementation of effective malaria control and prevention strategies, such as creating highly efficacious vaccines, affecting the ability to reduce the burden of the disease.
10. **Biological Factors:** Changes in the biology or behaviour of mosquito vectors or the malaria parasite can affect transmission dynamics and the effectiveness of control measures.
11. **Conflict and Instability:** Areas experiencing political conflict and instability can face disruptions in malaria control efforts, leading to increased transmission and outbreaks.
12. **Human Immunity Levels:** Variations in immunity levels among human populations can affect the susceptibility to malaria infections, influencing the spread and severity of the disease.
13. **International Travel and Trade:** Increased international travel and trade can facilitate the spread of malaria to new areas and contribute to the introduction of drug-resistant strains.
14. **Policy and Implementation Gaps:** Ineffectiveness and gaps in policy formulations and implementations can affect the success of malaria control and eradication programs.
15. **Environmental Factors:** Changes in ecosystems and environmental conditions, such as water bodies and vegetation, can influence mosquito breeding sites and malaria transmission patterns.

Each of these factors, individually or in combination, can significantly impact the prevalence, spread, and control of malaria in different regions. (WHO, 2023).

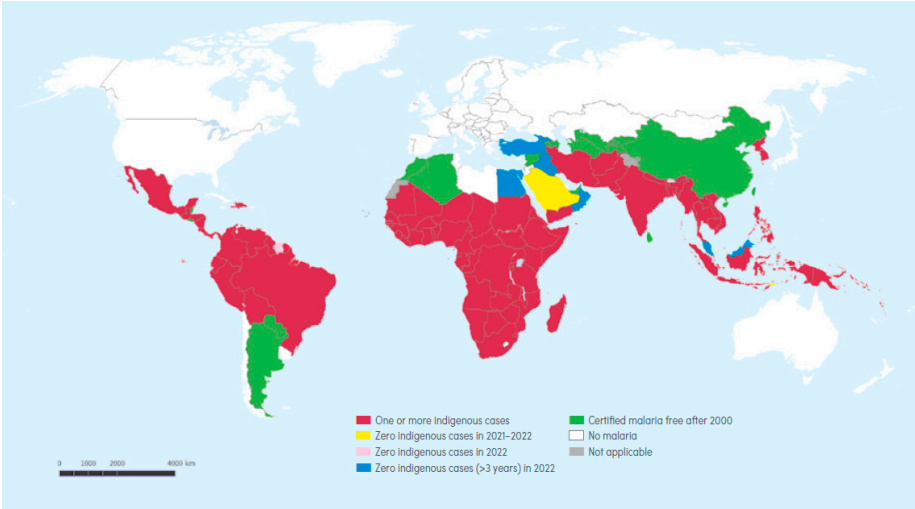

Figure 2. Estimation of *P. falciparum* global limits and endemicity in 2023 (World Health Organization, 2023).

1.3 Apicomplexan Protozoa

The different species of the genus *Plasmodium* belong to a wide group of protozoa that includes between 4000 and 5000 species called "Apicomplexans". The apicomplexan protozoa are unicellular parasites that have a unique structure called the "apical complex". Such structure, located at the apical end of the parasite, is organized from certain organelles e.g. micronemes and rhoptries which have a crucial role in enabling the parasite to invade host cells. *Toxoplasma spp.* and *Babesia* are among the other medically relevant apicomplexans. The parasitic species belonging to the apicomplexan group are also characterized by having complex life cycles.

1.4 Plasmodium Species

There are over a hundred species of *Plasmodium* that can infect a wide range of animals, such as monkeys, birds and rodents. There are at least six species of *Plasmodium* that are responsible for malaria in humans: *P. falciparum*, *Plasmodium vivax (P. vivax), Plasmodium ovale (P. ovale), Plasmodium malariae (P. malariae), Plasmodium knowlesi (P. knowlesi) and Plasmodium cynomolgi (P. cynomolgi).* They are all mainly transmitted through the bite of *Anopheles spp*. mosquitoes.

However, two species: *P. falciparum* as well as *P.vivax* are considered to be the dominant infecting species. *P. falciparum* is singled out as the most dangerous one, responsible for most of the human deaths due to malaria worldwide (Phillips *et al*., 2017). Historically, *P. falciparum* malaria has been a major global health concern, particularly in sub-Saharan Africa.

P. vivax is predominantly prevalent in South America and throughout Asia and is a substantial pathogen outside of Africa. *P. vivax* has traditionally been described as "benign tertiary malaria", however, this term is misleading as *P. vivax* has been reported to cause significant morbidity and in severe circumstances lethality (Barcus et al., 2007; Ric N Price, Douglas, & Anstey, 2009). *P. malariae* and *P. ovale* are considered relatively mild and infect humans less frequently. *P. knowlesi* was recognised as a fifth malaria recently and was found to be causing malaria in macaques but has also been shown to infect humans (Turkiewicz et al., 2023), as well as *P. cynomolgi,* a sixth malaria species originating from monkeys (Bykersma, 2021).

1.5 The Life Cycle of the Malaria Parasite

A malaria infection begins when a sporozoite-carrying female Anopheles mosquito injects about 10-150 sporozoites into the human skin. Upon injection, sporozoites may either migrate through the dermis or remain in the skin to form extrahepatic exoerythrocytic forms. The ones entering the bloodstream invade hepatocytes, remaining immune-exposed for a minimal duration before reaching the liver (Figure 3).

In the liver, sporozoites infiltrate hepatocytes, facilitated by surface co-receptors, and develop into schizonts, yielding 10,000-30,000 merozoites, with variations depending on the parasite species (Loubens et al., 2021). This pre-erythrocytic stage lasts approximately 5-16 days, varying by species. For example, *P. falciparum* takes 5-6 days, while *P. vivax* and *P. ovale* take approximately eight and nine days, respectively.

Post liver stage, merozoites are released into the bloodstream, marking the blood infection stage. In *P. vivax* and *P. ovale* malaria, some sporozoites (hypnozoites) remain dormant in the liver, developing into schizonts after weeks to months. Once in the bloodstream, merozoites rapidly invade RBCs, minimizing antigen exposure and subsequent immune response.

The parasites exhibit specificity in RBC invasion; *P. vivax* targets Duffy blood group-positive RBCs, while *P. falciparum* employs diverse receptors to invade all RBC types. Throughout their life cycle in RBCs, parasites undergo several

developmental stages, eventually releasing new merozoites to invade more cells. Non-sexual forms differentiate into gametocytes, crucial for disease transmission.

These gametocytes play a vital role in the parasite transmission to *Anopheles* mosquitoes, transforming into zygotes in the mosquitoes upon transfer. The development and appearance of gametocytes vary with the parasite species, affecting the densities and timing of the transmission stages (Tuteja, 2007).

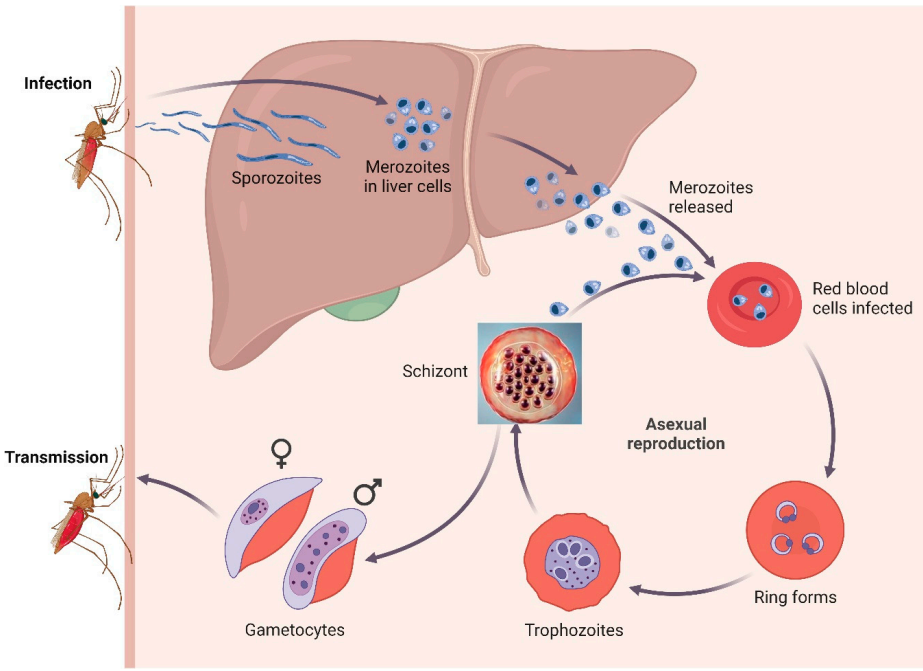

Figure 3. The *Plasmodium* parasite life cycle. Created with Biorender.

1.6 Clinical manifestation and management

The clinical findings in malaria are incredibly varied and can range from a mild headache to severe complications that can lead to death, especially in P. *falciparum* malaria. Because these complications can occur rapidly, every malaria patient must be rapidly evaluated and treated, and frequent observations are needed to watch for early signs of systemic complications. The initial symptoms of malaria, common to all types of malaria, are nonspecific and similar to a flu-like syndrome. The trademark of malaria is pyrexia. For up to two days (the fever is usually erratic at first, and the temperature increases with chills and mild shivering) before the onset

of fever, there may be prodromal symptoms such as nausea, vomiting, anorexia, malaise, headache, dizziness and back pain (Bartoloni & Zammarchi, 2012), and these symptoms may mislead physicians who rarely encounter malaria to diagnose it as influenza (particularly during the seasonal flu), dengue fever or viral hepatitis. Physicians should be aware of malaria epidemiology to prevent the missed diagnosis of this life-threatening disease. Rapid diagnosis and proper therapy are critical to avoid morbidity and fatal consequences. The duration of the incubation period varies among the different species of *Plasmodium*. It depends on several factors, including the infecting parasite (*P. falciparum* infections usually have the shortest and *P. malariae* the longest incubation time), the level of the previous immune status of the host, chemoprophylactic use of antimalarial drugs, and probably the density of the parasite inoculum. In regions where *P. falciparum* malaria endemicity is steady, severe malaria is most common in children up to 5 years of age and less common in older children and adults because of the acquisition of partial immunity.

On physical examination, splenomegaly can be seen during the acute episode but is more frequently noticed after the second week of the attack. An enlarged spleen rupture is a potential, albeit rare, complication. There may also be an enlarged and palpable liver. On examination of blood counts, thrombocytopenia is common, and occasionally mild leukopenia is present. Some degree of anaemia and reticulocytosis may occur because of the lysis of parasitized and non-parasitized RBCs. When infected with *P. vivax* and *P. ovale*, schizogony develops every 48 hours so that fever episodes appear each third day. During the breaks between febrile seizures, the affected person is usually fever-free and feels relatively well. If left without treatment, the spells may last from a few weeks to a few months and then spontaneously and gradually subside. Infection with *P. vivax* and *P. ovale* may relapse with recurrence of clinical symptoms and asexual parasitemia after a period of dormancy or latency. This occurs due to the re-invasion of merozoites into the blood, which occurs when hypnozoites awaken from dormancy and develop into hepatic schizonts. Recurrences may occur weeks, months, or sometimes years following primary infection, with timing varying according to the *P. vivax/ovale* strain, the geographic origin of illness, and previous insufficient antimalarial treatment. A relapse is similar to the first attack, except for a more abrupt onset and the lack of the initial period of irregular fever, as the infection tends to be more synchronous. The relapse is usually milder and of lesser duration than the first attack (Alexandre et al., 2010; R. N. Price et al., 2007).

In *P. falciparum* malaria, fever occurs after a couple of days with symptoms of prodrome that begin in the last days of the incubation period, with a typical range of 9-14 days. Initially, the fever is irregular but usually appears daily. It may be continuous or intermittent and exhibit no signs of periodicity until the illness persists for a week or more.

Routine laboratory tests may reveal mild or no anaemia in mild cases or other healthy individuals. When anaemia is seen, it is commonly normochromic and normocytic. Haptoglobin in serum may be undetectable, indicating haemolysis. However, the degree of anaemia can vary considerably and is not always indicative of the severity of the attack. White blood cell counts are usually low to normal during malaria, which could reflect the trapping of leukocytes in the spleen and other collaterals away from the peripheral circulation, and not a real depletion. Leukocytosis is typically reported in patients with concurrent infections and/or poor prognosis (McKenzie et al., 2005), as are serum transaminases and bilirubin concentrations, and lactic dehydrogenase that is usually mildly increased. The prothrombin time and partial thromboplastin times may be increased, and fibrinogen levels are also typically raised (Santoshi, Patel, Patel, Bansro, & Chhabra, 2022). According to some studies, the C-reactive protein (CRP) and procalcitonin levels are usually elevated in both uncomplicated and severe malaria, with levels correlating with parasitemia and prognosis (Wilairatana et al., 2021). Even if the affected person does not appear ill, severe complications can occur at any stage. In unimmune individuals, *P. falciparum* malaria can rapidly progress to severe malaria if appropriate treatment is not initiated. If the acute attack is quickly diagnosed and adequately treated, the prognosis for *P. falciparum* malaria is considered relatively good, although complications can still occur. Treatment usually works rapidly, resolving fever and most symptoms within three days. Relapse with clinical signs and parasitemia recurrence due to persistent erythrocytic forms is possible (Alexandre et al., 2010; R. N. Price et al., 2007).

1.7 Malaria Pathogenesis

P. falciparum merozoite invasion involves the secretory organelles of merozoites such as rhoptries, thick granules and micronemes that facilitate the passage of the merozoites into the erythrocytes. The connection between the parasite and the erythrocytes leads to a fast wave of disfigurement of the erythrocyte layer, forming a stable parasite-host cell intersection. Then the parasite penetrates the erythrocyte layer with the help of the actin-myosin apparatus and thrombospondin-related anonymous family protein (TRAP) family proteins and aldolase to form a parasitophorous vacuole to seal itself off from the host cell cytoplasm, and in this way it can survive inside the RBC (Hale et al., 2017). The detailed mechanism as well as the most recognized parasite and RBCs molecules involved in such interactions (Molina-Franky, Patarroyo, Kalkum, & Patarroyo, 2022) are detailed in **Figures 4**.

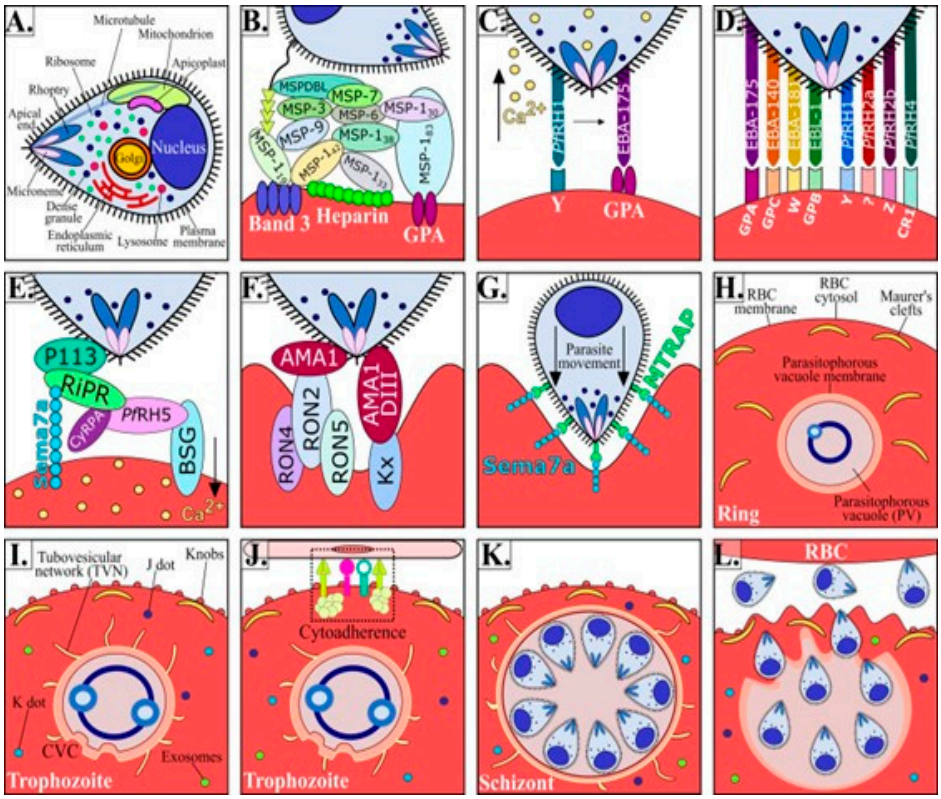

Figure 4. Invasion of RBCs by *P. falciparum* merozoites and substantial transformations in the infected RBCs. (A) Interaction of *P. falciparum* merozoites (B–G) with RBC, detailing the parasite ligands and RBC receptors that play roles in the initial attachment, repositioning towards the apex, and forming a secure junction during the merozoite's invasion of the RBC. (H) Depiction of the ring stage of *P. falciparum* as it develops within the parasitophorous vacuole membrane (PVM), highlighting the boundary between the parasite and the host's cytoplasm. At this stage, Maurer's clefts appear as flat, elongated membrane vesicles, showcasing mobility within the infected RBC (iRBC) cytoplasm. (I) During the trophozoite stage of *P. falciparum*, Maurer's clefts become connected to the membrane skeleton, and the parasite matures. The image also introduces the tubovesicular network (TVN), which extends from the PVM into the iRBC cytoplasm, as well as the caveola vesicle complex (CVC) that holds *P. falciparum* antigens, potentially playing a role in antigen transport, release, and plasma protein absorption. Additionally, structures like J dots, K dots, and exosomes are mentioned as means of transporting certain parasite proteins (e.g., *Pf*EMP-1) through the iRBC cytoplasm, and knobs are noted as a feature of the iRBC surface in the later stages of parasite development. (J) The cytoadherence of *P. falciparum* is illustrated, highlighting the interaction between varied surface antigens of the parasite and the receptors of the host. (K) Depiction of the schizont stage of *P. falciparum*. (L) Finally, the rupture of iRBCs and the subsequent release of merozoites, followed by their re-invasion into new RBCs, are illustrated (Molina-Franky et al., 2022). Re-printed with permission from the authors.

There are two main pathways of invasion pathways utilised by *P. falciparum*, namely the sialic acid (SA)-dependent and sialic acid-independent pathways, characterised by the capacity to invade erythrocytes in which SA has been cleaved

using the protease neuraminidase. SA-dependent invasion implicates the RBC glycophorin-binding proteins, EBA175, EBL-1 and EBA140 as well EBA181 and *Pf*Rh1 which have been demonstrated to bind SA but to an unidentified receptor. SA-independent invasion is predominantly dependent on the neuraminidase insensitive binding of *Pf*Rh4 (Stubbs et al., 2005). It is assumed that each parasite ligand binds to a distinct receptor. It is obvious that both *P. falciparum* reticulocyte binding protein-like homolog (*Pf*Rh) and erythrocyte binding antigen (*Pf*EBA) are legend classes capable of using alternative invasion pathways. Except for *Pf*Rh5, both classes are transmembrane proteins utilised by parasites for effective RBC invasion (Tham, Healer, & Cowman, 2012). The *Pf*Rh protein family is composed of five members namely *Pf*Rh1, *Pf*Rh2a, *Pf*Rh2b, *Pf*Rh4 and *Pf*Rh5. Some *Pf*Rh receptors are still unidentified but complement receptor 1 and basigin (BSG) have been appeared to serve as the RBCs receptors for *Pf*Rh4 and *Pf*Rh5, respectively (Crosnier et al., 2011; Tham et al., 2010).

Understanding the mechanisms of merozoite invasion into red blood cells (RBCs) is crucial for the development of effective vaccines. The complexity of the invasion process is partly due to the parasites having a wide array of genes that enable them to control the expression of different invasion pathways. This genetic diversity is likely a response to several factors:

- Hosts in endemic areas may during evolution develop resistance mechanisms that hinder the binding of the parasite to specific RBC ligands.
- The parasite must adapt to the variety of erythrocyte surfaces, which can vary in response to infection and available nutrients, making flexible invasion pathways a necessity for survival and the establishment of infection.

The ability of the parasite to use multiple invasion pathways improves its chances of successful infection (Figure 4).

The exit of merozoites from the parasitophorous vacuole (PV) and the host's cell membrane is mediated by some biochemical modifications and intracellular pressures in the mature late stage of the RBCs that result in instability of the cytoskeleton and an exploding rupture that will release merozoites (Molina-Franky et al., 2022). There are some proteins implicated in the excretion process, such as proteases. They are believed to be involved in two steps: the destruction and rupture of the parasitophorous vacuolar membrane, followed by destruction of the host cell membrane. Proteases involved in egress also include the aspartic protease plasmepsin II, the serine repeat antigen (SERA) family and a plant-like calcium-dependent protein kinase, which is expressed in merozoites (Counihan et al., 2017).

There are several mechanisms that *P. falciparum* use to induce surface modifications of the host erythrocytes, which have an essential role in the pathogenesis of cerebral malaria. The parasite exports and presents several different surface antigens on the surface of RBCs to hide from the host's immune response. The sequential expression of various surface antigens, referred to as antigenic variation, is an effective way for immune system evasion and is being mediated by several multigene families reconciled in *P. falciparum* such as repetitive interspersed family (RIFIN) proteins encoded by the rif genes, *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), which are encoded by the var genes, and STEVOR proteins that are encoded by the stevor genes. Proteins of the surface-associated interspersed gene family, known as SURFINs, are encoded by separate genes (Chan, Fowkes, & Beeson, 2014). A well-known phenomenon that has been well characterized is sequestration, when the parasite cause the infected RBC to attach to endothelial cells. This interaction may form clusters or rosettes, with the infected RBCs in the centre and surrounded by uninfected RBCs, known as rosetting. Sequestration in the brain can be severe, resulting in cerebral malaria.

1.8 Immunity to Malaria

General concepts of immunity

Innate immunity, also called nonspecific immunity, is defined as the natural resistance genetically established in the host. It comprises the mucociliary and epithelial barriers that repel swallowed or inhaled particles. Soluble proteins and small bioactive particles found in biological fluids confer also innate protection. In addition, chemokines, cytokines, and biological catalysts are released by activated cells. The cells involved in the innate immune responses include neutrophils, basophils, eosinophils, macrophages, and dendritic cells. Non-specific innate immunity involves also cell surface receptors that attach to expressed molecules of the attacking microorganisms.

Adaptive immunity is an acquired type of immunity. In contrast to the innate immune system, the adaptive immune system's responses are tailored to the specific pathogen and consequently take longer than the natural response to manifest. Adaptive immunity is either naturally or artificially acquired. Naturally acquired adaptive immunity results from coincidental contact with a pathogenic organism, while the artificially acquired results from deliberate manipulation of the immune response such as immunization. Naturally and artificially acquired immunity can be passive by introducing antibodies or activated T cells from an immune individual

into another host. Active immunity is generated in the host after his immune system comes into contact with an antigen. Based on the cells involved in the immune response, adaptive immunity can be characterized as either humoral immunity, which is mainly achieved through the production of antibodies by activation of B cells, or cell-mediated immunity, which is achieved by T cells. These antibodies can neutralize pathogens, mark them for destruction, or prevent them from infecting cells. The antibodies produced during humoral immune response, are the effector molecules causing the destruction of extracellular microorganisms and prevent the spread of intracellular infections. The activation of B cells and their differentiation into antibody-secreting plasma cells is triggered by antigens and usually requires helper-2 T cells (TH2).

Unlike humoral immunity, cell-mediated immunity does not depend on antibodies to achieve adaptive immune responses. The main drivers of cell-mediated immunity are macrophages, and secreted cytokines in response to an antigen. It is principally activated in response to intracellular pathogens such as viruses, intracellular bacteria, and intracellular fungi as well. The recognition of the intracellular target antigens by T cells participating in cell-mediated immunity is a crucial step that is mediated by antigen-presenting cells that express membrane-bound MHC class I antigens. The binding specificity of MHC proteins to the presented antigens drives the maturation and differentiation of naive T cells into either helper or killer T cells (Abbas, Lichtman, & Pillai, 2020).

Immunity against malaria

Despite the fact that the host remains susceptible to reinfection in response to natural parasite exposure, repeated infections elicit strong immunity against erythrocytic-stage parasitemia that is potent in preventing disease symptoms and clinical malaria symptoms (Cohen, McGregor, & Carrington, 1961; Edozien, Gilles, & Udeozo, 1962). As a result of continuous parasite exposure, this state of semi-immunity does not develop overnight, and only gradually develops over time, which explains the high disease susceptibility of infants and malaria-naive travellers.

Naturally acquired immunity to *P. falciparum* infection protects lots of individuals who are regularly exposed to infections from severe morbidity and mortality. There is no well-defined conception about the mechanism of such protection. Different modes of acquired or adaptive malaria immunity have been described: (i) anti-disease immunity, related to protection against clinical outcome; (ii) anti-parasite immunity, concerning protection against parasitemia, and influences the parasite density; and (iii) premunition, which is protection against disease and high numbers

of parasitemia through keeping a low-grade and asymptomatic parasitemia (Doolan, Dobaño, & Baird, 2009).

As far as the defence against *P. falciparum* is concerned, both cellular and humoral immune mechanisms play important roles. Despite this fact, it is important to keep in mind the fact that we haven't been able to establish clear correlates of protection yet (McCall, Kremsner, & Mordmüller, 2018). As a result, the relative contributions of the diverse immune mechanisms remain to be determined, and as such for the purpose of designing vaccination strategies against this complex parasitic disease, all aspects of the immune response remain under consideration in terms of their relationship to the immune response.

Innate immunity in malaria

The potential of innate immune responses in the defence against malaria has often been overlooked. Such innate reactions play a crucial role in managing the initial stages of malaria in humans. These immune mechanisms are activated once the parasite density surpasses a certain threshold, leading to the partial removal of infected cells, capping the peak parasite density. Remarkably, these density-dependent mechanisms seem to restrict the expansion of all blood-stage parasites, regardless of their species or strain. This suggests that innate immunity is triggered by molecular elements common across various *Plasmodium* species and strains. This phenomenon could also shed light on why mixed species infections are commonly seen in areas where multiple *Plasmodium* species are prevalent (Stevenson & Riley, 2004).

In contrast to other intracellular infections where the contribution of innate immunity has been thoroughly investigated, the innate immunity to malaria remains less explored. A fundamental unresolved issue in this field is determining the specific antigen-presenting cells (APCs) responsible for activating T cells, especially CD4+ TH1 cells that produce IFN-γ and facilitate the shift to protective cytophilic antibody subclasses such as IgG1 and IgG3 in humans, during acute malaria infections (Su & Stevenson, 2002). Although malarial ligands that trigger innate responses are still being characterized, Dendritic cells and macrophage activation might be one of the initial responses to malaria. Toll-like receptors (TLRs), a key pattern-recognition receptor class, are crucial in activating innate immunity and modulating adaptive responses to malaria, though their role in malaria immunity is still under investigation. Some studies have shown that *Plasmodium* parasites might inhibit normal Dendritic cells maturation, while others indicate no perturbation in Dendritic cells maturation and activation by malaria parasites. This discrepancy suggests that an initial phase of conventional APC/Dendritic cells

activation may be followed by a period where pro-inflammatory signals are diminished or actively downregulated to prevent pathology. Nonetheless, significant pro-inflammatory cytokine responses occur during malaria infections, underscoring the need for further studies to clarify APC function induction and modulation by malaria (Stevenson & Riley, 2004; Yap, Lundie, Beeson, & O'Keeffe, 2019).

Macrophages play a pivotal role in malaria's innate immunity, not only as APCs but also through their ability to phagocytose infected erythrocytes independently of cytophilic or opsonizing malaria-specific antibodies. Recent studies suggest that scavenger receptors like CD36 are crucial for such phagocytosis in non-immune individuals. This interaction, likely involving CD36 binding to *Pf*EMP1 on infected cells, does not lead to pro-inflammatory cytokine production by monocytes/macrophages. However, macrophages might play a more significant role during adaptive immunity as effector cells, producing anti-parasite molecules post-activation by CD4+ T-cell-derived IFN-γ (Ozarslan, Robinson, & Gaw, 2019; Stevenson & Riley, 2004).

The role of Natural killer T (NKT) cells in anti-malarial immunity, especially against pre-erythrocytic parasites in hepatocytes, has been demonstrated. However, whether NKT cells are essential for immunity to liver-stage parasites remains unresolved. Malaria-responsive γδ T cells, like NKT cells, seem to serve as a bridge between innate and adaptive immune responses, showing significant expansion during acute *P. falciparum* and *P. vivax* infections. These cells produce substantial amounts of IFN-γ and exhibit anti-parasite functions. The role of γδ T cells in immunity to different malaria stages varies across models (Burrack, Hart, & Hamilton, 2019; Stevenson & Riley, 2004).

Adaptive, humoral immunity in malaria

There is a coordinated collaboration between innate and adaptive immunity to ensure an effective immune response against infections. The adaptive immune system takes over a few days after clonal maturation of antigen-specific T and B cells (Abbas et al., 2020).

In the context of *P. falciparum*, humoral immunity plays a crucial role in controlling and preventing infection. The humoral immune responses that occur naturally in the body can target both freely circulating parasites, as well as infected host cells that display parasite antigens on their outer surface. In areas where malaria is endemic, repeated exposure to *P. falciparum* can lead to the development of partial immunity. This immunity doesn't prevent infection but can reduce the severity of disease. This

is believed to be due, in part, to the acquisition of a broad repertoire of antibodies against different parasite antigens. Naturally acquired immunity against malaria depends on both age and exposure. Thus, a child born in a malaria endemic area may acquire a higher degree of clinical immunity depending on the number of infections with malaria (Doolan et al., 2009). It is expected that this immunity becomes stronger with age, which translates into milder episodes of clinical malaria until no more symptoms of the disease appear in adolescence, despite constant exposure to infected mosquito bites (Crompton et al., 2014). The most studied aspect of humoral immunity against *P. falciparum* involves antibodies that target the different stages of the parasite. Understanding antibodies play a crucial role in the immune defence against malaria. Immunoglobulins are also known as antibodies and are crucial for the immune response against different pathogens. They recognize and bind to specific antigens. There are several types of immunoglobulins, including IgA that can be found mainly in the mucous membranes and body secretions like saliva and tears. It plays a role in mucosal immunity. IgG is the most abundant immunoglobulin in the blood. It provides long-term immunity against pathogens. IgM is the first antibody produced in response to an infection. It provides immediate but short-lived protection (Abbas et al., 2020). When the body encounters a pathogen, the production of specific immunoglobulins against that pathogen increases, leading to elevated levels of these antibodies in the blood. This increase aids in neutralizing and eliminating the pathogen (Abbas et al., 2020). While there are challenges due to the complexity of the *Plasmodium* life cycle and its immune evasion strategies, a deeper understanding the body's immune response against the parasite, particularly the role of antibodies, is crucial for vaccine development, more effective therapies, and disease control (Aleshnick, Florez-Cuadros, Martinson, & Wilder, 2022). Antibodies achieve their protective functions by different approaches. Antibodies can facilitate ”opsonization”, assisting the uptake of merozoites by phagocytes, leading to their destruction (Leitner, Haraway, Pierson, & Bergmann-Leitner, 2020). Antibodies can also contribute to blocking cytoadherence, preventing infected RBCs from adhering to endothelial cells, a mechanism associated with cerebral malaria (Craig, Khairul, & Patil, 2012). Antibodies can also achieve their protective actions through indirect effects. By promoting the release of inflammatory mediators, antibodies can bolster other immune responses against the parasite (Perkins et al., 2011).

It is important to understand how children acquire immunity to malaria to be able to control the disease. Newborn babies and young infants are relatively protected from symptomatic malaria compared to adults. This has primarily been attributed to maternal antibodies present in the first few months of life and that are transferred from the mother to the child (Dobbs & Dent, 2016). Children under the age of five

are most susceptible to infection as maternally derived antibodies decrease, followed by gradual acquisition of antibodies in response to infections, which leads to natural acquired immunity to malaria among young children (Dobbs & Dent, 2016; Doolan et al., 2009).

In areas with high prevalence of malaria, the levels of IgG are often high, with IgG1 and IgG3 isotypes being higher among chronically infected individuals and asymptomatic infections (Vigan-Womas et al., 2010; Q. Wang et al., 2016). The high IgG among asymptomatic carriers of *P. falciparum* may indicate a role in natural acquired immunity to malaria (Adamou et al., 2019). However, IgG4 was associated with symptomatic carriers of *P. falciparum* (Saavedra-Langer et al., 2018). IgG3 and IgG2 has been shown to be present in both asymptomatic and symptomatic individuals (Saavedra-Langer et al., 2018).

Immunoglobulin A is one of the five classes of antibodies found in mammals and plays a crucial role in mucosal immunity. Its presence in various bodily fluids like saliva, tears, and breast milk makes it vital for the protection of mucous membranes covering the mouth, airways, and digestive tract. IgA directed against *P. falciparum* has been found in breast milk (Leke, Ndansi, Southerland, Quakyi, & Taylor, 1992), and IgA levels were high during a controlled malaria infection study with minimal mosquito bites inducing malaria (Taghavian et al., 2018).

IgM levels have been shown to be increased in acutely infected patients and closely associated with IgG and IgA levels (Taghavian et al., 2018). However, IgM has been reported to be less indicative of the progression of clinical disease towards chronicity than IgG or IgA (Odegbemi & Williams, 1995). IgM was comparable to IgG in functional assays such as inhibiting *P. falciparum* growth and enhancing phagocytosis of *P. falciparum* by monocytes in vitro (Hopp et al., 2021).

Immunoglobulins levels may also reflect the number of exposures to infection. IgM and IgA were predominant in individuals with five or less previous malaria infections, while IgG was high in those with more than 5 episodes of malaria exposure (Taghavian et al., 2018). This indicated that IgG can be protective, even though later studies have doubted the effectiveness of antibodies as a treatment plan for malaria in endemic areas (Pirahmadi et al., 2019). However, monoclonal antibodies cannot be ignored as a new therapeutic potential.

While humoral immunity is an essential component of the immune response against *P. falciparum*, the complexity and variability of the parasite make it a challenging target. One of the challenges in studying humoral immunity against *P. falciparum* is the sheer number of antigens expressed by the parasite. Identifying which of these antigens that are the most important targets for protective immunity is a major research focus. The protective role of the antibody response faces several challenges

including strain-specific immunity (Gonzales et al., 2020; Raghavan et al., 2023) where due to antigenic diversity, presented by the parasites as “antigenic variation”, antibodies against one strain might not protect against another. Also, antibodies usually confer only short-lived protection (Rogers, Vijay, & Butler, 2021). Their levels can decline rapidly, leading to reduced protection against reinfection. This property, the relatively short-lived nature of the humoral response, represent a major challenge in developing a vaccine against *P. falciparum*. While people living in endemic areas can develop immunity over time, this immunity can wane if they move to non-endemic areas (Blank et al., 2020). Individuals who leave an endemic malaria area to live in a non-endemic country show that immunity declines relatively rapidly if there is no continuous reinfection (Mischlinger et al., 2020).

There are also some changes that occur to naturally acquired immunity against malaria during pregnancy. Despite the presence of immunity from growing up specifically in an endemic area, pregnancy seems to remove protection from malaria and make women again susceptible to severe disease, especially during their first and second pregnancies (Fried & Duffy, 2017). This is most likely due to the general suppression of cell-mediated immunity during pregnancy (Feeney, 2020).

Antibodies directed against antigens of different Plasmodium stages:

1. **Antibodies against merozoites**: The asexual phase of *P. falciparum* is the source of all known malaria symptoms; therefore, antibodies that target the parasite at this phase can potentially diminish or eliminate the disease's clinical manifestations. During this phase, merozoites are released into the bloodstream and invade RBCs initiating a rapid multiplication cycle. Being in the bloodstream makes the merozoites more vulnerable to the immune system, and studies have shown the presence of naturally produced antibodies against various merozoite proteins and organelles.

   The antibodies that target the merozoites can inhibit the growth of the parasite, prevent its invasion into RBCs, or opsonize infected RBCs for clearance by the immune system (Gonzales et al., 2020). As a result of changes in the expression of their surface proteins, merozoites that come out from the liver are not efficiently recognized by antibodies to sporozoites (Lasonder et al., 2002; Le Roch et al., 2003). Aside from this, merozoites also possess highly variable surface proteins that aid in their escape from humoral immune responses (Belachew, 2018). Antibodies can bind to merozoite surface proteins (MSPs), preventing them from entering RBCs (Beeson et al., 2016). Merozoite Surface Protein 1 (MSP1) is particularly abundant and crucial for RBC invasion. While some research links anti-MSP1 antibodies to protection against malaria, other studies do not. These antibodies can block RBC invasion, promote parasite

phagocytosis by monocytes, and support complement fixation. However, naturally occurring antibodies may also disrupt these protective processes, highlighting the complexity of the immune response to malaria.

Despite this complexity, some proteins like Apical Membrane Antigen 1 (AMA1) and various Erythrocyte Binding Antigens (EBAs) have shown promising results in eliciting protective antibodies, though their effectiveness can be strain specific. Other proteins like those from the Reticulocyte-Binding Homologue (Rh) family also play critical roles in RBC invasion and have been shown to be targets of protective immune responses (Mian et al., 2022).

However, the immune response to malaria is highly variable and influenced by genetic factors. Large-scale genetic studies are needed to fully understand how human genes have evolved in response to malaria and how they influence the immune response. Achieving sustained protection against malaria in endemic regions will likely require a comprehensive immune response targeting multiple parasite antigens (Kwiatkowski, 2005).

2. **Antibodies against sporozoites**: Some antibodies target the sporozoite stage of the parasite, which is the form injected by the mosquito during a bite. These antibodies can prevent sporozoites from reaching and infecting the liver (Flores-Garcia et al., 2018). From the time that the sporozoites are injected into the skin until the infection of the hepatocytes occurs, antibodies are capable of interfering with the development of sporozoites (Behet et al., 2014). Antibodies can target the circumsporozoite protein (CSP) on sporozoites, potentially inhibiting liver invasion (Fabra-García et al., 2022). In response to mosquito-transmitted infections associated with *P. falciparum*, sterilizing immunity was only reported after non-natural exposure to the sporozoite, indicating that neutralizing antibody titers against injected sporozoites might not be efficiently induced as a result of mosquito-transmitted infections (Julien & Wardemann, 2019).

3. **Antibodies against gametocytes**: Gametocytes are the sexual stages of the parasite that are taken up by mosquitoes. Antibodies targeting gametocytes can potentially block transmission of the parasite from humans to mosquitoes (de Jong et al., 2020). The majority of studies have shown that antibodies against the sexual stage *P. falciparum* proteins from human and animal models can reduce the transmission of the parasite to subsequent hosts when these antibodies are transferred to the Anopheles mosquito vector with a blood meal (Farrance et al., 2011; Nikolaeva et al., 2020).

4. **Antibodies against the surface-expressed antigens on infected RBCs:** One of the main Variant surface antigens (VSAs) of malaria parasites that influence

the host-parasite interaction is the *Pf*EMP1 antigen. *Pf*EMP1 molecules are expressed on the surface of infected RBCs and is crucial for both cytoadhesion and immune evasion, enabling the parasite to establish and maintain a chronic infection in the host. They are encoded by a diverse family of var genes, undergo rapid switching and are central to the parasite's clonal antigenic variation. This involves changing the proteins on the surface of the iRBCs, making it difficult for the immune system to recognize and attack the parasite. Such strategies of antigenic variation are also considered a major challenge for vaccine development (Rajneesh et al., 2023). Despite this, there's a suggestion that this diversity might be finite, based on observations like the restricted diversity of VSAs in severe malaria cases and the genetic structuring of the var gene repertoire in the *P. falciparum* genome (Bull et al., 2005). PfEMP1 binds to various receptors on the host cells, such as CD36, ICAM-1, and EPCR. These interactions are critical for the parasite's survival and evasion of the host's immune system (Gowda & Wu, 2018; Theander, 1992; Wahlgren, Goel, & Akhouri, 2017).

Other significant surface targets for antibodies include antigens such as RIFINs, SURFINs and STEVOR, which, like *Pf*EMP1, are expressed on the surface of iRBCs and their presence on the iRBC surface adds to the crucial role in the parasite's ability to cause severe malaria (Kanoi et al., 2020).

Cell-mediated immunity in malaria

Cell-mediated immunity is an essential arm of the adaptive immune system, primarily involving various types of immune cells that act directly against pathogens without the need for antibody production. This mechanism is particularly important in the defence against intracellular pathogens, such as parasites, viruses and some bacteria, as well as in the response to tumour cells and in organ transplantation. In the context of malaria infection, cell-mediated immunity plays a crucial role through the activation and coordination of immune cells by cytokines (Abbas et al., 2020). CD8+ T cells (Cytotoxic T lymphocytes) are crucial for detecting and killing infected host cells. They recognize peptides presented by MHC class I molecules on the surface of infected cells. Upon recognition, they release perforin and granzymes, leading to the apoptosis of the infected cell. In malaria, CD8+ T cells are important for killing liver-stage parasites (Cockburn et al., 2013; Reyes-Sandoval et al., 2011). CD4+ T cells (Helper T cells) play a pivotal role in the immune response by helping other cells in the immune system to respond to infection. Upon activation, they proliferate and differentiate into various subtypes, including Th1, Th2, Th17, and regulatory T cells (Tregs), each producing different cytokines that orchestrate the

immune response. In malaria, Th1 cells are particularly important, as they produce cytokines like IFN-γ and TNF, which activate macrophages and enhance the killing of parasites (Chatzileontiadou, Sloane, Nguyen, Gras, & Grant, 2021). While B cells are primarily known for their role in humoral immunity by producing antibodies, they also contribute to cell-mediated immunity. They can present antigens to T cells and produce cytokines that modulate T cell responses (Abbas et al., 2020). In malaria, antibodies produced by B cells are crucial for controlling the blood stage of the infection, but the interaction between B cells and T cells also influences the overall immune response to the parasite (Kurup, Butler, & Harty, 2019). NK cells are a type of lymphocyte that can kill cells infected with viruses or other pathogens without prior sensitization. They are activated by cytokines such as IFN-γ produced by T cells and macrophages. NK cells also produce cytokines that can enhance the adaptive immune response. In malaria, NK cells contribute to the early control of parasite replication by producing IFN-γ, which inhibits the replication of the parasite in the liver (Stevenson & Riley, 2004). Dendritic cells are antigen-presenting cells that are crucial for the initiation of T cell responses. They capture antigens, process them, and present them on their surface to T cells, along with costimulatory signals that are necessary for T cell activation. In malaria, dendritic cells are important for initiating the immune response to the parasite by presenting antigens derived from the parasite to T cells (Yap et al., 2019).

Cytokines have been identified as small proteins which are generated by cells of the immune system. Raised levels during malaria may be the cause of the harmful shedding of parasites from cells. Lymphotoxin, a less studied cytokine, is known to upregulate TNF. This Lymphotoxin may increase the expression of vascular cell adhesion molecule 1 (VCAM-1) and intracellular cell adhesion molecule 1 (ICAM-1), suggesting that these cytokines augment cellular adherence to the endothelium (Jamil Al-Obaidi & Desa, 2024). Elevated IL-6 levels are noted in severe malaria and seem to be a poor prognostic factor. On the contrary, IL-10, found at elevated circulating levels in malaria, has been reported to oppress the severity of the disease by suppressing the inflammatory effects of TNF (M'Bondoukwé et al., 2022). In human-controlled malaria infection, high IFN-γ responses were associated with a lower rate of parasite multiplication (Mpina et al., 2017). Also, prospective field studies have shown that parasite-induced IFN-γ responses are linked to a lower risk of fever and clinical malaria episodes (Sylvester et al., 2018).

1.9 Autoantibodies in Malaria

Autoantibodies during the blood stage of malaria represent a fascinating area of research that touches upon both potential protective mechanisms and the risk of contributing to disease severity. The balance between beneficial and harmful effects of these autoantibodies, along with the broader immunological responses to malaria infection, requires further investigation to fully understand and leverage this knowledge towards improving malaria prevention and treatment strategies (Mourão, Cardoso-Oliveira, & Braga, 2020a). The link between malaria and autoantibodies is a topic with limited research performed so far. The presence of autoantibodies against RBC antigens has been commonly observed in areas endemic for malaria. Autoantibodies in malaria, particularly during the blood stage of the infection, can play a complex role that involves both potential harm and, intriguingly, possible benefits to the host. *Plasmodium falciparum* is known to induce host autoimmune responses that have been associated with some of the malaria symptoms, like for example anemia, acute kidney injury and thrombocytopenia (Perkins et al., 2011; Rivera-Correa & Rodriguez, 2020). Autoantibodies have a huge role of destructing non-iRBCs during malaria where anti-RBC IgM and IgG decrease uninfected RBC deformability and induce erythrophagocytosis *in vitro* (Mourão, Cardoso-Oliveira, & Braga, 2020b; Mourão et al., 2016). Autoantibodies that are targeting RBCs may induce complement-mediated cell lysis and phagocytosis. Another mode of action inducing anemia is when anti-phosphatidylserine antibodies bind to uninfected RBCs, mediating their phagocytosis (Fernandez-Arias et al., 2016). In *P. falciparum* malaria, patients with anemia demonstrate that atypical memory FcRL5+T-bet+ B-cells increase and associate with both higher levels of anti-PS antibodies in plasma and with development of anemia (Rivera-Correa, Mackroth, et al., 2019). These self-reactive immunoglobulins have been shown to increase in vitro phagocytosis and in vivo clearance of uninfected RBCs, contributing to malarial anemia in a murine model (Fernandez-Arias et al., 2016). Furthermore, the level of anti-PS antibodies and hemoglobin levels has been reported to be negatively correlated in both *falciparum* and *vivax* malaria (Barber et al., 2019; Rivera-Correa, Conroy, et al., 2019). It has also been exhibited that certain atypical B cells populations, expressing CD11c and T-bet, secrete anti-PS antibodies. The stimulation of such cells has been demonstrated to be dependent on parasite DNA and different receptors have been suggested to be involved such as interferon-γ receptor (IFN-γR), B-cell receptor (BCR) and Toll-like receptor 9 (TLR9) (Rivera-Correa et al., 2017). However, the exact role of these atypical cells in the pathogenesis of human malaria is still undefined. Relatively recent evidence has emerged from a study conducted with *P. falciparum*-infected returning travelers (Rivera-Correa, Conroy, et al., 2019; Rivera-Correa, Mackroth, et al., 2019). The study has demonstrated that

FcRL5+T-bet+ B-cells are expanded in acute malaria. Moreover, it has been noticed that naïve peripheral blood mononuclear cells can produce anti-PS antibodies when activated by *P. falciparum*-infected RBCs lysates, emphasizing the role of atypical memory B cells as a major promoter of autoimmune anemia related to malaria. These findings highlight the fundamental role of such atypical memory B-cells as well as anti-PS autoantibodies in anemia in humans with malaria (Mourão et al., 2020a).

One of the beneficial roles of autoantibodies in malaria is the ability of these antibodies to target various components of the RBC surface, which is crucial for the invasion by the malaria parasite. Studies in areas of intense malaria transmission have shown that antibodies against Band 3-related neoantigens, which become exposed during the intracellular development of *P. falciparum*, can block cytoadherence and reduce parasite density (Hogh, Petersen, Crandall, Gottschau, & Sherman, 1994). Band 3, an erythrocyte membrane protein, plays a crucial role in the invasion of red blood cells by malaria parasites. These antibodies are thought to contribute to the clearance of infected red blood cells, thereby playing a role in the body's defense against malaria. Furthermore, the glycosylphosphatidylinositol-anchored micronemal antigen (GAMA), a protein involved in malaria parasite invasion of erythrocytes, has been shown to bind specifically to the extracellular loop of Band 3 on the erythrocyte surface. These findings suggest that exposure to malaria parasites induces immune responses that can confer a degree of protection against the disease (Hogh et al., 1994). Antibodies against this loop can reduce the binding activity of GAMA to erythrocytes and inhibit the invasion of *P. falciparum* merozoites into human erythrocytes. This discovery underscores the importance of the interaction between GAMA and Band 3 in the malaria parasite's lifecycle and highlights potential targets for vaccine development or therapeutic interventions aimed at blocking parasite invasion.

In conclusion, more studies are needed to understand the mechanisms and functions of autoantibodies, especially their action against RBCs in connection to malaria.

1.10 Changes in Plasma Proteins and Malaria

Inflammation is a key immune response to infections and other harmful stimuli, playing a crucial role in the body's attempt to heal itself. When an infection occurs, the body's inflammatory response is triggered, aiming to eliminate the cause of the harm, clear out damaged cells, and initiate tissue repair.

Plasma proteins, which are primarily produced in the liver, serve various vital functions in the body. These proteins, which include for example albumin, globulins, and fibrinogen, play roles in clotting, immune responses, and in maintaining the osmotic pressure.

During inflammation in general, irrespective of cause, the synthesis of many of proteins can change, leading to an alteration in their levels in plasma. For instance: C-reactive protein (CRP) is a protein that increases rapidly in the blood in response to inflammation. It can serve as an indicator of inflammation in the body. Elevated levels of CRP can be seen in infections, inflammatory diseases, and other conditions. Albumin is the most abundant plasma protein and helps maintain the body's osmotic balance. During inflammation, the production of albumin can decrease, leading to lower levels in plasma. Haptoglobin is a protein that binds free hemoglobin and prevents its oxidative activity. Its levels decrease in conditions of intravascular hemolysis, such as in certain infections like malaria (Golubeva, 2023). However, if there is no hemolysis but only inflammation, the level of haptoglobin will increase. α-1-antitrypsin is another protein that will increase during infections and inflammation, and it is an important inhibitor of various proteases. Orosomucoid (also called Alpha-1-acid glycoprotein, AGP), is also an acute-phase protein that can increase during inflammation.

Malaria parasites often elicit an inflammatory effect on their hosts. As a result, an elevated amount of biomarkers in the human host may signal a worsening or an already worsened disease state.

In the context of malaria, the inflammatory response can be both protective and pathological. While it aims to control and eliminate the parasitic infection, excessive inflammation can contribute to some of the clinical manifestations of the disease, such as fever, anemia, and complications like cerebral malaria. Monitoring the levels of specific plasma proteins and immunoglobulins can provide insights into the severity of the infection, the body's immune response, and the prognosis of the disease (Popa & Popa, 2021). It was for example noted that the levels of α-1-antitrypsin increased with the progression and severity of the infection (Lewis, 2012), and the levels were elevated in both *P. vivax and P. falciparum* (Ray et al., 2012). For CRP, the concentration was noted to increase with elevated parasitemia (Addai-Mensah et al., 2019). The inflammatory markers such as CRP could potentially be used as supplementary diagnostic and prognostic biomarkers for *plasmodium* parasite infections (Wilairatana et al., 2021). However, it may not be useful for diagnostic purposes due to low PPV and NPV for low and moderate parasitemia (Addai-Mensah et al., 2019).

During infection, low albumin levels are common due to hepatic dysfunction that can be associated with a malaria infection (Daneshvar et al., 2009). However, levels between the infected and non-infected may not be significant enough to use as a diagnostic test (Saad et al., 2012). Down regulation of albumin has been observed during both *P. falciparum* and *P. vivax* malaria (Ray et al., 2012).

Hemolysis play a major role during malaria and it will cause haptoglobin levels to go down during both *P. falciparum* and *P. vivax* malaria (Ray et al., 2012). On the other hand, higher levels of orosomucoid were associated with severity of the disease likely due to the anemia associated with malaria, and CRP and orosomucoid levels were highly correlated and increased during infection (Beesley et al., 2000; De Moraes et al., 2018). Orosomucoid, also known as alpha-1-acid glycoprotein (AGP), is an acute-phase protein that is synthesized in the liver. Its levels in the blood increase in response to inflammation, infection, and stress. In malaria, orosomucoid can be part of the body's immune response to the infection. Elevated levels of orosomucoid have been observed in patients with malaria, which reflects the inflammatory state induced by the infection.

The connection between anemia and orosomucoid in malaria can be understood through the role of orosomucoid in modulating the immune response and inflammation. Inflammation is a key driver of the pathogenesis of anemia in malaria. Cytokines and other inflammatory mediators can suppress erythropoiesis and promote hemolysis, contributing to the development of anemia. Orosomucoid, by virtue of being an acute-phase reactant, can influence the inflammatory process. It has several functions, including modulating the immune response, influencing the binding and transport of drugs, and possibly protecting tissues from damage during inflammation.

1.11 Malaria Vaccines

Over the last 60 years, much hard work has been put into the development of malaria vaccines. A journey that began in the early 1960s was inspired by the remarkable success of vaccines against diseases such as polio, measles, diphtheria, tetanus, rabies, and other diseases that were present at the time. This approach has proven to have the capability of reducing the global burden of infectious diseases by eradicating smallpox from humans for the first time in human history (Sallusto, Lanzavecchia, Araki, & Ahmed, 2010).

In the beginning, attempts to develop a malaria vaccine resulted in a great deal of frustration. In recent years, researchers have realized that vaccines for this disease

is extremely difficult to develop, and as time went on, it became increasingly clear that this disease is caused by a smart parasite. In order to achieve a successful malaria vaccination, a number of factors must be taken into account. The main challenge was overcoming the extremely complex biology, life cycle, and genome of the malaria parasite (*P. falciparum*), as well as the parasite's ability to evade the human immune system and the absence of sterile immunity to the disease (Lorenz & Karanis, 2011).

Historically, efforts to combat malaria through vaccination have faced challenges, which has resulted in limited success. Attempts have been made for example using viral vector vaccines to deliver *Plasmodium* antigens, boosting both T-cell and antibody responses (McCann, O'Connor, Lambe, & Pollard, 2022; Travieso, Li, Mahesh, Mello, & Blasi, 2022) or different protein-in-adjuvant vaccines focusing on combining multiple parasite antigens to induce broader immunity (Hodgson et al., 2014).

Broadly, malaria vaccine approaches can be classified into three categories:

Pre-erythrocytic vaccines: These target the parasite in its sporozoite stage before it enters the liver. The goal is to prevent the infection entirely. RTS,S/AS01 (Mosquirix™) is an example of pre-erythrocytic vaccine targeting the circumsporozoite protein (CSP) found on the sporozoite's surface of *P. falciparum.* It's the first broadly used vaccine, and it has showed a moderate efficacy of around 30% (Praet et al., 2022). Notably, a 12-month study conducted in African countries found that the RTS,S vaccine's efficacy declines over time, necessitating booster doses for sustained effectiveness (Laurens, 2020). However, it isn't a standalone solution. There's a rich history and extensive literature detailing the evolution and challenges faced during its development. While this vaccine offers hope, it's essential to continue researching and developing other potential candidates.

The R21/Matrix-M vaccine, a promising development from the University of Oxford and Novavax, has recently received an endorsement from the WHO. Positioned as a successor to the RTS,S/AS01 vaccine, R21 has shown a remarkable 77% efficacy in Phase 2b trials in Burkina Faso, targeting children aged 5-17 months. However, this efficacy was over 24 months (Datoo et al., 2022). With Phase 3 trials underway, it stands as a beacon of hope in the fight against malaria. WHO's support for R21 stems from its demonstrated safety and effectiveness, along with its ability to bridge the supply-demand gap present with the existing RTS,S vaccine.

However, since there is no blood stage protection in any of the current vaccines, and if resistance against the vaccine will start to occur the risk is high for a fast failure of the vaccines.

Blood stage vaccines: These focus on parasite antigens on merozoites and aim to target merozoites. One of the most promising candidates in this category is MSP3 (Merozoite Surface Protein 3). MSP3 is part of the parasite's surface and plays a key role in its ability to invade human erythrocytes. By targeting this protein, the vaccine aims to elicit an immune response that can recognize and neutralize the parasite before it can invade red blood cells, thus blocking the progression of the disease (Alves, Guimarães, Almeida, & Mariúba, 2022). As of the last update, MSP3 was in phase 2b clinical trials. Phase 2b trials are designed to evaluate the efficacy of the vaccine in providing protection against the disease under natural disease conditions, as well as to further assess its safety profile. Successful outcomes from this phase could pave the way for phase 3 trials, which involve a larger group of participants and aim to confirm the vaccine's efficacy, monitor side effects, compare it to commonly used treatments, and collect information that will allow the vaccine to be used safely (Alves et al., 2022). Moreover, AMA1 (Apical Membrane Antigen 1) is essential protein for merozoite invasion of RBCs. Antibodies targeting AMA1 can inhibit invasion, making it a promising vaccine candidate. Rh proteins (Rh5, Rh2b, Rh4) are involved in the process of RBC invasion. Rh5 is essential for binding to the RBCs surface during invasion. EBA (Erythrocyte Binding Antigens) antigens are involved in the binding of merozoites to RBCs. EBA175 and EBA140 are examples of EBA proteins that have been investigated as vaccine candidates. MSP (Merozoite Surface Protein) are involved in the process of merozoite invasion and are expressed on the surface of merozoites. Various MSPs, such as MSP1, MSP2, and MSP3, have been studied as potential vaccine targets. RONs (Rhoptry Neck Proteins) proteins are involved in the formation of the moving junction during invasion. RON2 is a promising vaccine candidate due to its role in establishing tight junctions between the merozoite and the host cell. SERAs (Serine Repeat Antigens) are a family of proteins expressed in the blood stages of *Plasmodium* parasites. They play roles in parasite replication and egress from infected RBCs. SERA5 has been investigated as a vaccine candidate. GLURP (Glutamate-Rich Protein) is a protein expressed on the surface of merozoites and has been studied as a potential vaccine candidate due to its role in invasion and its immunogenicity. Exp-1 (Exported Protein-1) is involved in the export of proteins into the RBCs during the blood stage of the parasite's lifecycle. It has been investigated as a potential vaccine target.

However, other components of the parasite's life cycle within the bloodstream could also be targeted by vaccines. For instance:

- Infected Red Blood Cells (iRBCs): Some vaccines may target antigens expressed on the surface of iRBCs. These antigens could potentially interfere with the ability of infected cells to sequester in tissues or avoid immune detection, thus reducing disease severity.

- Gametocytes: These are the sexual stage of the malaria parasite that develop in the human bloodstream. Targeting antigens expressed on gametocytes could potentially block transmission of the parasite to mosquitoes, thus helping to interrupt the malaria life cycle and prevent its spread.
- Host Factors: Some vaccines may aim to stimulate the host's immune response in a way that enhances the clearance of iRBCs or reduces the severity of symptoms, rather than targeting specific parasite antigens.

Transmission-blocking vaccines: These vaccines aim to halt the transmission of gametocytes from an infected individual, but these vaccines have not reached as far as the sporozoite vaccines yet.

1.12 General aim

The main aim of this work was to deepen our knowledge on understanding antibodies directed against RBC antigens in the context of malaria.

Specific aims

The specific aims were as follows:

- To investigate whether RBC autoantibodies are present in malaria endemic areas, and if so, explore which antigens these antibodies are directed against (Study I, II, III, IV).
- To investigate the presence of autoantibodies directed against a selection of specific RBC antigens among healthy Ugandan males (Study I).
- To understand which RBC antibodies could be beneficial by inhibiting the parasite life cycle (potential treatment against severe malaria anemia), (Study II).
- To understand the immunologic profile in immune, healthy individuals and correlate this to autoantibodies against RBCs (Study III).
- To determine the correlation between levels of anti-PS antibodies in pregnant women and the parameters of importance in development of anemia and immunity (Study II).
- To investigate the dynamics of IgM and IgG anti-PS antibodies in a longitudinal cohort of mother-baby pairs living in a malaria endemic area in Uganda (Study IV).

2 Methods

We conducted experiments using a variety of methods in this thesis. My goal here is to discuss the importance, advantages, and limitations of the methods.

2.1 Samples and data collections (Study I, II, III, IV)

The research described involves the collection and analysis of blood samples from distinct groups across different regions, primarily for studying health indicators and disease prevalence among specific demographics during a certain period. Here's a summarized interpretation focusing on the locations, reasons for sample collection, and the selection of participant groups:

1. **Uganda (Kampala and Wakiso districts)**: Blood samples were collected from 149 healthy adult male blood donors during the dry season. These samples were used to establish a baseline for healthy individuals in this specific geographic and climatic context. The choice of healthy males minimizes variables that could affect blood parameters, such as pregnancy-related formation of antibodies against RBCs.
2. **Sweden**: As a control group, 20 blood samples from healthy adult male donors were used. These were selected to provide a comparative baseline against the samples collected in Uganda.
3. **Nigeria (Ibadan, Oyo State)**: This study included pregnant women, with samples collected from 281 volunteers across four hospitals. This subgroup was chosen to explore health indicators specific to pregnancy and the impact of regional health practices, like intermittent preventive treatment in pregnancy (IPTp). The collection sites were selected to represent a range of healthcare settings within the region, from teaching hospitals to local clinics, providing a comprehensive overview of maternal health in this area.
4. **Uganda (Kasangati)**: This part of the study focused on a longitudinal analysis of mothers and infants, examining changes over time from childbirth through the first nine months postpartum.

Overall, the selection of diverse geographical locations and specific demographic groups (healthy adult males, pregnant women, and mother-infant pairs) allows the research to address various health questions across different populations. The choice of locations like Uganda and Nigeria were because Uganda and Nigeria carry a disproportionately high burden of malaria, which continues to be a major public health challenge. In 2022, the WHO African Region, where both countries are located, was home to approximately 94% of all malaria cases (233 million) and 95% of malaria deaths (580,000) globally. Children under 5 years old are particularly vulnerable, accounting for about 80% of all malaria deaths in the region. Nigeria alone accounted for 26.8% of all global malaria deaths, the highest in the world, while Uganda accounted for 5.1%, making them critical focal points in the fight against malaria(World Health Organization, 2023).

Malaria transmission in Uganda is primarily caused by the *P. falciparum* parasite, which accounts for 98% of infections. The country experiences stable, perennial malaria transmission across 90-95% of its territory, with transmission peaks aligning with the rainy seasons. Despite efforts, certain populations in Uganda face significant challenges in accessing malaria prevention and treatment services due to economic, social, and contextual barriers. These vulnerable groups include children under five years, pregnant women, people living with HIV, and others facing specific economic or geographic disadvantages.

In response, Uganda has developed comprehensive strategies aimed at ensuring equitable access to malaria services, guided by principles of human rights, gender equality, and health equity. These strategies include adopting the WHO guidelines for Intermittent Preventive Treatment in Pregnancy (IPTp) and expanding the ownership and use of insecticide-treated nets (ITNs). From 2016 to 2018, there was a notable improvement in the percentage of pregnant women receiving the recommended doses of IPTp, and household ownership of ITNs increased significantly.

Nigeria, on the other hand, represents the highest global burden of malaria, with an estimated 68 million cases and 194,000 deaths in 2021 alone. The entire population of Nigeria is at risk of malaria year-round, making it a priority for global malaria eradication efforts.

Both Uganda and Nigeria's high burden of malaria underlines the importance of sustained international and local efforts in malaria control and elimination. This includes improving access to prevention methods like ITNs and medication, enhancing healthcare infrastructure, and addressing social and economic barriers to healthcare access. The WHO and other international organizations continue to support these countries through various initiatives, including the introduction of

malaria vaccines and preventive chemotherapies, aimed at reducing the incidence and severity of the disease.

2.2 Direct antiglobulin test (DAT) (Study I, III)

A DAT-test is a simple method and a routine clinical laboratory assay used to detect antibodies bound to RBCs. By pipetting the RBC suspension into a gel card well, plasma samples were tested with anti-human IgG in the gel card system. After centrifugation, the result was read over a light box. Based on the degree of agglutination, the results were grouped as follows: 1+, 2, 3, and 4. The medical laboratory only conducted the assay once, as is standard practice in the routine laboratory **(Figure 5).**

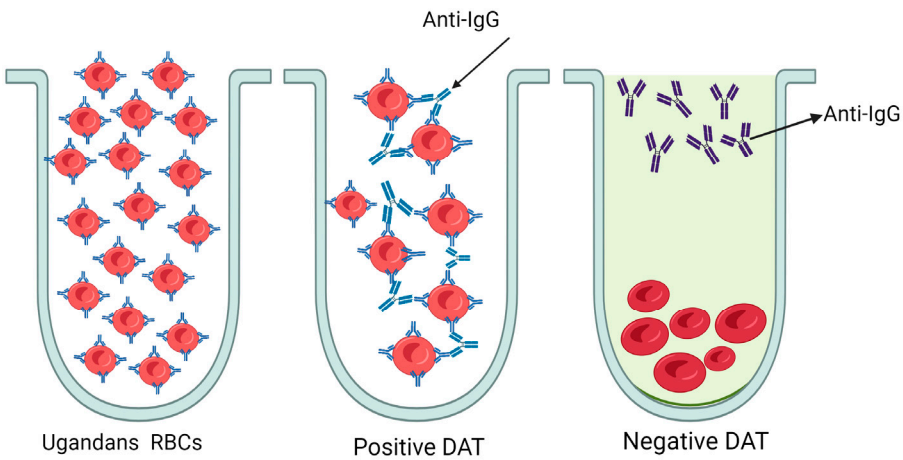

Figure 5. A schematic overview of a Direct antiglobulin test, DAT.

2.3 Antibody screening by the gel-indirect antiglobulin test (gel-IAT) (Study I, III)

In routine clinical blood bank laboratories, antibody screening is used to identify the existence of antibodies that may cause a transfusion reaction to occur when they are not expected. In this study, all samples were screened for unexpected IgG antibodies by testing the plasma with reagent RBCs from three well-characterized reagent RBC samples, each derived from an individual healthy blood donor of blood group O (to

avoid the appearance of anti-A and anti-B agglutination). We used the gel-indirect antiglobulin technique (gel-IAT) in order to detect the presence of these antibodies. The plasma was pipetted into a suspension of RBCs in wells of the gel cards before the samples were placed in the incubation chamber. We incubated the cards, then centrifuged them at the end of the process. There was a light box that was used to read the results of the assays and the results were scored as follows: (agglutination from 1+ to 4+). RBCs were tested both untreated and after being treated with papain, which is a protease frequently used to boost the antibody-antigen response in screenings of RBCs. We treated the RBC with papain before testing: The RBC were centrifuged to compact them, then combined with a solution containing papain dissolved in phosphate-buffered saline (PBS). This mixture was then allowed to incubate for a duration of 5 minutes at a temperature of 37°C. After this incubation period, the RBCs were washed using PBS, then resuspended once again in papain before undergoing further testing. The papain-treated RBCs were then compared to the untreated ones according to the procedures previously described **(Figure 6).**

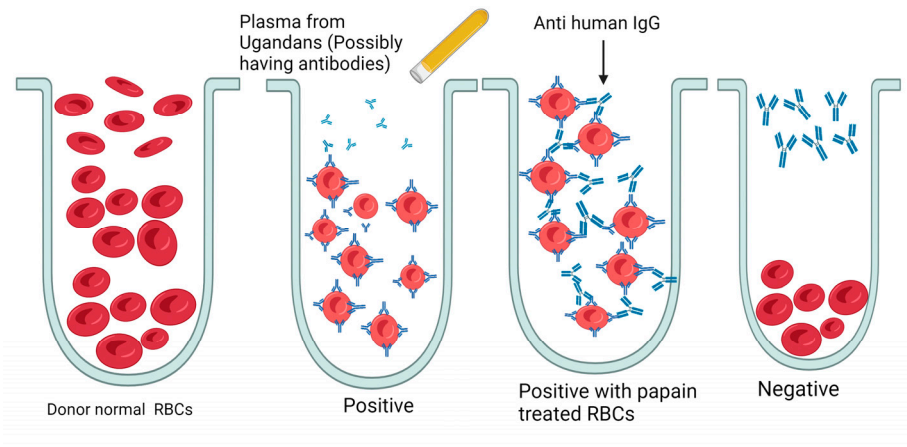

Figure 6. Schematic picture of antibody screening by the gel-indirect antiglobulin test.

2.4 Antibody identification (Study I)

Antibody identification is performed as part of routine transfusion medicine laboratory processes as soon as an unexpected antibody is detected as part of routine transfusion medicine laboratory services. In order to carry out all of this testing, we had to use a test a panel of reagent RBCs (in our case, we used 11 different RBCs) from different blood donors. As far as antibody screening and antibody

identification is concerned, all tested RBC (both for antibody detection and antibody screening) have phenotypes (presence or absence of a particular antigen) for the following antigens (blood group system in brackets): D, C, E, c, e, CW (Rh), M, N, S, s (MNS), P1 (P1PK), K, k, Kp^a, Kp^b (Kell), Le^a, Le^b (Lewis), Fy^a, Fy^b (Duffy), Jk^a, JK^b (Kidd), Lua, and Lu^b (Lutheran). As part of the identification process, plasma reactivity patterns are matched with the antigen profiles associated with the tested RBCs. We performed the same gel-IAT test as described previously, but we used other RBCs (such as those of blood group O) for this test. During the antibody identification process, samples which were DAT-positive or plasma that had reacted with at least one of the antibody screenings (untreated or papain-treated cells) were selected for further testing. It was generally the case that the antibody identification panel was tested only once, however, if the specificity of the antibody was unclear, for example it could potentially have been fibrin in the plasma that interfered with interpretation, a retest was conducted. An example of this would be a small clot visible at the top of the gel agglutination column, and this could be resolved by centrifuging the plasma sample again **(Figure 7).**

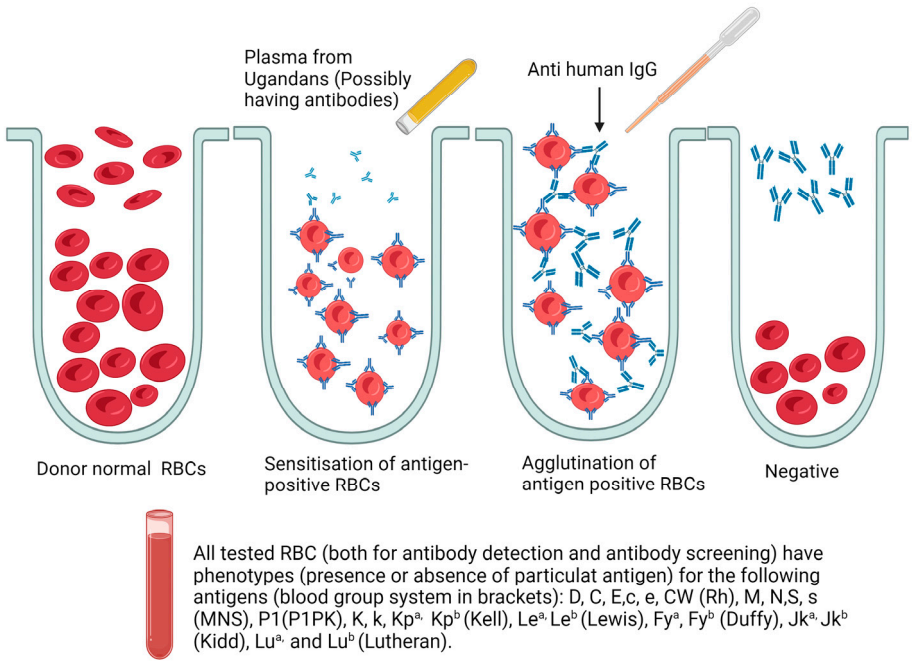

Figure 7. Schematic picture of antibody identification by the gel-indirect antiglobulin test.

2.5 Test of rare phenotypes (Study I)

Plasma samples that responded with all test RBCs in the 11-cell panel were assumed to have an antibody directed against a common antigen present on all RBCs. As many RBCs with rare phenotypes as were available in our rare RBC inventory were thawed and tested. These included the following rare phenotypes (absence of a particular antigen): Rh_{null}, Cr (a-), Di (b-), En (a-), Fy(ab-), Ge:-2.3, Gy (a-), JMH, Jo (a-), Jr (a-), Js (b-), Kn/McC-, K0-, Lan-, U-, Vel-. The different RBCs were prepared as suspensions and tested with the gel-IAT as previously described. The objective was to determine whether any of the plasma that had reacted with the 11-cell antibody identification panel were non-reactive with any of the rare phenotype RBCs, which would indicate antibody specificity for that membrane protein **(Figure 8).**

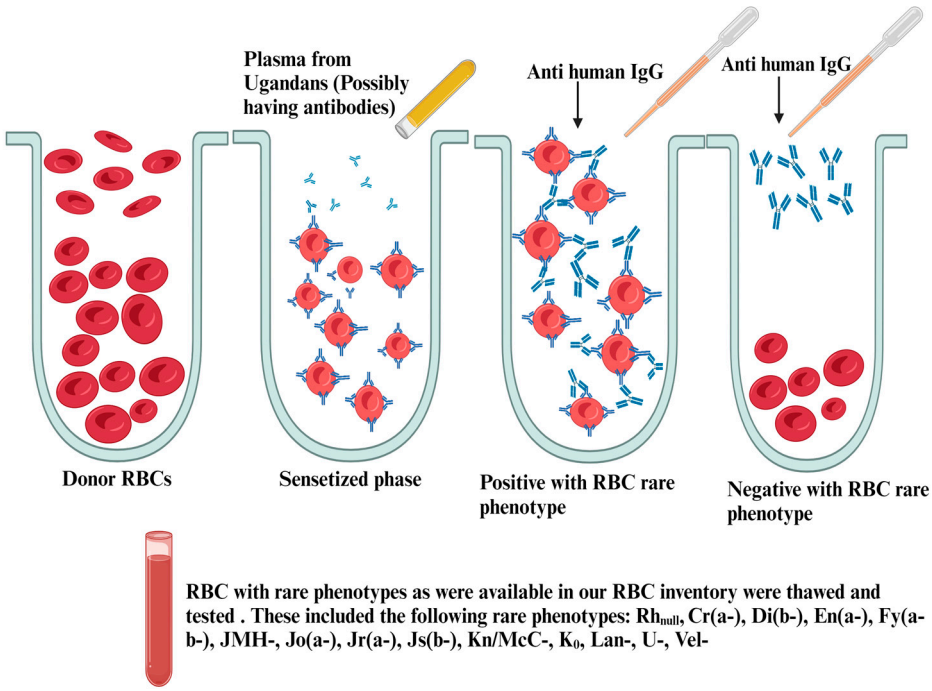

Figure 8. Schematic picture of test of rare phenotypes by the gel-indirect antiglobulin test. Created with Biorender.

2.6 Lewis inhibition studies (Study I)

In three samples, an apparent anti-Le^a was identified. However, to be sure that the test result really was due to antibodies against Lewis, a confirmatory assay was performed. This confirmatory assay was necessary since soluble Lewis antigen could be found in saliva and other body fluids, including plasma, which can lead to a false positive detection of these antibodies. Briefly, plasma and Lewis agent were incubated at room temperature and then retested with the antibody identification panel (**Figure 9**).

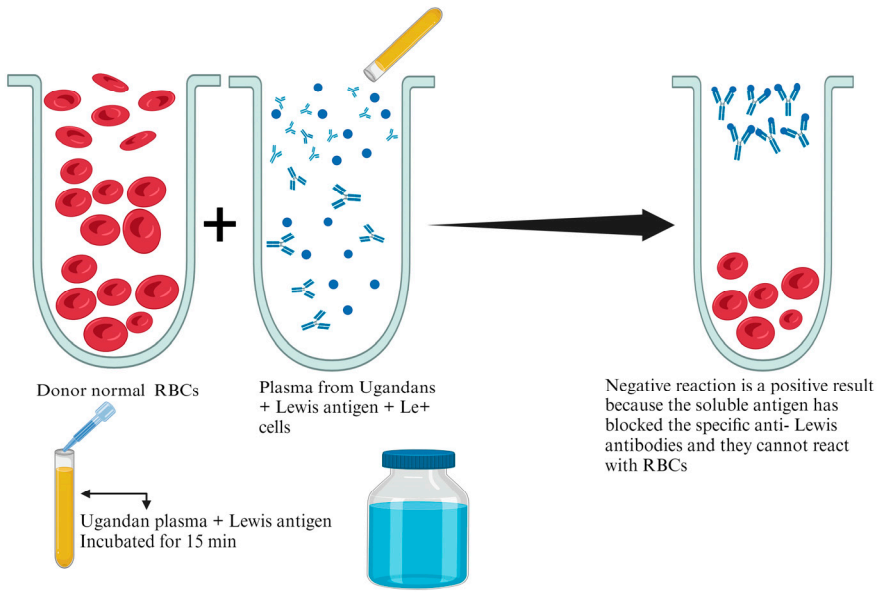

Figure 9. Schematic picture of Lewis inhibition assay by the gel-indirect antiglobulin test. Created with Biorender.

2.7 Measurement of IgG directed against *P. falciparum* schizont extract in ELISA (Study I, III)

In an ELISA assay, plasma samples from Ugandan and Swedish individuals were analyzed to determine if there were IgG antibodies present that targeted schizont extract prepared from FCR3S1.2 parasites. If there were antibodies present, this would be a sign that the individuals had been exposed to *P. falciparum*, but it can´t be used to determine if a single individual is immune or not. The extract was coated in ELISA plates overnight, washed with PBS-Tween and blocked with casein in PBS-Tween. In the following step, plasma samples were placed in casein-PBS

Tween solution for incubation. We washed the plates and added goat anti-human IgG conjugated to Alkaline phosphatase. Following washing, the color was developed with p-nitrophenylphosphate disodium hexahydrate and the absorbance was read at 405 nanometer. All incubations took place at room temperature. There was a duplicate analysis of all samples and the average values were used for the analysis of the samples that were further analysed (**Figure 10**).

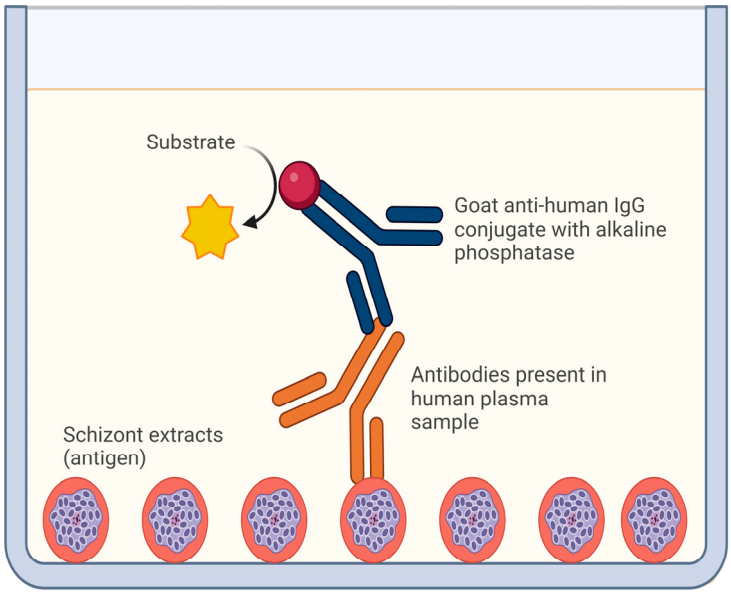

Figure 10. Schematic picture of measurement of IgG directed against *P. falciparum* schizont extract in ELISA. Created with Biorender.

2.8 Plasma protein analysis (III)

To determine the extent of inflammation present in the Ugandan individuals, a plasma protein analysis was performed at the routine clinical chemistry laboratory at Skåne University Hospital in Malmö, Sweden. It was done on the Roche platform and the following proteins were analyzed: CRP, α-1-antitrypsin, albumin, haptoglobin, orosomucoid, IgA, IgG and IgM.

Haptoglobin measurement (Study II)

Plasma haptoglobin levels were determined using an ELISA kit by following the manufacturer's instructions. This method was used here due to small volumes of samples.

2.9 Measurement of IgG directed against RBC antigens in ELISA (Study III)

In this study, plasma samples from Uganda and Sweden were tested for the presence of IgG targeting RBCs or recombinant RBC antigens by ELISA. An ELISA was conducted by coating 96 well plates with RBCs of blood group O from blood donors in coating buffer or with the recombinant proteins JMH, Cromer or Kell in PBS, and then incubating them overnight. Following the washing of the plates in PBS containing Tween-20 and blocking them with BSA in PBS-Tween for one hour, the plates were washed and incubated for one hour with plasma in PBS-Tween before being washed and incubated again for one hour with plasma. In the next step, goat anti-human IgG (specific for the ɣ chain) conjugated to alkaline phosphatase was added. Afterwards, the color was developed with p-Nitrophenyl Phosphate disodium hexahydrate (pNPDHE) and the absorbance was measured at 405 nm to determine the final color intensity. The incubations took place at room temperature throughout the entire process. A duplicate analysis of all samples was carried out and the mean values were then used as a basis for further analysis **(Figure 11 and 12).**

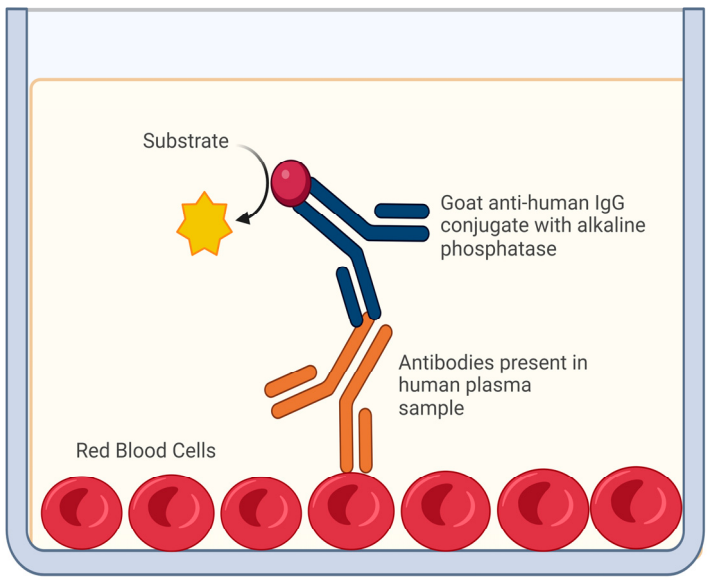

Figure 11. Schematic picture of measurement of IgG directed against RBC in ELISA. Created with Biorender.

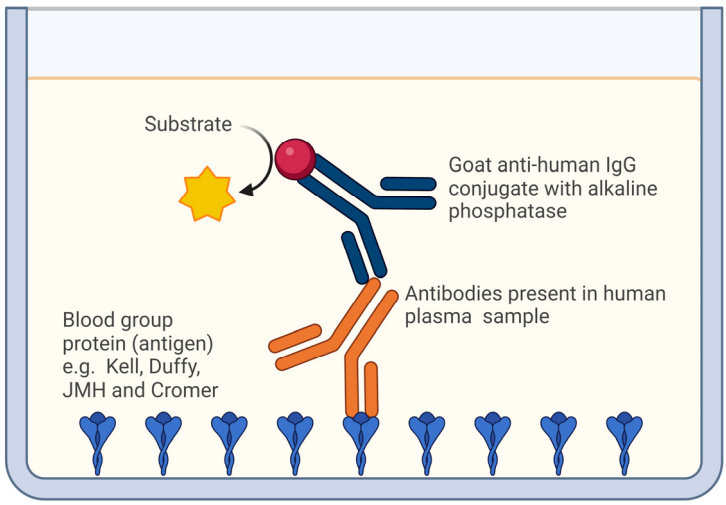

Figure 12. Schematic picture of measurement of IgG directed against RBC antigens in ELISA. Created with Biorender.

2.10 Growth Inhibition assay (Study III)

This assay was performed to investigate the potential function of antibodies present in plasma. The growth/invasion inhibition assay used in this study has been described elsewhere (Tijani et al., 2017). Generally, plasma samples were placed into 96-well U-bottom plates along with trophozoite-stage 3D7 *P. falciparum* parasites. The plasma samples were initially filtered over a 40K molecular weight filter to remove small molecules but still leave the immunoglobulins present. As inhibitors and growth controls, heparin and PBS were inserted on each plate. At around 40 hours of culture, each well was replenished with fresh culture medium. As part of the experiments, the cells were colored with acridine orange at approximately 80 to 90 hours after the experiment began (which is approximately two life cycles). Following washing of the stained cells with PBS, the cells were fixed with formaldehyde/glutaraldehyde. An Accuri C6 (BD) flow cytometer was used to determine parasitemia. We used the software FLOWJO (BD) for the analysis of the data. A moist candlelight cabinet was used to maintain all cultures at 37°C in a moist atmosphere **(Figure 13).**

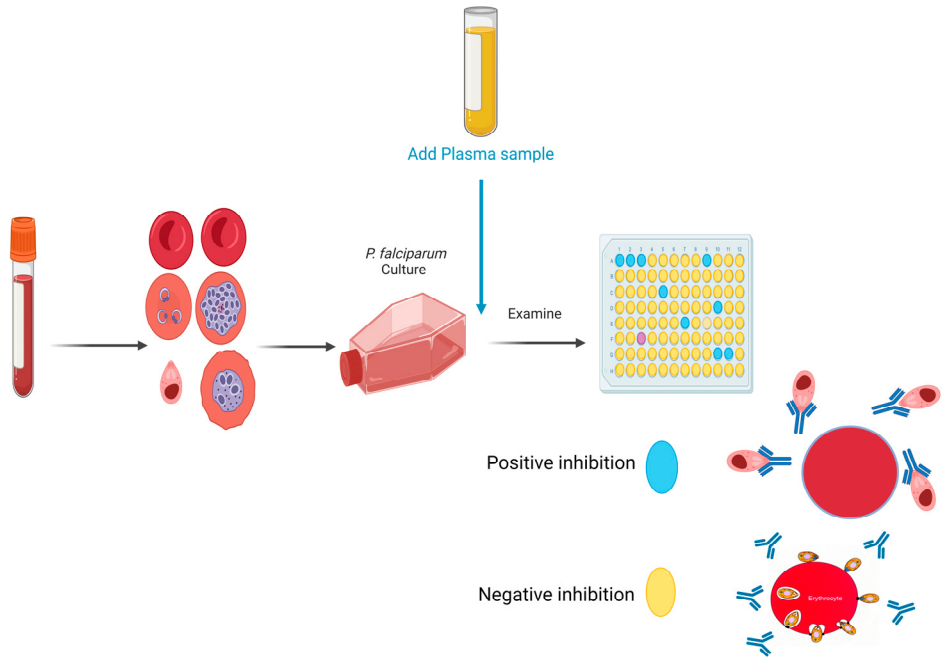

Figure 13. Schematic picture of growth inhibition assay. Created with Biorender.

2.11 DNA Extraction, Polymerase chain reaction (PCR) and Gel electrophoresis analysis (Study III)

In order to decide whether the individuals in our study were carrying low levels of parasites or not, we performed PCR. We used a QIAamp DNA Blood Mini Kit according to the manufacturer´s instructions to extract DNA from 149 Ugandan men using 200 µL of EDTA blood per individual, and then stored them at -20°C until usage. The extracted DNA was used to amplify a 430bp fragment of the *P. falciparum* cytochrome B gene. In order to conduct a positive control of this experiment, DNA was prepared in-house from cultured *P. falciparum* FCR3S1.2 in addition to a negative control of water. In order to conduct the PCR test, a master mix based on primer 1, PlasMtR forward, and primer 2, PlasMtR reverse, together with Platinum II Hot-start Green PCR Master Mix and H_2O was made. The following steps in the PCR programme were run: 95°C 4 min and 95°C 15s, 60°C 90s for 45 cycles, and then samples were run on large agarose gels and stained **(Figures 14 and 15).**

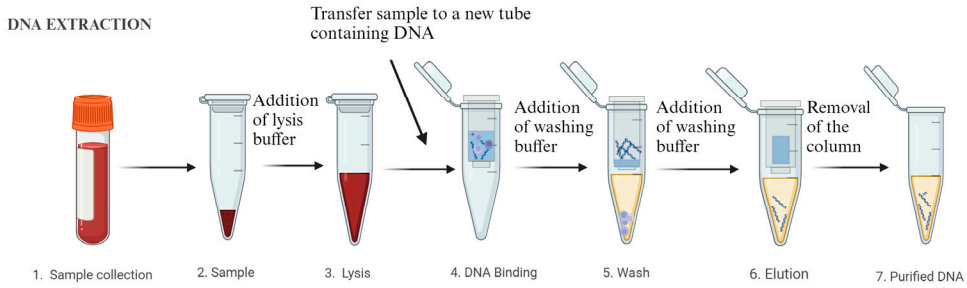

Figure 14. Schematic picture of DNA extraction steps process. Created with Biorender.

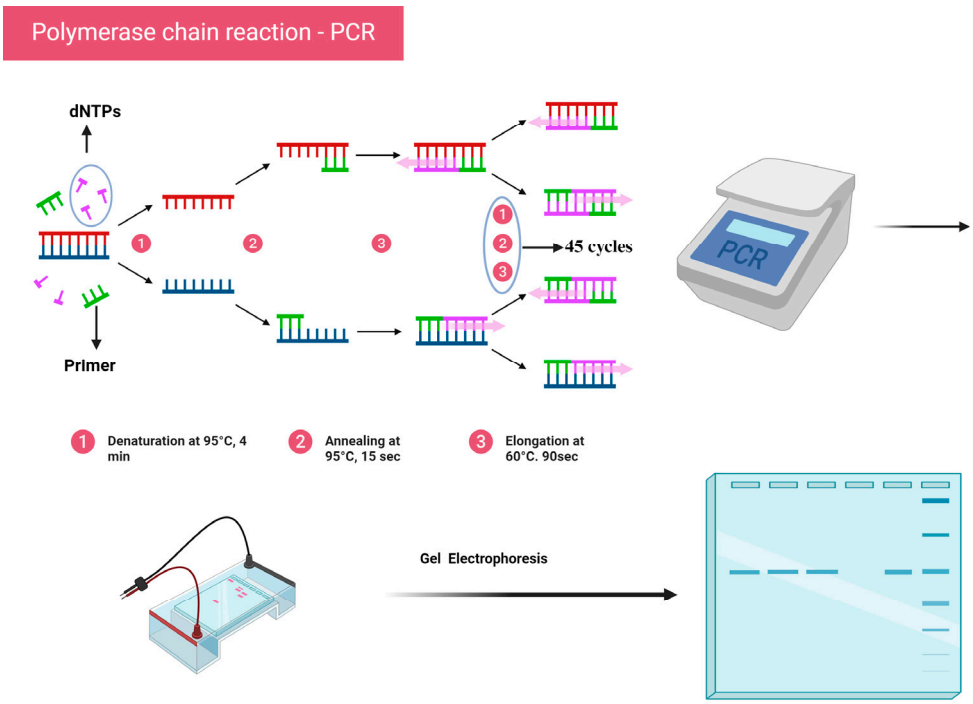

Figure 15. Schematic picture of Polymerase chain reaction (PCR) and Gel electrophoreses analysis. Created with Biorender.

2.12 Measurement of anti-PS by Enzyme Linked Immunosorbent Assay (ELISA) (Study II, IV)

In study II and IV, anti-PS IgG/IgM antibodies were detected using an ELISA kit and were measured according to the instructions provided by the manufacturer. Basically, we diluted the samples, calibrators, and controls and added them to PS-precoated wells, which were then allowed to incubate for 30 minutes. After washing, incubation for 15 minutes was carried out and HRP-labelled anti-human IgG/IgM was added to the wells. Adding TMB (3,3',5,5'-tetramethylbenzidine) and incubating for 15 minutes were the next procedures. The reaction was stopped by adding stop solution, then absorbance was measured at 450 nm. An anti-PS antibody standard curve was used in order to determine the concentration of anti-PS antibodies **(Figures 16 and 17).**

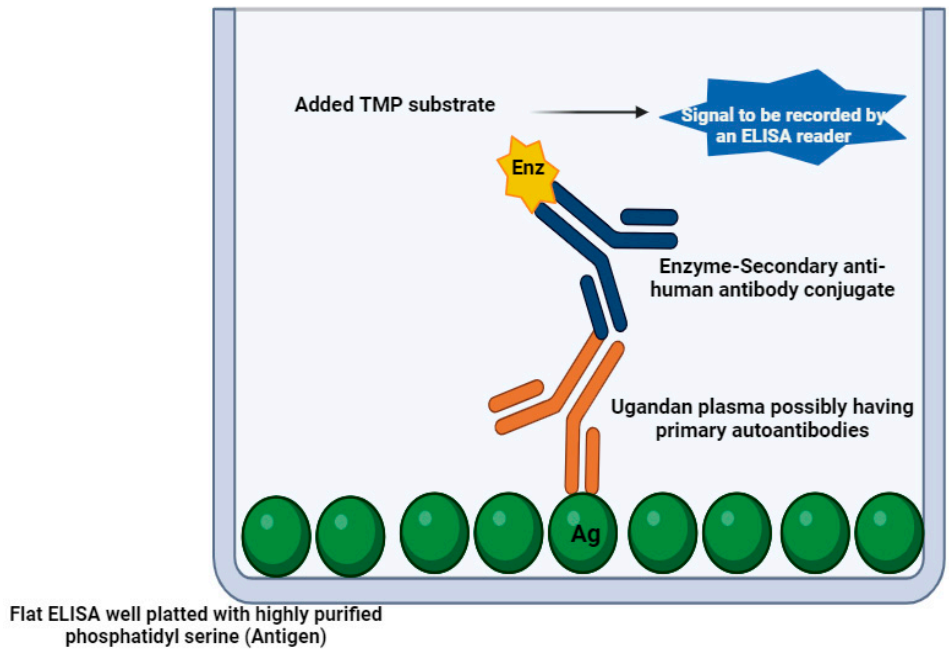

Figure 16. Schematic picture of measurement of anti-PS IgG by Enzyme Linked Immunosorbent Assay (ELISA). Created with Biorender.

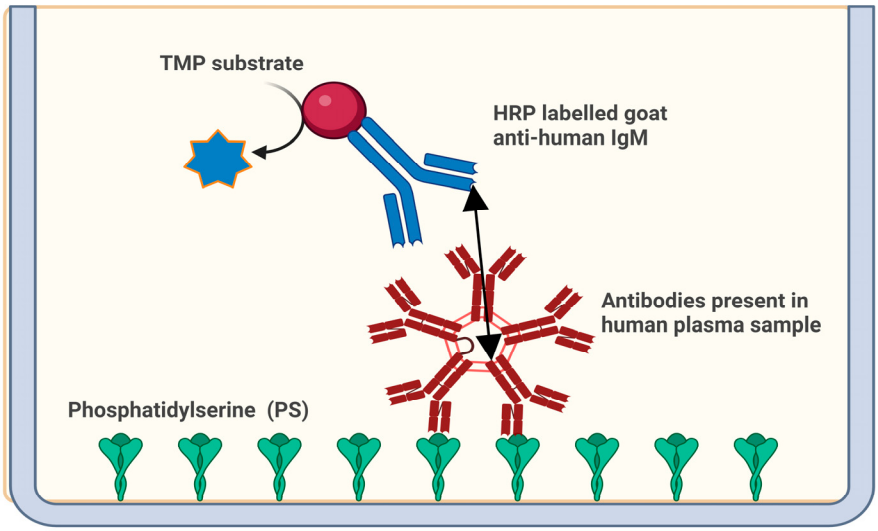

Figure 17. Schematic picture of measurement of anti-PS IgM by Enzyme Linked Immunosorbent Assay (ELISA). Created with Biorender.

2.13 Parasitological examination (Study II)

Thick and thin smears made directly from collected blood samples were stained with Giemsa. Parasitemia was calculated based on the number of parasites counted against 500 leukocytes and assuming 8000 leukocytes/µL of blood.

2.14 Measurement of anti-VAR2CSA by ELISA (Study II)

Anti-VAR2CSA (VAR2CSA is a 350 kDa protein that is composed of six Duffy-binding-like domains and mediates infected RBC sequestration by binding to oncofetal chondroitin sulphate, which is exclusively expressed by the syncytiotrophoblasts in the placenta) IgG antibodies in the plasma samples were measured by standard ELISA. Immunoplates were coated overnight at 4°C with VAR2CSA in PBS. Plates were washed using PBS/Tween and then blocked with skimmed milk/PBS/Tween 20 for 2h. Washing was repeated and plasma samples in skimmed milk/PBS/Tween 20 were added in duplicates followed by incubation for 1 h. Plates were washed again and HRP-conjugated goat anti-human IgG diluted in

skimmed milk/PBS/Tween 20 was added and incubated for 1 h. After washing, ABTS was added and color was allowed to develop for 30 min in the dark before absorbance was read at 405 nm.

2.15 Statistical data analysis (all studies)

Statistical Software and Versions: Data analysis was conducted using GraphPad Prism (version 10.0) and R Statistical Software (version 4.2.2). GraphPad Prism was specifically used in nonparametric tests and graphical analysis, while R was used for its general statistical computing capabilities.

Statistical Tests and Models Used:

- **Mann-Whitney tests** were frequently utilized to compare two independent groups across various parameters.
- **Spearman's correlation** was employed to assess the correlation between parameters.
- **Kruskal-Wallis tests** were mentioned for comparing medians across multiple groups.
- **Multivariate regression analysis** was used to explore associations involving multiple variables like antibody levels, age, and gravidity.
- **Wilcoxon matched pairs signed rank test** was specified for comparing paired samples.
- **Linear mixed models** were applied to analyze longitudinal data, specifically to model antibody levels over time and to adjust for maternal antibody levels at birth. These models were also used to investigate the impact of high versus low antibody levels at birth on the trajectory of infant antibody responses.
- **Pearson correlation** was utilized for assessing correlations at specific time points, separately for mothers and infants.
- **Adjustments for Multiple Comparisons**: The false discovery rate adjustment method was mentioned to control for multiple comparisons in statistical testing.

Significance Levels and Analysis Groups: A significance level of $p < 0.05$ was consistently acknowledged across the analyses as the threshold for statistical significance.

2.16 Ethical considerations

For study II, ethical approval was obtained from the Oyo State Ministry of Health Ethical Review Approval Committee (AD13/479/833), the Ethical Committee Board of Our Lady of Apostle Hospital, Oluyoro, Okeofa, Ibadan (OCH/EC/18/08) and the Swedish Ethical Review Authority (2022-00777-01). Permission was also obtained from the administrative heads of each hospital where samples were collected. A signed consent form was obtained from each participant before samples were collected. Study participation was voluntary and refusal did not attract any penalty with regards to the benefits of the study. The participants were given the opportunity to ask questions before enrolment.

For study IV, the ethical approvals for this study were obtained from the Makerere University School of Medicine Research and Ethics Committee, The Uganda National Council of Science and Technology (approval Uganda 2007-045), and Regionala Etikprövningsnämnden in Stockholm, Sweden (2011/132-31/3). All participants signed the informed consent forms before samples were collected. Study participation was voluntary, and refusal did not attract any penalty with regards to the benefits of the study. The participants were given the opportunity to ask questions before enrolment.

For the Ugandan and Swedish adult samples, they were from blood donors who agreed to the use of the left over blood for research and these individuals were completely anonymous to us and we could not trace back who it came from. Therefore, no ethical permission is needed.

3 Results

3.1 Study I

In this investigation, we examined Ugandan samples to gain insights into the potential presence and specificities of autoantibodies against RBCs in healthy individuals living in a malaria endemic area. The Ugandan cohort comprised 149 adult male blood donors, while the Swedish group consisted of 20 male blood donors.

The results show that when we used DAT, 15% of the Ugandan samples displayed positive reactions, with 23 positive cases. In contrast, all Swedish RBCs tested negative in the DAT **(Table 1).**

In antibody screening tests, we found 15 reactions among the Ugandan individuals when using untreated RBCs, while papain-treated RBCs yielded 62 reactions. In total, 44% of the Ugandan samples exhibited antibody reactivity against the screening RBCs.

When adding those positive in DAT to those positive in antibody screening tests, we found 78 positive samples where we continued with further tests to identify the antibody specificity in more detail. Of these, 14 samples displayed specific antibodies, while 6 exhibited partial specificity **(Table 2).** Another 58 samples demonstrated antibodies but without precise identification. 34 samples exhibited a faint reactivity, but we couldn't pinpointed it further. Notably, 19 samples positive in antibody screening didn't show reactivity in antibody detection, possibly due to instability upon freezing. Among DAT-positive samples, five lacked detectable antibodies, indicating that all circulating antibodies were bound to the individual's RBCs and were undetectable in plasma. When we run Rare RBC Panel Testing, samples reacting with the initial panel were tested with rare RBCs. The results are shown in Table 1 and 2.

Table 1. Summary of DAT (Direct Antiglobulin test) and antibody screening results for Ugandan (malaria endemic area) and Swedish (non-endemic area) samples.

	Ugandans (%)	Swedes (%)
Number of individuals tested	149 (100%)	20 (100%)
Total positive DAT[1]	23 (15%)	0
Positive DAT only	13 (9%)	0
Total positive antibody screen	65 (44%)	1 (5%)
Antibody screen positive only	55 (37%)	1 (5%)
Both DAT and antibody screen positive	10 (7%)	0
Total positive for DAT and/or antibody screen	78 (52%)	1 (5%)

Furthermore, we tested antibody responses against *P. falciparum* schizont extract, and found that 99% of the Ugandan samples exhibited a positive reaction (indicating that they had been exposed to parasites), while there was no reactivity in the Swedish samples. Interestingly, no significant correlations emerged between schizont extract ELISA and autoantibody tests against RBCs.

In conclusion, specific autoantibodies against erythrocytes are common in a malaria endemic area.

Table 2. Results of the antibody identification studies performed on 78 Ugandan samples that had a positive antibody screen and/or a positive DAT[2].

Table 2a. Results of the antibody identification studies: 14 plasma samples contained specific antibodies to blood group antigens.

Samples (n)	Antibody specificity	Blood group system	Membrane component that antibodies are directed against
1	Anti-En[a]	MNS	GPA[3]
2	Anti-M	MNS	GPA
1	Anti-S	MNS	GPB[4]
4	Anti-U	MNS	GPB
1	Anti-U, -E	MNS, Rh	GPB, RhCE
1	Anti-Le[a], -E	Lewis, Rh	Glycolipids, RhCE
2	Anti-Le[a]	Lewis	Glycolipids
1	Anti-H	H	N-glycans on membrane proteins
1	Anti-Kp[a]	Kell	Kell

1 Direct Antiglobulin test (DAT).

2 Direct antiglobulin test (DAT).

3 Glycophorin A (GPA).

4 Glycophorin B (GPB).

Table 2b. Results of the antibody identification studies partial specificity could be identified in 6 pan-reactive samples. Di (Diego), Cr (Cromer), Fy (Duffy), Ge (Gerbich), GPA (Glycophorin A), GPB (Glycophorin B), GPC (Glycophorin C), ACKR1 (Atypical Chemokine Receptor 1).

Samples (n)	Phenotype of the nonreactive rare RBC	Implicated membrane components that antibodies are directed against
1	Di(b-), Cr(a-), Fy(a-b-)	Band 3, CD55, ACKR1, GPA
1	Di(b-), Cr(a-), Fy(a-b-), En(a-)	Band 3, CD55, ACKR1, GPA
1	Di(b-), En(a-), Fy(a-b-)	Band 3, GPA, ACKR1
1	Di(b-), Fy(a-b-), Lan-, Ge:-2,3	Band 3, ACKR1, ABCB6, GPC
1	Di(b-), En(a-), Ge:-2,3	Band 3, GPA, GPC
1	Rhnull, Fy(a-b-)	RhD/RhCE/RhAG , ACKR1

3.2 Study II

In this study, 281 pregnant women living in Nigeria participated, with ages ranging from 18 to 43 years, and an average age of 30 years. The study population was further categorized based on gravidity, including primigravidae, secundigravidae, and multigravidae (3-7 pregnancies) **(Figure 18 and 19)**.

We measured IgG antibodies against PS and found a decrease in PS-specific antibody levels as gravidity increased ($p<0.001$). Notably, both primigravidae and secundigravidae had substantially higher median antibody levels compared to multigravidae. This intriguing trend remained consistent regardless of the presence of peripheral infection, as no significant difference was noted in anti-PS antibody levels between infected and uninfected pregnant women ($p=0.33$). This suggests a potentially unique immunological response in multigravidae.

Moreover, the comparison of anti-PS and anti-VAR2CSA IgG antibody levels among anemic pregnant women shed light on the differences between these two groups. While anti-PS antibody levels were notably higher in anemic women ($p = 0.007$), there was no significant difference in anti-VAR2CSA antibody levels ($p = 0.18$) **(Figure 19).** This underscores the potential association between anemia and anti-PS antibody response.

When anti-VAR2CSA IgG antibodies were analyzed by gravidity, multigravidae pregnant women displayed higher median VAR2CSA antibody levels compared to primigravidae and secundigravidae, but this difference did not reach statistical significance ($p = 0.35$). This suggests that gravidity might not be the sole determining factor in the anti-VAR2CSA antibody responses.

To further elucidate relationships among various parameters, a Spearman correlation analysis was conducted. There was no correlation observed between anti-PS and anti-VAR2CSA antibody levels (r=-0.03, p=0.62), suggesting an independent regulation of these two antibody types. However, anti-PS antibodies displayed an inverse correlation with age (r =-0.16, p=0.01), but then older women have had more pregnancies compared to younger women. However, when a multivariate regression analysis was conducted, gravidity (b = 0.42) emerged as a more influential factor on PS antibody levels than age (b = 0.1).

Furthermore, comparing pregnant women who enrolled in IPTp and those who did not showed no significant difference in mean anti-PS antibodies levels (p = 0.49), indicating that IPTp does not affect the anti-PS antibodies.

The outcomes of Study II provide valuable insights into the complex relationships between anti-PS and anti-VAR2CSA antibody levels, their associations with anemia, gravidity, and age. The most interesting finding is that anti-PS antibodies, which are associated with anemia, decrease with more pregnancies.

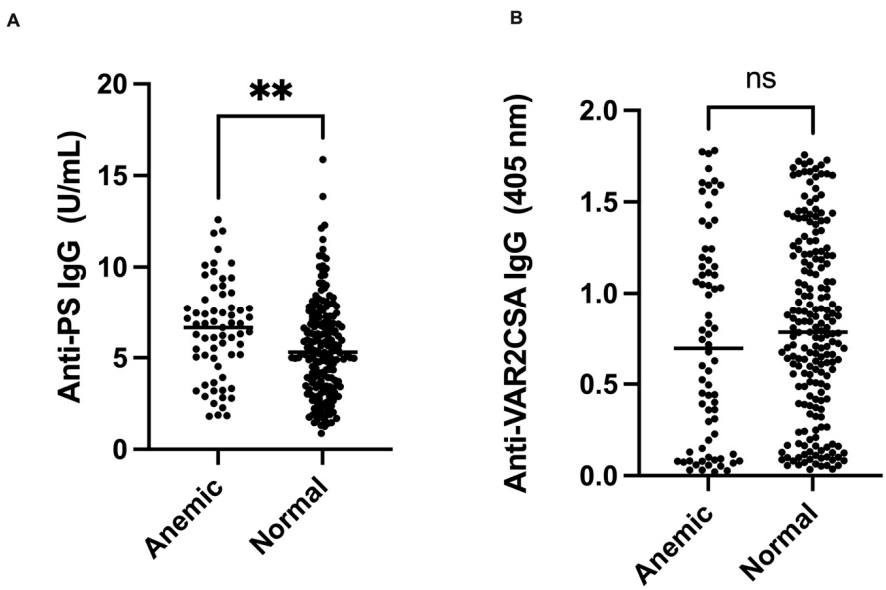

Figure 18. Comparison of anti-PS and anti-VAR2CSA antibody levels in pregnant women comparing anemic to those with normal PCV. (A) The median anti-PS antibody level was significantly lower in anemic pregnant women (** represent ≤ 0.01). (B) There was no difference in median anti-VAR2CSA antibody levels between anemic and normal pregnant women (p = 0.18).

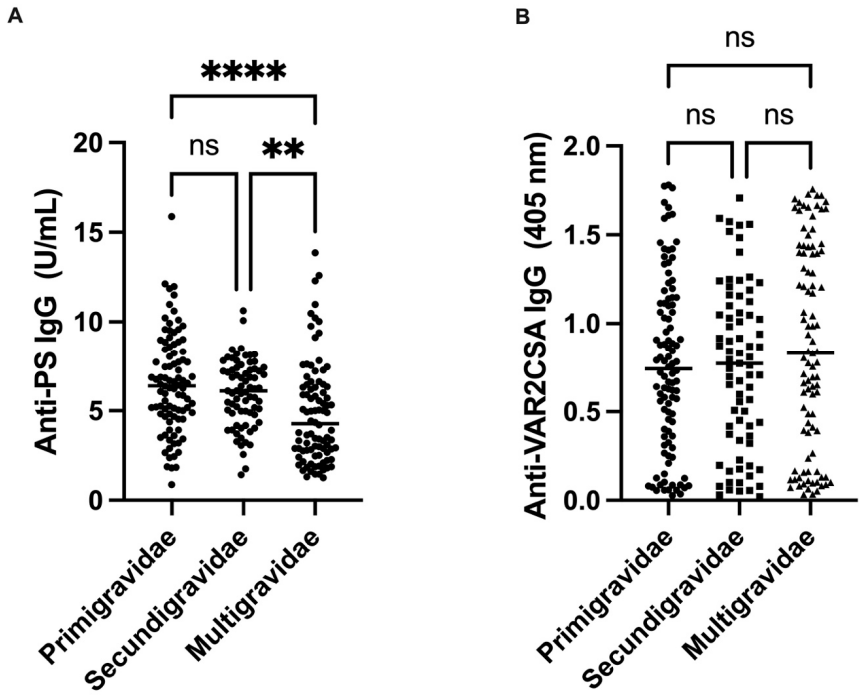

Figure 19. Comparison of anti-PS and anti-VAR2CSA antibody levels by gravidity. (A) Anti-PS levels were low in multigravidae when compared to both primigravidae and secundigravidae (** and **** represent $p \leq 0.01$ and $p \leq 0.0001$, respectively). (B) Anti-VAR2CSA antibody seemed to be highest in multigravidae, but it was not statistically significant ($p = 0.35$).

3.3 Study III

In this study, we wanted to continue our studies of antibodies against RBCs, and to understand how natural immunity against malaria is maintained, and what could possibly be important for this. We used the same Ugandan samples (n=149) from adult blood donors as in study I, and also included Swedish samples (n=10) for comparison.

IgG directed against RBC in ELISA

We measured binding of IgG antibodies from plasma to three different Swedish donors of blood group O RBCs in ELISA. For all three RBCs, we could see clear differences between the Ugandan and Swedish individuals. The Ugandan samples showed varying but relatively high and significantly different binding when compared to the Swedish samples.

IgG directed against JMH, Cromer and Kell

We measured presence of IgG directed against recombinant proteins of the RBC surface antigens JMH, Cromer and Kell in plasma from the Ugandans and compared this to Swedish blood donors. The results showed a highly significant difference between Ugandan and Swedish samples for antibodies directed against JMH ($p<0.0001$), with higher levels of anti-JMH antibodies among the Ugandans. No such difference was evident regarding Kell ($p= 0.88$) or Cromer ($p= 0.48$).

Plasma protein analysis

For the 149 Ugandan males the following plasma proteins were measured: CRP, α-1-antitrypsin, albumin, haptoglobin, orosomucoid, IgA, IgG, and IgM. The results were compared to Swedish reference intervals since we did not have access to Ugandan reference intervals. The most striking results were the high levels of IgG, which is known to be common in areas where multiple infections including malaria are often present. We also found almost 20% of the individuals to have low levels of haptoglobin, and slightly more than half to have low levels of orosomucoid. 14 individuals had elevated levels of CRP, of which most were only slightly elevated. So in conclusion, the results show almost no signs of inflammation, except for elevated levels of IgG.

Polymerase chain reaction (PCR) results

To know whether low levels of parasitemia were present in the Ugandan samples, these were tested by PCR by amplifying a fragment of 430 bp from the malaria cytochrome B gene. 41 samples (28 %) were found to be positive. As a control, six Swedish serum samples were run and they were all negative.

Growth/invasion inhibition results for PCR positive and PCR negative samples

We used plasma from 133 Ugandan individuals in growth/invasion inhibition assays and compared the results between PCR positive (n=41) and PCR negative (n=92) individuals. Those that were PCR positive inhibited the parasite growth more than those that were PCR negative ($p = 0.0185$).

IgG against *P. falciparum* schizont extract for PCR positive and PCR negative samples

We have in this study compared those that were PCR positive to those that were PCR negative and found that the PCR positive individuals had higher levels of antibodies against schizont extract.

Other comparisons between PCR positive and PCR negative samples

For the Ugandan samples, when the results from both study I and this study were put together, we had access to results from IgG directed against untreated and papain treated RBC, DAT, gel-IAT, IgG directed against the recombinant RBC antigens JMH and Cromer, and plasma proteins (α-1-antitrypsin, Albumin, CRP, haptoglobin, orosomucoid, IgG, IgA, IgM). When PCR positive and PCR negative results were compared for all these results, we did not find any significant differences (p-values from 0.13-0.97).

Correlations between all 24 measured parameters

To enhance our comprehension of how antibodies targeting RBCs in Ugandan samples relate to various immunological and inflammatory factors, we performed an analysis to determine the relationships among all evaluated metrics. We wanted to know which factors in development of immunity that could possibly be of importance and how these correlates to each other, and we therefore produced a heatmap. In general, having a high level of one inflammatory marker such as α-1-antitrypsin correlated positively with higher levels of other parameters, such as haptglobin, orosomucoid, IgG and IgA. Furthermore, higher levels of CRP were positively correlated with higher levels of orosomucoid and haptoglobin.

There was a positive correlation between results when antibodies against RBCs in ELISA were tested for the three different donors of RBCs. When antibodies against schizont extract was investigated, they showed a positive correlation with PCR and a negative correlation with growth/invasion.

Discovering antibodies against a single donor's RBCs ELISA was associated with the presence of antibodies against the other two donors tested. The analysis revealed a very strong linkage among the three donors when using untreated RBCs, with the average correlation being notably high. This strong connection persisted when employing papain-treated RBCs, with the average correlation slightly higher than with untreated RBCs. Similarly, comparing results for untreated and papain-treated RBCs demonstrated significant correlations.

Furthermore, the Ugandan samples were assessed for antibodies targeting specific RBC proteins: Kell, JMH, and Cromer. There were minor associations between antibodies against the Cromer antigen and other measured parameters, although antibody levels against Cromer were generally low, suggesting limited conclusiveness from these findings. Conversely, a relatively strong positive association was observed for antibodies against Kell and JMH.

When evaluating antibodies against *P.falciparum* (schizont extract) in correlation to all other parameters, a positive correlation was found with higher levels of parasitemia as determined by PCR and a negative correlation with the outcomes of growth/invasion inhibition assays. This indicates that individuals with higher levels of anti-parasite antibodies may be more effective in inhibiting parasite growth.

3.4 Study IV

Anti-PS IgG and IgM antibody levels in infants over time

The study on infants' immune responses revealed diverse reactions in their antibody levels against PS. For anti-PS IgM antibodies, the general trend was an increase from birth until 9 months, despite a small decrease at 6 months and an earlier anomaly at 2.5 months. The levels of anti-PS IgG antibodies largely remained stable during the same period. Researchers adjusted the data to account for the influence of antibodies passed from mothers to infants, which confirmed the observed trends.

Further examination identified infants as either high or low responders based on their initial antibody levels. Low responders showed a notable increase in antibodies at 2 months, which then stabilized at higher levels through to 9 months. High responders for anti-PS IgM experienced a decrease by 6 months, followed by an increase at 9 months. Meanwhile, high responders for anti-PS IgG saw a decline in levels, especially by 9 months, with a minor and insignificant rise from the beginning. Adjustments for maternal antibody levels didn't alter these outcomes, indicating a clear pattern of response based on initial antibody levels among infants **(Figure 20).**

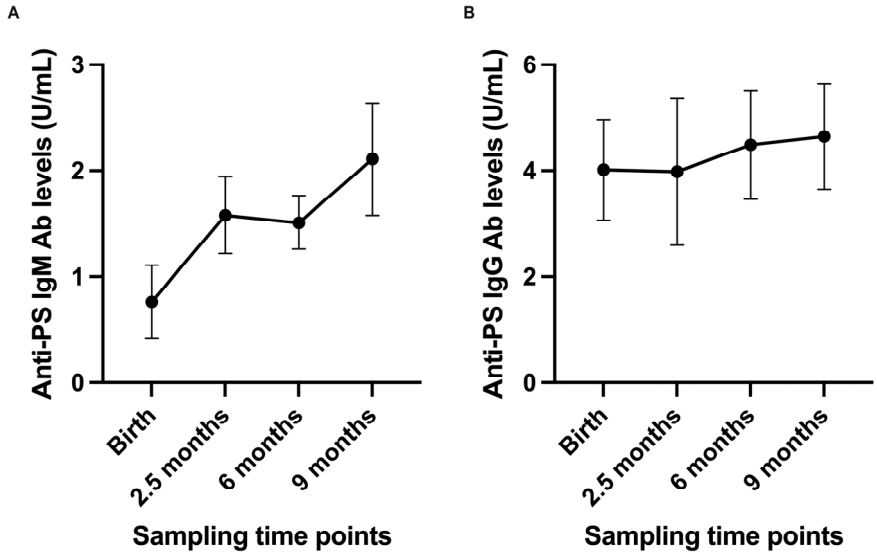

Figure 20. Longitudinal anti-PS IgM (A) and IgG (B) antibody profiles in infants from birth to 9 months postpartum. Dots represent estimated mean and whiskers represent 95% confidence intervals. Using the values at birth as references the changes in anti-PS IgM antibody levels were 0.036 (2.5 months), 0.067 (6 months) and 0.001 (9 months) while for IgG the p values were 0.971 (2.5 months), 0.737 (6 months) and 0.624 (9 months).

Comparisons of infants' and mothers' anti-PS antibodies at birth and 9 months post-partum

The study explored how childbirth affects the production of certain antibodies (anti-PS, IgM, and IgG) in mothers both at childbirth and nine months later. It was found that for mothers, the levels of these antibodies didn't change much during this period. Interestingly, newborns had much lower levels of IgM antibodies compared to their mothers at birth, but their anti-PS IgG antibodies differed from their mothers'. By the time these infants were nine months old, their levels of both IgM and IgG antibodies had reached similar levels to those of their mothers at the same post-partum stage. This suggests that the initial differences in antibody levels observed at birth tend to disappear as the infants grow older **(Figures 21, 22 and 23).**

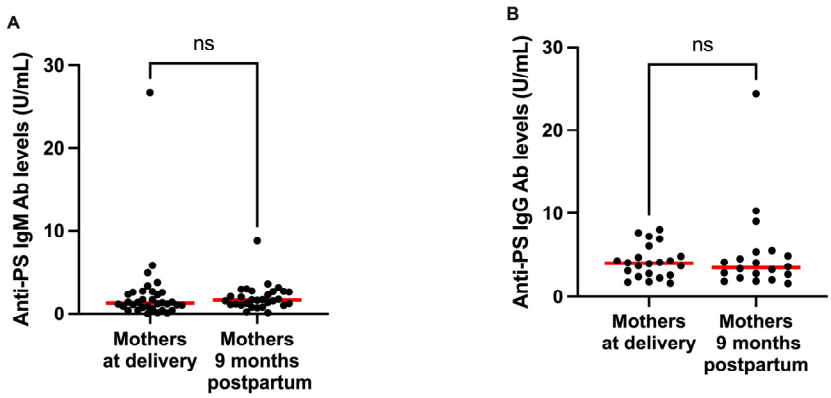

Figure 21. Comparison of median anti-PS IgM (A) and IgG (B) antibodies of mothers at delivery and 9 months postpartum. Red lines represent the median and 'ns' signifies not statistically significant (Wilcoxon matched pairs signed rank).

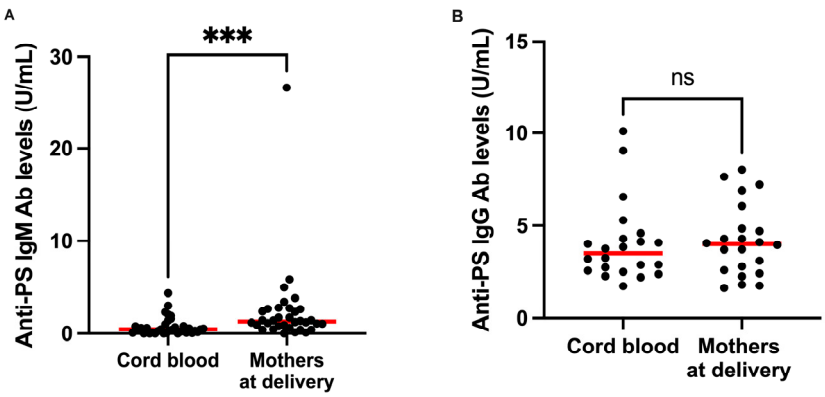

Figure 22. Comparison of median anti-PS IgM (A) and IgG (B) antibodies in cord blood and mothers at delivery. Red lines represent the median, 'ns' signifies not statistically significant (Mann-Whitney test) and *** represents statistical significance at $p < 0.001$ (Mann-Whitney test).

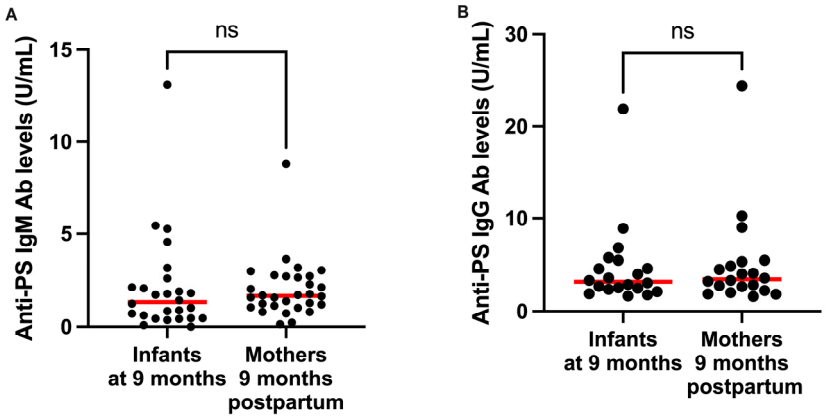

Figure 23. Comparison of median anti-PS IgM (A) and IgG (B) antibodies of infants and mothers 9 months postpartum. Red lines represent the median and 'ns' signifies not statistically significant (Mann-Whitney).

Association between B cell subtypes and anti-PS IgM and IgG antibody levels

The study done by Lugaajju et al. (2017) delves into how our body's immune response to malaria may intertwine with the development of autoimmune reactions, focusing on the interplay between various B cell subtypes and antibodies against phosphatidylserine (anti-PS IgM and IgG). Initially, the study uncovered a notable connection right after birth between the levels of anti-PS IgM antibodies and several B cell categories, including IgG+ memory B cells and various atypical and *P. falciparum*-specific B cell types. This relationship, however, shifted with age. By 2.5 months, only the links to atypical B cell groups persisted, and by 6 months, these associations vanished. They reemerged at 9 months, but this time only with non-IgG memory B cells and *P. falciparum*-specific total B cells. In contrast, the anti-PS IgG antibodies showed a different pattern, with no significant connections at birth or 6 months, but by 2.5 and 9 months, significant associations were noted with atypical B cells and *P. falciparum*-positive plasma cells. This study highlights the dynamic nature of the immune response to malaria, suggesting that it might influence or trigger autoimmune antibody responses at different life stages.

4 Discussion

4.1 Autoantibodies in malaria – good or bad?

Individuals living in endemic areas of malaria, and survive the disease, ultimately develop immunity after repeated exposures. Comprehending the mechanisms underlying the development of natural immunity is crucial to be able to make a valid malaria vaccine. In the meantime, novel therapy, to guard against the severe anemia affecting young children, is crucially required. The present status where blood transfusion is the only existing therapeutic approach is not ideal due to deficiency of blood banks in many tropical regions, as well as the unavoidable possibility of blood-transmitted infections.

The majority of destructed RBC during *P. falciparum* malaria are the uninfected cells (White, 2018). Therefore, it is logical to assume a possible involvement of immune mechanisms. Some reports have related autoantibodies to anemia development, whereas other studies suggested a protective role of such self-antibodies (Castro-Gomes et al., 2014; White, 2018). This vague dual role of autoantibodies complicates the efforts to accurately characterize their protective role. An additional challenge is to know whether the antibodies are protective, or only markers of exposure.

4.2 Autoantibodies in malaria – the good ones?

In literature, during active malaria, there's an observed increase in autoantibody production, such as those against double-stranded DNA (Adebajo, Charles, Maini, & Hazleman, 1993). Comparable autoantibodies are commonly found in Systemic Lupus Erythematosus (SLE) (Cozzani, Drosera, Gasparini, & Parodi, 2014; Dema & Charles, 2016), whereas SLE-prone mice exhibit reduced susceptibility to cerebral malaria (Waisberg et al., 2011). Despite the low incidence of autoimmune diseases in Africa, particularly due to high malaria exposure, genetic predisposition to autoimmunity, especially among females of African descent, may confer

advantages in severe malaria (Greenwood, Herrick, & Voller, 1970; Molokhia & McKeigue, 2006).

Autoantibodies against RBCs remain underexplored, and their role in malaria-endemic settings remains unclear. Investigating individuals with repeated malaria episodes could shed light on the mechanisms behind natural immunity development. In our studies, we focused on assessing if individuals with lifelong malaria exposure developed antibodies against RBCs, crucial targets for malaria parasites. We examined blood samples from asymptomatic adult males, excluding potential antibody generation through pregnancies. Using anti-*P. falciparum* schizont extract antibodies as a marker for malaria exposure, we found that almost all tested individuals had such antibodies. Additionally, a significant portion of the samples exhibited antibodies reactive to RBCs, a phenomenon uncommon in non-malaria-endemic populations. This suggests a probable association between malaria exposure and the production of anti-RBC autoantibodies.

Although the exact role of these autoantibodies remains ambiguous, they might hinder healthy RBC infection by merozoites through steric hindrance. Moreover, they could potentially increase RBC clearance in the spleen, leading to enhanced RBC turnover, which may aid in combating RBC-invading parasites. Notably, certain antibodies targeting RBCs exhibited specificity to membrane components implicated in parasite invasion, such as glycophorins. Genetic variants of these glycophorins have been associated with malaria resistance, indicating their potential role in natural immunity (Louzada et al., 2020; Reid et al., 2012).

While some samples demonstrated specificity for certain RBC antigens, characterizing the exact specificity of all antibodies proved challenging. Pan-reactivity against a panel of papain-treated test RBCs was observed in some samples, suggesting a complex interplay between parasite and RBC membrane complexes in antibody production. This complexity parallels the mechanism of drug-induced antibody formation (Doolan et al., 2009).

In another study, we investigated antibodies against the RBC surface antigen JMH, indicating potential relevance in malaria protein binding. We found elevated levels of such antibodies, suggesting a possible role in inhibiting merozoite invasion. Given the prevalence of these autoantibodies in immune adults, we hypothesized that they might hinder merozoites from invading new RBCs. Nevertheless, when we attempted to correlate the findings from various autoantibody measurement techniques to results in growth/invasion inhibition assays, the correlation wasn't as precise or evident as anticipated. This discrepancy may stem from the possibility that individuals classified as parasitemia negative would test positive if a more sensitive PCR method had been available. It might also be because immune

individuals have antibodies against so many several targets and it could be difficult to see the effect of single autoantibodies.

The high malaria exposure risk in Uganda underscores the importance of understanding the immune response dynamics. While our study participants exhibited low levels of inflammation, they demonstrated elevated IgG levels, possibly due to high exposure to pathogens like malaria. The positive correlation between antibodies against schizont extract and parasitaemia suggests recent exposure to infection. However, the lack of correlation with other parameters like inflammation or anti-RBC antibodies highlights the complexity of the immune response.

One of the beneficial roles of autoantibodies in malaria is the ability of these antibodies to target various components of the RBC surface, which is crucial for the invasion by the malaria parasite. Studies in areas of intense malaria transmission have shown that antibodies against Band 3-related neoantigens, which become exposed during the intracellular development of *P. falciparum*, can block cytoadherence and reduce parasite density (Hogh et al., 1994). Band 3, an erythrocyte membrane protein, plays a crucial role in the invasion of red blood cells by malaria parasites. These antibodies are thought to contribute to the clearance of infected red blood cells, thereby playing a role in the body's defense against malaria. Furthermore, the glycosylphosphatidylinositol-anchored micronemal antigen (GAMA), a protein involved in malaria parasite invasion of erythrocytes, has been shown to bind specifically to the extracellular loop of Band 3 on the erythrocyte surface (Lu et al., 2022). These findings suggest that exposure to malaria parasites induces immune responses that can confer a degree of protection against the disease. Antibodies against this loop can reduce the binding activity of GAMA to erythrocytes and inhibit the invasion of *P. falciparum* merozoites into human erythrocytes. This discovery underscores the importance of the interaction between GAMA and Band 3 in the malaria parasite's lifecycle and highlights potential targets for vaccine development or therapeutic interventions aimed at blocking parasite invasion.

In conclusion, more studies are needed to understand the mechanisms and functions of autoantibodies, especially their action against RBCs in connection to malaria. Further longitudinal studies are necessary to elucidate the dynamic changes in the immune response over the course of infection. Understanding this intricate interplay is essential for developing improved diagnostics, treatments, and vaccines for malaria. Moreover, recognizing the expected immune response in immune adults with incidental malaria findings is crucial for accurate diagnosis and management.

4.3 Autoantibodies in malaria – the bad ones?

Autoantibodies against phosphatidylserine (PS) have been associated with anemia (Fernandez-Arias et al., 2016; Rivera-Correa, Conroy, et al., 2019; Rivera-Correa & Rodriguez, 2020), suggesting their potential harm to RBCs. Complement-mediated lysis also targets RBCs, but neither antibody levels nor specificities reliably predict infection pathogenicity (Rivera-Correa & Rodriguez, 2020; Waitumbi, Opollo, Muga, Misore, & Stoute, 2000), indicating gaps in understanding anemia pathways.

PS-specific antibodies occur in autoimmune diseases and various infections, including malaria, Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA) and HIV/AIDS (Barber et al., 2019; Elkon & Silverman, 2012; Skouri, Gandouz, Kraiem, Harrabi, & Ben Said, 2008). Parasitemia level doesn't correlate with anti-PS antibodies (Barber et al., 2019), suggesting past rather than current infection influences antibody levels. CD11c+T-bet+ B cells, producers of PS, initiate antibody production upon parasite DNA contact (Rivera-Correa, Conroy, et al., 2019; Rivera-Correa et al., 2017).

Higher anti-PS antibodies in anemic individuals align with the notion that PS antibodies induce anemia through phagocytic clearance of opsonized RBCs (Fernandez-Arias et al., 2016). An inverse correlation exists between PS-specific antibody titres and hemoglobin levels (Barber et al., 2019; Rivera-Correa, Mackroth, et al., 2019). Haptoglobin level, expected to decrease in intravascular hemolysis, shows no correlation with anti-PS antibodies, possibly due to acute-phase reactant regulation (in the acute phase haptoglobin becomes elevated) by factors other than malaria (Eluke, Eluke, Okereke, Okwuosa, & Ufelle, 2018).

Malaria prevention strategy involving the administration of sulfadoxine-pyrimethamine (SP) during antenatal care visits in malaria-endemic areas. SP is the drug commonly used for IPTp due to its safety and efficacy in preventing malaria in pregnant women. Artemisinin-based combination therapies (ACTs), which include artemisinin derivatives combined with other antimalarial drugs, are primarily used for the treatment of malaria, including uncomplicated and severe cases.

Artemisinin is not typically used as part of IPTp Artemisinin but can be used as part of IPTp in the current study area, which has been linked to PS exposure on RBCs (Fasanya et al., 2023). Phosphatidylserine translocation to the erythrocyte surface takes place as an event in RBC apoptosis induced by the drug (Alzoubi et al., 2014), however no difference was observed in anti-PS antibody levels between pregnant women who participated in IPTp and those who did not (Fasanya et al., 2023). Anti-

full-length VAR2CSA antibodies show no correlation with anti-PS antibodies, suggesting different B cell origins (Fasanya et al., 2023).

Postpartum, anti-PS IgM increases in infants, potentially due to their own antibody development from malaria exposure (Boyle et al., 2019; Mourão et al., 2020a). Anti-PS IgG levels in infants remains comparable to maternal levels, indicating that anti-PS IgG autoantibodies are placentally transferred to neonates in individuals living in a malaria endemic area (Fasanya et al., 2023; Leonard et al., 2023).

Infant antibody profiles suggest homeostatic regulation, possibly linked to atypical B cell sources of anti-PS antibodies (Rivera-Correa, Mackroth, et al., 2019). Correlation between atypical B cells and anti-PS antibodies, especially IgM, indicates early malaria exposure (Rivera-Correa, Mackroth, et al., 2019). Plasma cell correlations suggest broader B cell compartment involvement in anti-PS production (Jenks, Cashman, Woodruff, Lee, & Sanz, 2019).

However, autoantibodies other than anti-PS antibodies have proven to correlate with deleterious effects that take place during the course of malaria. Anti-dsDNA antibodies directed against double-stranded DNA and are commonly associated with autoimmune diseases like systemic lupus erythematosus (SLE). They can contribute to tissue damage and inflammation (Arbuckle et al., 2003). In a cohort study on Ugandan children having severe *falciparum* malaria, anti-PS antibodies as well as anti-dsDNA antibodies were associated with acute kidney injury, post-discharge mortality and morbidity, and also mediated severe anemia (Rivera-Correa, Conroy, et al., 2019)

5 Conclusions and future prospectives

We conclude that novel insights into the molecular basis of autoantibody reactions directed against RBC antigens have been established. This dissertation has shed light on crucial aspects of autoantibody responses against RBC antigens and their potential implications for both malaria immunity and pathogenesis.

This thesis report has centered on studying asymptomatic *P. falciparum* infections trying to better understand the immune mechanisms present in a well-developed state of immunity. We found a clear presence of antibodies against RBCs in parallel with high levels of IgG and almost no signs of inflammation, even though many individuals were carrying parasites. On the other hand, anti-PS-specific antibody levels correlated with anaemia indicated by PCV on one hand but were not associated to VAR2CSA-specific antibodies in pregnant women. The severity of pathological malaria complications is usually less among multigravidae possibly because of the expected protective effect of VAR2CSA-specific antibodies, but also in combination with the reduction of anti-PS antibodies. Moreover, we have provided evidence that indicates that anti-PS IgG autoantibodies are placentally transferred to neonates in individuals living in a malaria endemic area. We also demonstrated the dynamics of these autoantibodies during the early part of life of infants and their relationship with different B cell populations.

RBC antigens play an important role in this process because the malaria parasite goes through multiple stages in the human host interacting with various host factors, including the RBC. Enhancing host immunity or modulating host-pathogen interactions could complement traditional anti-malarial treatments and reduce the likelihood of resistance development or diminish the risk of severe anemia. Future work in developing combination therapy against resistant malaria could focus on several key areas including identification of novel drug targets.

For future studies, it would be interesting to design an age-stratified cohort to explore the chronological production of autoantibodies against RBCs, and if they are produced concurrently with anti-parasite antibodies. Investigating whether these antibodies exacerbate or mitigate the risk of complications such as maternal anemia

or adverse birth outcomes could guide the development of interventions to improve maternal and neonatal health.

In conclusion, this thesis has clearly shown that immune individuals living in malaria endemic areas harbor antibodies against RBCs. We speculate that in immune individuals having both good and bad autoantibodies, good antibodies could overcome the deleterious effects of bad ones. Good autoantibodies attain high levels and have the ability to affect many targets such as the RBC membrane components involved in *plasmodium* invasion. This speculation is supported by the fact that most individuals in malaria-endemic regions exhibit an asymptomatic form of the disease.

Future studies elucidating how the autoantibodies against RBCs contribute to protection or pathogenesis could enhance the development of targeted interventions, such as vaccines or immunomodulatory therapies and have the potential to contribute significantly to global efforts to control and ultimately eliminate malaria as a public health threat.

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- Prophet Mohammad

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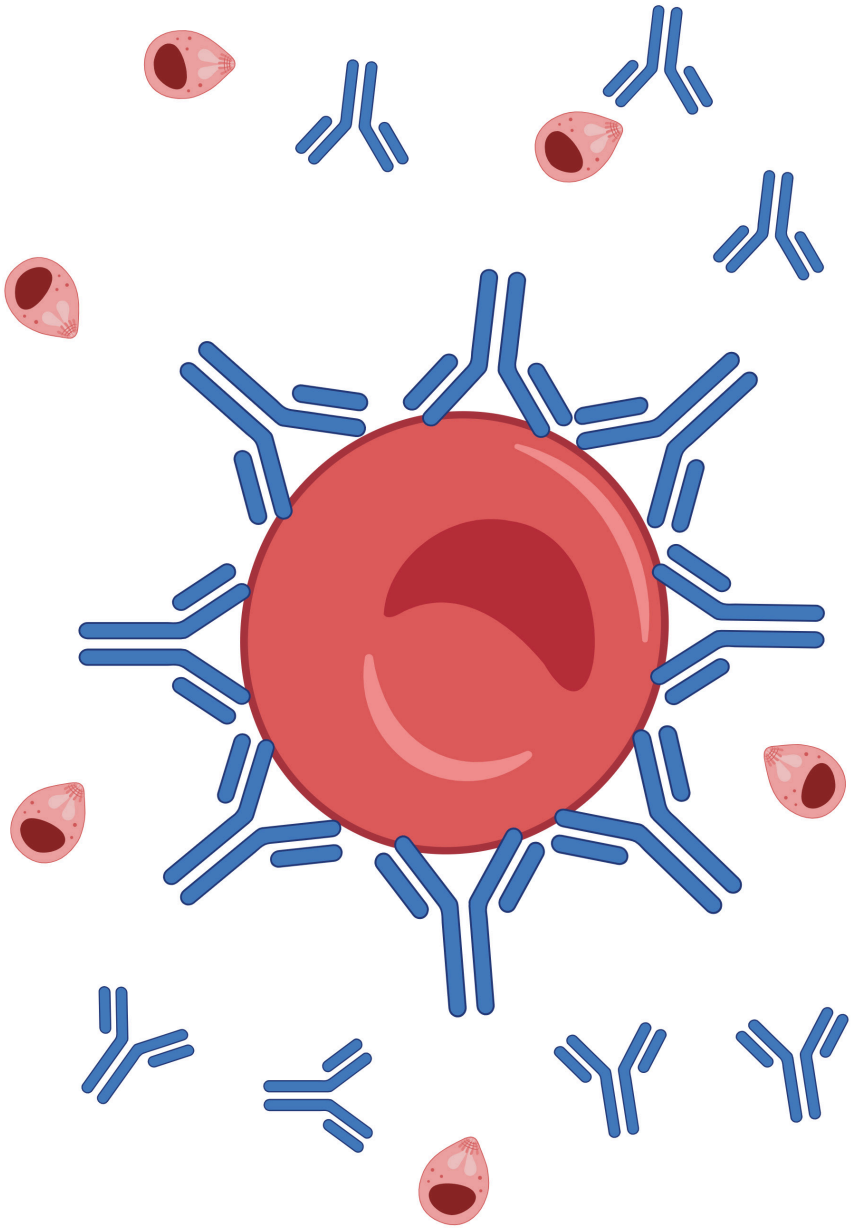

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