# Characterization of Tissue Tropism in *Plasmodium Falciparum* malaria

A Thesis

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by

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# Certificate

This is to certify that this dissertation entitled **"Characterization of tissue tropism in *Plasmodium falciparum* malaria"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Shantanu Hutke at Indian Institute of Science Education and Research under the supervision of Dr. Krishanpal Karmodiya, Assistant Professor, Department of Biology, during the academic year 2022-2023.

Dr. Krishanpal Karmodiya

(Supervisor)

Committee:

Name of your Guide: Dr. Krishanpal Karmodiya

Name of Your TAC: Dr. Kundan Sengupta

**This thesis is dedicated to my Aai and Baba for their love and support…**

# Declaration

I hereby declare that the matter embodied in the report entitled **"Characterization of tissue tropism in *Plasmodium falciparum* malaria"** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Krishanpal Karmodiya and the same has not been submitted elsewhere for any other degree

Shantanu Hutke

Date: 10/04/2023

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# Abstract

Cytoadhesion and sequestration of *Plasmodium falciparum* Infected Erythrocytes (IEs) are the key modes of parasite virulence. Var gene family produces a huge repertoire of the PfEMP1 proteins, which are transported to the IE surface, and interact specifically with the human endothelial receptors, leading to multiple organ complications in severe malaria. Tissue tropism is the ability of a pathogen to invade a tissue or an organ in a specific manner. *P. falciparum* is well recognized for its ability to sequester in the deep microvasculature of vital organs such as kidneys, lungs, hearts, and gastrointestinal tract, but the tissue tropism in malaria is not well understood. To investigate the host-parasite interactions at the vascular endothelia, we have performed static cytoadherence assays with the primary human brain endothelial cell line (HBEC-5i). Specific binding was observed with the *P. falciparum*-infected RBCs to the brain endothelial cell line. To learn more about the interaction specificity of the PFEMP1 proteins for the endothelial receptors, in order to unleash the tissue tropism exhibited by the parasite, we searched for the human endothelial receptors expressed across different tissues and associated with cytoadhesion. We have overexpressed some of them in the HEK293T cell line. PF3D7_0420900, a PfEMP1 protein from the var subgroup B has been observed to be enriched in the CD151 adhered IEs. It could be a potential interacting partner of CD151. Cytoadhesion assays with the rest of the endothelial receptors followed by RNA sequencing of the adhered IEs would reveal the PfEMP1 specificity. This study would pave the way for therapeutic research and help in controlling the spread of malaria.

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# Contributions

| Contributor name | Contributor role |
|---|---|
| SH, BD, KK | Conceptualization Ideas |
| SH, BD | Methodology |
| SH | Software |
| SH | Validation |
| SH | Formal analysis |
| SH | Investigation |
| SH | Resources |
| SH | Data Curation |
| SH | Writing - original draft preparation |
| BD, KK | Writing - review and editing |
| – | Visualization |
| BD, KK | Supervision |
| KK | Project administration |
| KK | Funding acquisition |

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# Chapter 1: Introduction

## 1.1 Malaria the disease overview

Malaria is an ancient vector-borne life-threatening disease primarily transmitted through the bites of female Anopheles mosquitoes. Six different species of the protozoan parasite of genus *Plasmodium* are known to cause the disease. These species include *P. falciparum,* two different forms of *P. ovale, P. malariae, P. vivax,* and *P. knowlesi*. (William et al., 2013, Antinori et al., 2012). The symptoms of malaria include fever, headache, and chills which typically occur around ten days after the infection. The symptoms might be variable from mild to severe, as per the complexity of the manifestation. Serious complications of malaria include severe anemia, cerebral and pregnancy-associated malaria. It is one of the most lethal parasitic diseases of humans, which has enormous medical, economic, and emotional impacts on the world. Malaria is known to cause the most significant number of deaths worldwide (Talapko et al., 2019).

In 2021, malaria had a devastating impact worldwide. Reportedly, there have been around 247 million documented cases and 619,000 deaths. Malaria is a threat to almost half of the world's population (W.H.O.). There has been almost a century of global efforts and research being carried out to prevent, diagnose, and treat the disease. Despite these massive efforts, the disease continues to be a serious public health concern and requires attention to prevent and control its spread.

Malaria is majorly endemic in regions of the world that have tropical and subtropical climates. Its incidence is exceptionally high in Africa. Pregnant women and children, especially those who are below the age of five years, are highly susceptible to the disease. The World Malaria Report 2022 suggests India bears approximately 3% of the global malaria burden. On the other hand, India bears the highest malaria burden in the region of South East Asian. Despite this, it has demonstrated a substantial decline of 49% in the reported cases of malaria and 50.5% in deaths attributed to the disease as compared to the statistics from 2017. Over the past few years, India has made remarkable progress in reducing the incidence of malaria. Encouragingly, the World Malaria Report has documented a remarkable reduction in the number of

malaria cases from India (Figure 1), with an estimated reduction of 24% in 2017 as compared to 2016 and a further decrease of 28% in 2018 as compared to 2017(WMR 2022). Amongst the six species of *Plasmodium*, the infections caused by *Plasmodium falciparum* are considered to be the deadliest (Lapp et al., 2022).

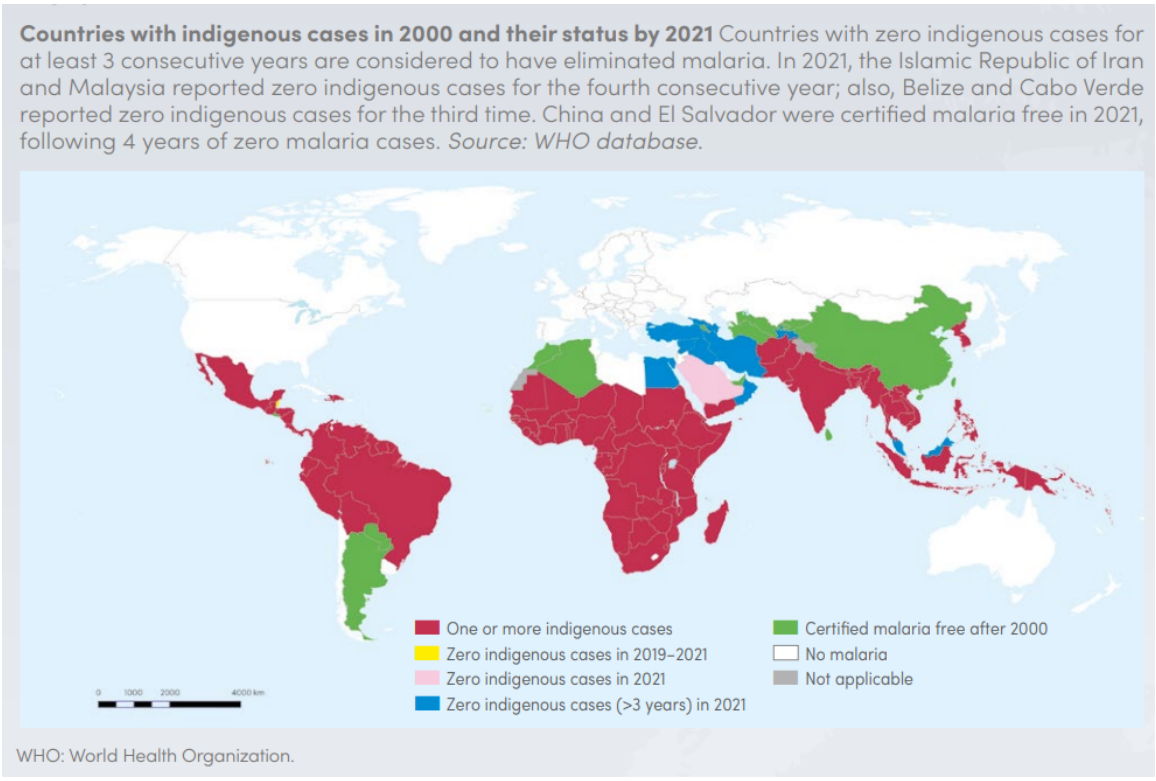


**Figure 1.** The prevalence of malaria across the globe (World Malaria Report 2022).

### 1.2 The life cycle of *Plasmodium falciparum*

*P. falciparum* life cycle is slightly complex which depends on two different hosts, female Anopheles mosquitoes and humans (Figure 2). It comprises sexual and asexual stages, being carried out in the mosquito and the human, respectively. The incidence of infection occurs when the mosquito injects the sporozoites into the skin while feeding on human blood. It also releases some vasodilators to increase the chance of entering the blood vessel. These motile extracellular forms migrate rapidly to the liver through the bloodstream and invade hepatocytes (Loubens et al., 2021), thereby escaping host immunity (Tavares et al., 2013). After the invasion, each

sporozoite undergoes a process of differentiation and mitotic division, eventually giving rise to thousands of merozoites. These are potent of further invasion in the red blood cells (RBCs), which leads to the onset of a most crucial intra-erythrocytic cycle (Cowman and Crabb 2006). During this stage, it becomes possible to detect the clinical symptoms of malaria (Tangpukdee et al., 2009). After the invasion inside red blood cells, the merozoites undergo a sequence of developmental stages, starting with the formation of ring-shaped structures. These rings eventually mature to form trophozoites and schizonts, further ends up releasing new merozoites to repeat the cycle of invasion. Some merozoites from the population differentiate to form male and female gametocytes. Subsequently, a healthy female anopheles mosquito picks up these gametocytes. The parasites undergo sexual reproduction event inside the mosquito and release sporozoites that get stored in the mosquito's salivary glands. However, during the next meal of the mosquito, these are released into the human host. *P. falciparum* infections progress from uncomplicated to severe forms of malaria, which include cerebral malaria and multiple organ complications.

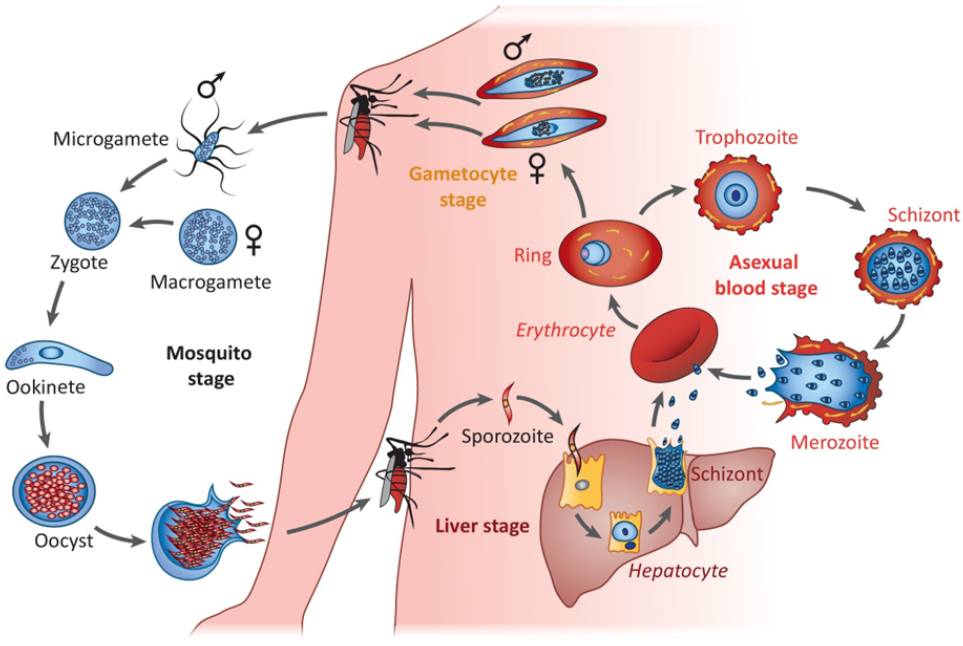


**Figure 2.** The life cycle of malaria parasite ; *Plasmodium falciparum* (Maier et al., 2018)

### 1.3 Uncomplicated malaria

The symptoms and signs of uncomplicated malaria are not unique and can be similar to those of other febrile conditions (Grobusch et al., 2005). These include fever, muscle pains, fatigue, vomiting, abdominal discomfort, etc.

### 1.4 Severe malaria

The infections that are accompanied by multiple organ complications and further leading to organ failure best characterizes the condition of severe malaria. Severe malaria involves various complications such as cerebral malaria, liver failure, acute renal failure and jaundice, pulmonary edema, severe anemia, acute respiratory distress syndrome (ARDS), and bleeding. These complications may develop rapidly and can potentially result in death within a short period of time. The microvascular sequestration of infected RBCs and thereby causing the obstruction to blood flow, is responsible for developing multiple organ complications (Trampuz et al., 2003).

### 1.5 Cerebral malaria

Cerebral malaria is a condition that is clinically identified by a state of unarousable coma with a Blantyre coma score ≤ 2, considering there are no other potential causes of coma, such as meningitis, accompanied by a very high level of parasitemia caused by *Plasmodium falciparum* (Luzolo et al., 2019). Cerebral malaria is considered to be one of the important causes of lethality amongst *P. falciparum* infections. The disease pathology has been linked to the sequestration of parasite-infected erythrocytes in the deep microvasculature of the brain. Multiple organ complications, such as retinopathy and kidney failure, are also found to be related with cerebral malaria.

### 1.6 Cytoadherence

The *P. falciparum* virulence is majorly incidental with its propensity to attach infected erythrocytes (IEs) to the inner lining of blood microvessels in various organs, a process recognized as cytoadhesion (Figure 3). This attachment enables the IEs to sequester in the microcirculation, which leads to blockage of blood flow and induces

inflammation (Miller et al., 2002). The occurrence of this phenomenon is most notable during the parasite's intraerythrocytic cycle. The adherence of *P. falciparum* on the host cells is modulated by three different groups of parasite-derived antigens, which are referred to as variant surface antigens (VSAs). These VSAa are encoded by the multigene families of parasites. These groups comprise the *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), the Repetitive Interspersed Family (RIFIN) proteins, and Subtelomeric Variable Open Reading frame (STEVOR) proteins. The binding of PfEMP1 to various receptors present on different endothelial cells suggests its role in immune system evasion and modulation of the immune response. Therefore it is considered to be the most important of the VSAs (Lee et al., 2019).

The IEs can adhere to endothelial cells, placental syncytiotrophoblasts, leukocytes, platelets, and uninfected red blood cells (rosetting). Cytoadhesion serves as an immune evasion strategy, as the parasites escape from splenic clearance, ensuring the prolonged survival of the parasite.

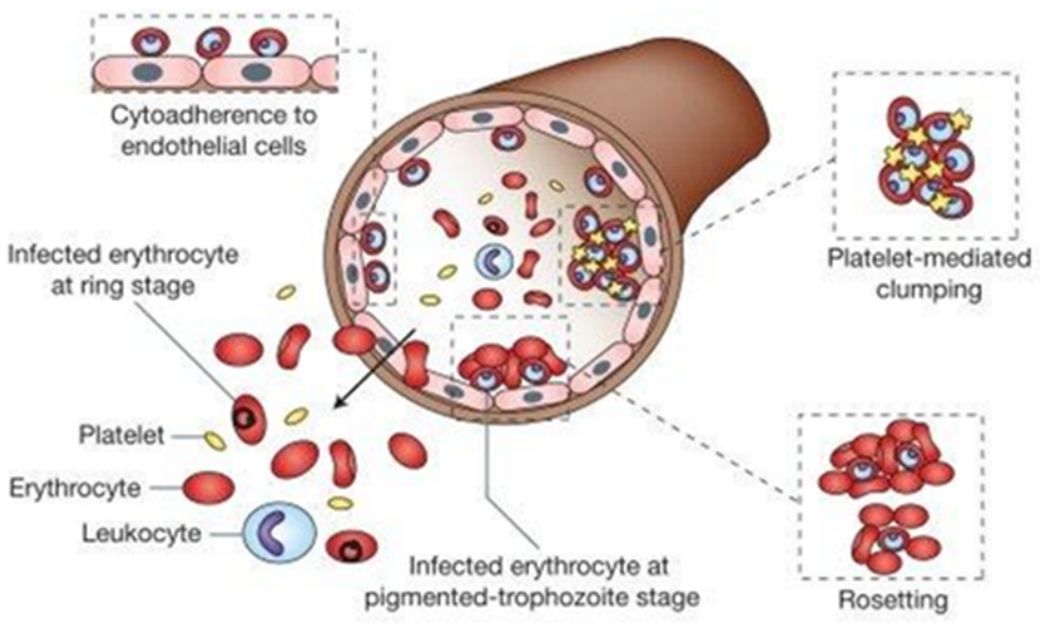


**Figure 3.** Cytoadhesion of *Plasmodium falciparum-infected* erythrocytes to the endothelium of blood vessels (Rowe et al., 2009).

## 1.7 PfEMP1 Proteins

*Plasmodium falciparum* Erythrocytic Membrane Protein 1 (PfEMP1) plays a crucial role in both antigenic variation and the binding of IEs to the endothelial cells of the host. This makes it a key molecule in this process. It is the most comprehensively studied VSA. PfEMP1 protein is recognized as an adhesive molecule that can attach to a diversified endothelial receptors of humans. The genome of *P. falciparum* harbors around 60 two-exon var genes, which are known to encode the PfEMP1 family of proteins. These proteins have high molecular weight (200-450 kD) and are trypsin-sensitive transmembrane proteins (Gardner et al., 2002). The exon I of the var gene shows a high degree of interclonal and intraclonal sequence variation. It encodes for an extracellular part of the protein, which consists of cysteine-rich interdomain regions (CIDRs) along with 2-10 Duffy-binding-like (DBL) domains (Figure 4 (A)) that confer the binding property (Smith et al., 2013). CIDRs and DBL domains are further divided into three and seven sequence classes, respectively. These domains undergo nonrandom recombination in different PfEMP1 proteins to form 21 different domain trains, which are also referred to as Domain Cassettes (DCs). The domain cassettes are known to determine the receptor specificity of the PfEMP1 proteins (Figure 4 (B)) (Haviid and Jensen 2015). The exon II is relatively conserved and codes for the acidic intracellular terminal segment (ATS) and the short transmembrane domain (Rask et al., 2010).

PfEMP1 proteins get exported to the IE surface and are confined in a membrane protrusion called a knob. These knobs usually start appearing on the IE surface approximately 16 hours after the invasion and are at their peak density around 20 hours (Ortolan et al., 2018). These time points coexist with the trophozoite stage of the parasitic life cycle. Therefore the trophozoite stage is considered a crucial stage in cytoadhesion and pathogenesis.

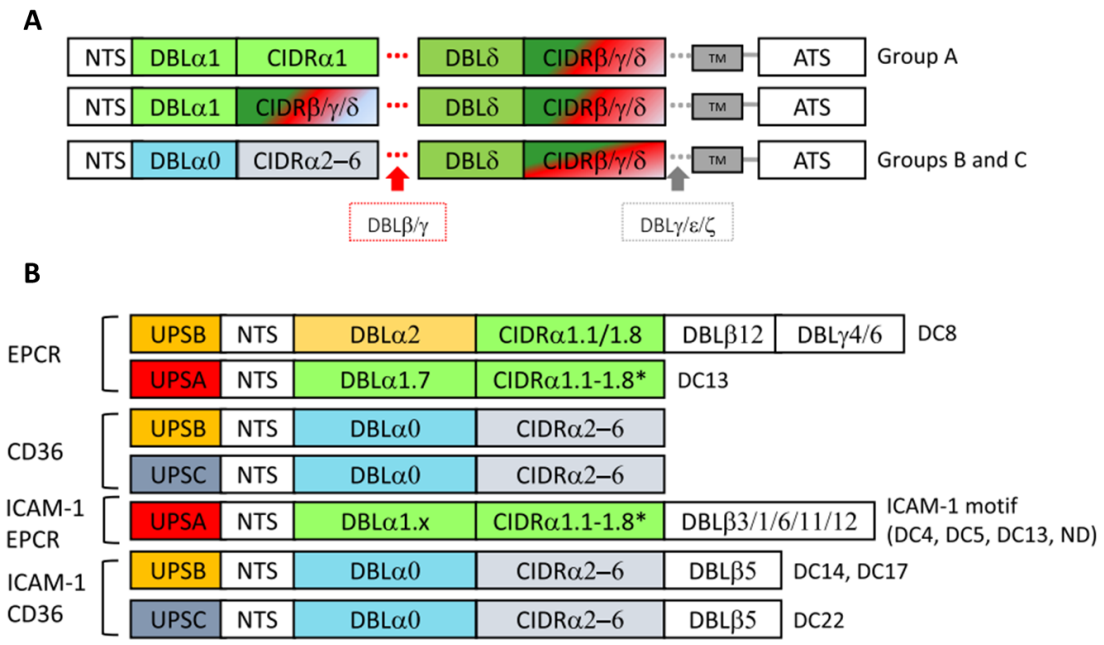


**Figure 4.** Structure of a PfEMP1 protein (A) PfEMP1 proteins are composed of different types of DBL (Duffy-binding-like) and CIDR (Cysteine-rich interdomain region) domains. Typically, PfEMP1 proteins belonging to groups B and C possess a structure of four-domain, whereas the larger PfEMP1 proteins may contain more DBL domains. (B) The *Plasmodium falciparum* genome encodes tandem domain cassettes (DC) which are responsible for specific adhesion phenotypes (Jensen et al., 2020).

### 1.8 Var genes

The genome of *P. falciparum* comprises a highly diverse class of around 60 var genes, which encode for PfEMP1 virulent proteins. The var genes continuously undergo recombinations to generate a vast repertoire in nature (Flick et al., 2004). This gene family was discovered in 1995, a decade after the discovery of PfEMP1 proteins, and was given the name var(variant) (Baruch et al., 1995, Su et al., 1995, Smith et al., 1995). The size of var genes is generally large (6-13kb). These are present mostly in the subtelomeric region of nearly all the chromosomes (Gardner et al., 2002). The classification of var genes can be done depending on their upstream promoter sequence into three major groups (var A, var B, var C) and two intermediate groups, var B/A and var B/C (Lavsten et al., 2003).

## 1.9 Clonal antigenic variation in var genes

Parasitic microorganisms encounter various obstacles in their quest to establish a stable presence within their host. These include physical clearance, immune detection, and destruction. In due course of time, *P. falciparum* has evolved with a strategy of controlled variation of surface-exposed antigenic determinants to escape the immune system and maintain chronic infections despite constant immune pressure from the hosts. Multiple proteins are exported on the surface of IEs during the intraerythrocytic cycle. Amongst them, the var gene family of parasites implements this strategy. Only one of the PfEMP1 variants gets expressed on the surface of any given IE at any particular time. This expressed PfEMP1 variant can change from one cycle to the another (Jensen et al., 2020). This pattern of mutually exclusive expression is also referred to as clonal antigenic variation.

According to a review by Guizetti and Scherf (2013), the transcription of single var gene starts at the ring stage right after the merozoite invasion event in erythrocytes. As trophozoite and schizont stages approach, the expression becomes poised and ready for activation in the next cycle, which can lead to a switch to a different var gene variant. This switching process can result in the alteration of immune responses and a change in the pattern of adhesion phenotypes. The regulation of mutually exclusive patterns of expression involves both genetic and epigenetic factors.

## 1.10 Multiple organ complications in malaria

The excessive sequestration of parasites in deep microvascular beds may cause localized inflammation and lead to organ complications and dysfunction (Miller et al., 2002). Previous studies have shown that *Plasmodium falciparum* induces multiorgan complications in severe malaria(Balaji et al., 2020). Cerebral malaria and placental malaria are the most lethal and well-studied examples of such organ complications (Brabin et al., 2004, White et al., 2013). The fully developed forms of parasitic infected erythrocytes (IEs) exhibit a sequestered state within a range of microvascular beds. These include the heart, lungs, gastrointestinal tract, kidney

spleen, and subcutaneous adipose tissue the skin (Figure 5) (Spitz, 1946; MacPherson et al., 1985; Milner et al., 2014; Milner et al., 2015).

Although the gastrointestinal tract has been traditionally viewed as a location with low pathogenicity, recent research suggests that it can be a significant site for the sequestration of parasites. Moreover, there are growing pieces of evidence indicating that higher levels of parasitic infestation may lead to compromised gastrointestinal barrier function. In fatal cerebral malaria cases, the gastrointestinal tract was considered to be the major sequestration site (Seydel et al., 2012). The kidney usually does not serve as a major site for IEs sequestration, but kidney injuries are often observed in cerebral malaria cases. Renal impairment is a frequently observed complication in severe malaria cases among adults, with a prevalence of up to 37%. This condition is associated with an increased mortality risk, with a four-fold increase in death rates reported in affected individuals (Dondorp et al., 2009). The lung is also considered a site for IEs sequestration(Spitz et al., 1946; Seydel et al., 2015). A notable proportion of individuals suffering from severe *falciparum* malaria, specifically up to 25% of adults and 40% of children, are susceptible to the development of respiratory distress. Acute respiratory distress syndrome (ARDS) and Acute lung injury (ALI)are complex medical conditions that can have diverse underlying causes. Severe *Plasmodium falciparum* infections are commonly associated with ARDS (Acute Respiratory Distress Syndrome), with incidence rates ranging between 5% to 25% in adults and up to 29% in pregnant women. However, it is noteworthy that ARDS is an infrequent occurrence in young children.(Taylor et al., 2012). Apart from this, the sequestration of parasites has also been observed in heart and liver microvasculature. But it remains poorly characterized (Ortolan et al., 2018).

### 1.11 Tissue tropism

The ability of a given pathogen to interact with different tissues or organs in a specific manner is called tissue tropism. Parasites exhibit varying degrees and levels of tropism, targeting different aspects of the host organism, such as specific tissues, individual cells, and even subcellular compartments. The factors influencing a pathogen's selection of a particular tissue for infection are often not well understood and are likely to be multifaceted. However, the tropism of a pathogen for a specific

tissue can directly affect its life cycle, potentially promoting its persistence within the host and enhancing its capacity for transmission. Recently reviewed, Pereira et al. 2019, suggest that *Plasmodium* shows an affinity for almost all the organs such as CNS, lungs, liver, spleen, intestine, kidney, lymph nodes, skin, bone marrow, adipose tissue, and placenta. The PfEMP1 proteins of *P. falciparum*, which are responsible for cytoadhesion, incorporate the specificity for multiple host tissues (Figure 5 (B)). The antigenic variation in var genes leads to the formation of a diverse repertoire of PfEMP1 receptors, which generates an avenue for the parasite to perform interaction with multiple endothelial receptors and cause sequestration in diverse microvascular beds.

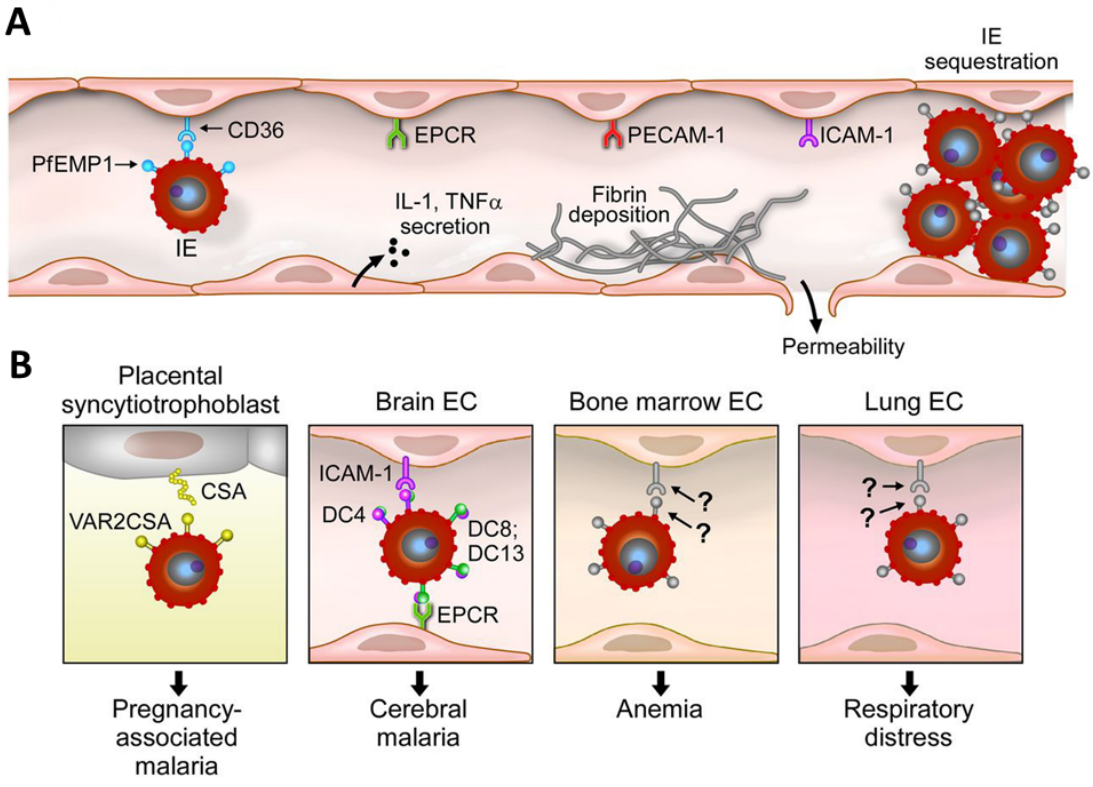


**Figure 5.** Tissue tropism in *P. falciparum* malaria (A) Differential expression of endothelial receptors and downstream events after the parasite IE sequestration. (B) Tissue specific sequestration of different DCs of PfEMP1 proteins. (Aird et al., 2014)

## 1.12 Endothelial receptors

Vascular endothelium shows diversity in the cell population that expresses diverse transmembrane receptors. The well-known endothelial cell receptors that are involved in cytoadhesion of *P. falciparum* IEs are CD36 (Barnwell et al., 1989), EPCR (Turner et al., 2013), and ICAM-1 (Berendt et al., 1989), PECAM-1. Apart from this, there are more than ten receptors have been found as PfEMP1 interactors, which include P Selectin, E Selectin, CD9, CD151, thrombospondin, complement C1Q binding protein, and VECAM-1, etc. These receptors are expressed in the microvasculature of vital organs. Despite having evidence, the interactions of these receptors with PfEMP1 proteins have not been well characterized.

The objective of this project is to conduct a comprehensive investigation into the interactions that result in tissue tropism in *Plasmodium falciparum* and to characterize them. The primary objective is to identify the critical endothelial receptors associated with cytoadhesion and sequestration of malaria parasites. To achieve this, we plan to perform cytoadhesion assays under static conditions using the laboratory strain of *P. falciparum*, 3D7. Subsequently, we will investigate the upregulation of PfEMP1, specifically in the 3D7 population, that interacts with the targeted endothelial receptor. This approach will generate a comprehensive map, revealing the key players involved in the tissue tropism of *P. falciparum*. The resulting information will help identify potential therapeutic targets for inhibiting tissue tropism and, ultimately, controlling the spread of malaria.

Overall, this project aims to pave the way for future therapeutic research focused on *P. falciparum* malaria. By identifying the essential endothelial receptors that contribute to tissue tropism, we can better understand the pathogenesis of the disease and develop more effective treatments to combat its spread.

# Chapter 2: Materials and Methods

## 2.1 In vitro culture of *Plasmodium falciparum*

The *Plasmodium falciparum* strain 3D7 was cultured in vitro using RPMI1640 medium. The medium was supplemented with 0.5% AlbuMAX I, 25 mM HEPES, 11 mM glucose, 100 µM hypoxanthine, 1.77 mM sodium bicarbonate, and 12.5 µg/ml gentamicin sulfate. The culture was maintained in a 5% CO2 incubator at 37 °C with humidified conditions and subculturing was done every two days, by means of splitting the flask to ensure a parasitemia of around 5%. The hematocrit level was maintained at 3% by adding freshly washed human RBCs (O +ve) obtained from healthy human donors. The parasitemia level was monitored by staining thin blood smears with Giemsa.

## 2.2 HBEC-5i in vitro culture

The primary cell line HBEC-5i, a cerebral microvascular endothelial cell line, was kindly provided by Dr. Hem Chandra Jha (IIT Indore). The HBEC-5i were initially cultured in Dulbecco's Modified Eagle Medium: nutrient Mixture F-12 (DMEM-F12 HAM). The medium was supplemented with 40 µg/mL endothelial cell growth supplement (ECGS), 10% FBS, and 1% antibiotic anti-anti. The cell culture plates were monitored for confluency and given passages when 80% confluent.

## 2.3 HEK293T in vitro culture

HEK293T is an immortalized human embryonic kidney cell line. It was kindly provided by SG lab. The culturing of cells was done in Dulbecco's Modified Eagle Medium: nutrient Mixture F-12 (DMEM-F12 HAM). The medium was supplemented with 10% FBS. The cell culture plates were monitored under the bright field inverted Zeiss microscope and were given passages after 100% confluency. The cells were further used for overexpression studies.

## 2.4 Plasmids

The plasmids mentioned below in (Table 1) were obtained from addgene. These contain the human endothelial receptors of our interest.

| Sr. No. | Human Endothelial receptor | Plasmid name | Size(Base pairs) | Plasmid ids |
|---|---|---|---|---|
| 1. | CD36 | mCherry-CD36-C-10 | 6139 | 55011 |
| 2. | ICAM-1 | Human ICAM-1 | 5831 | 8632 |
| 3. | PECAM1 | pLPCX-PECAMTS | 10,044 | 4585 |
| 4. | P Selectin | SELP-bio-His | 8951 | 51639 |
| 5. | CD9 | CD9-mEGFP | 5388 | 182864 |
| 6. | CD151 | mApple-CD151-7 | 5476 | 54873 |
| 7. | Thrombospondin | THBS1-bio-His | 10148 | 53417 |
| 8. | Complement C1Q binding factor | C1QBP-bio-His | 7484 | 53330 |
| 9. | EPCR | pGEM-T | 3000 | HG13320-G |

**Table 1.** The list of plasmids obtained from Addgene harboring the genes for human endothelial receptors of interest.

## 2.5 Generation of overexpression cell lines

The HEK293T cell line was used to overexpress human endothelial receptors through transfection. The transfection process employed Lipofectamine P3000 reagent (Invitrogen), and a single 10cm cell culture plate with 80-90% confluency was used. Firstly, media removal was done from the plate, and the cells were then

incubated with nutrient-deficient OptiMEM medium for half an hour at 37°C. To achieve this, 2.5ml of OptiMEM per 10cm plate was utilized.
Simultaneously, a DNA-Lipofectamine mixture was prepared using 12µl of Lipofactamine, 15µl of P3000, and 10.5µg of plasmid DNA in 2.5ml of OptiMEM per 10cm of HEK293T. It was then subjected to incubation at RT for around 12 minutes. After the half-hour incubation period, the DNA-Lipofectamine mixture was added drop by drop to the plate.
Subsequently, the cells were subjected to incubation with a transfection medium for 12-14 hours at 37°C, following which they were provided with DMEM media. These overexpression lines were then used for cytoadhesion assays.

### 2.6 Static cytoadhesion assay

In 1995, Cooke and Coppel et al. standardized static cytoadhesion assays for studying the cytoadhesion of *Plasmodium falciparum*-infected erythrocytes. The procedure involved using a HEK293T cell culture plate with 90% confluency, which was incubated with 10ml of a 3D7 parasite culture for 1 hour at 37°C. The culture contained mature-stage parasites, specifically trophozoites, at 10% parasitemia and 3% hematocrit. Subsequently, unbound red blood cells (RBCs) were washed off using 1x DPBS with a standardized washing procedure, and the bound RBCs were removed with stringent washes and collected in a falcon tube. The collected material was then spun at 1200rpm for four minutes, and the supernatant was removed, leaving behind a pellet that was subjected to further RNA isolation procedures.

### 2.7 CF11 cellulose filtration

Venkatesan et al. (2012) developed a method for reducing human DNA contamination from *P. falciparum*-infected whole blood samples, which we adapted to remove HEK293T DNA from our samples. To accomplish this, we used a 10ml disposable syringe with the plunger removed and inserted a 1 $cm^2$ square of lens paper at the bottom of the syringe barrel to cover the tip opening. Approximately 11 ml of loosely packed cellulose powder was added to the syringe barrel, ensuring that the lens paper fully covered the syringe opening and that the cellulose powder did not fall out. The plunger was then inserted, and the cellulose powder was packed

down to approximately the 5.7 ml mark. Next, we passed 7ml of 1x PBS through the column to make it wet, allowing the flowthrough to come out by gravity.

The cell pellet, which consisted of IEs adhered to HEK293T cells, was diluted in equal volumes of 1x PBS/RPMI and then passed through the column, allowing the flowthrough to come out by gravity. Once the column was empty, an additional 5-7 ml of PBS/RPMI was passed through the column, and the plunger was inserted. Finally, all the stuck sample was taken out by forcibly pressing the plunger.

By utilizing this method, we were able to significantly reduce the presence of HEK293T DNA in our samples, thereby improving the quality and reliability of our data.

**2.8 RNA isolation using TRIzol method**

The harvested cell pellets were subjected to homogenization using 500µl of TRIzol (Biorad) reagent. The TRIzol suspension was mixed thoroughly with 0.2 times its volume of chloroform and allowed to incubate at room temperature for 12 minutes. Afterward, the samples were subjected to centrifugation at 14,000 RPM for 25 minutes at a temperature of 4ºC. The upper aqueous phase was then collected meticulously into a separate tube.

To cause RNA precipitation from the upper aqueous phase that was collected, isopropanol at a volume of 0.5 times the upper phase was added to the tubes. The mixture was then thoroughly mixed and allowed to incubate at room temperature for 15-20 minutes. The next step involved centrifuging the samples at 14,000 RPM for 12 minutes at a temperature of 4℃ to form a pellet of RNA. The supernatant was removed, and the RNA pellet was washed with 70% ethanol once. The RNA pellet was air-dried and then resuspended in nuclease-free water. The concentration of RNA in the resulting solution was determined using a nanodrop instrument.

**2.9 Quantitative Real-Time PCR**

The study utilized 1 microgram of RNA that was free of DNAse to generate cDNA via the ImProm-II Reverse Transcription System (Promega), using random primers. Real-time PCR was performed using the CFX96 Real-Time PCR detection system (Biorad), with the internal controls being P. falciparum seryl-tRNA synthetase and Human GAPDH. To measure the expression, SYBR green dye was employed to quantify fluorescence. Each experiment consisted of three technical replicates.

# Chapter 3: Results

## 3.1 Tissue-wise distribution profile for PfEMP1 receptors

We wanted to study the interactions between PfEMP1 proteins and the endothelial receptors across the whole body. Therefore we conducted a comprehensive literature survey to locate and identify the well-known PfEMP1-interacting endothelial receptors that are predominantly expressed in the microvasculature of vital organs and are believed to cause severe malarial complications. It revealed that CD36 is mainly expressed in hepatocytes, adipose tissues, and heart cells, as reported in studies by Kazem Zibara et al. 2002 and Lehner et al. 2016. ICAM-1 demonstrates the highest expression on brain microvasculature, as reported in a study by Cunningham et al. 2017. Endothelial protein C receptor (EPCR) is predominantly present in the brain and placenta. PECAM-1 is present in the brain and lungs and is known to cause malaria-associated pulmonary complications, as reported in a study by Berger et al. 2013. CD9 is majorly expressed in the esophagus. P Selectin is majorly present in the kidney and urinary bladder, as well as in the respiratory tract. Thrombospondin 1 demonstrates expression in the lungs and gall bladder. In contrast, CD151 shows predominant expression in the heart and lungs. Complement C1q binding protein is present in the kidneys (Metwally et al., 2017). Overall, these findings highlight the specific expression patterns of PfEMP1-interacting endothelial receptors across various microvascular beds (Figure 6), providing insights into the molecular mechanisms underlying severe malarial complications in different organs to develop targeted therapeutic interventions for severe malaria. We further investigated the effect of cytoadherence on these receptors, as it has been shown to play a significant role in severe malaria pathogenesis.

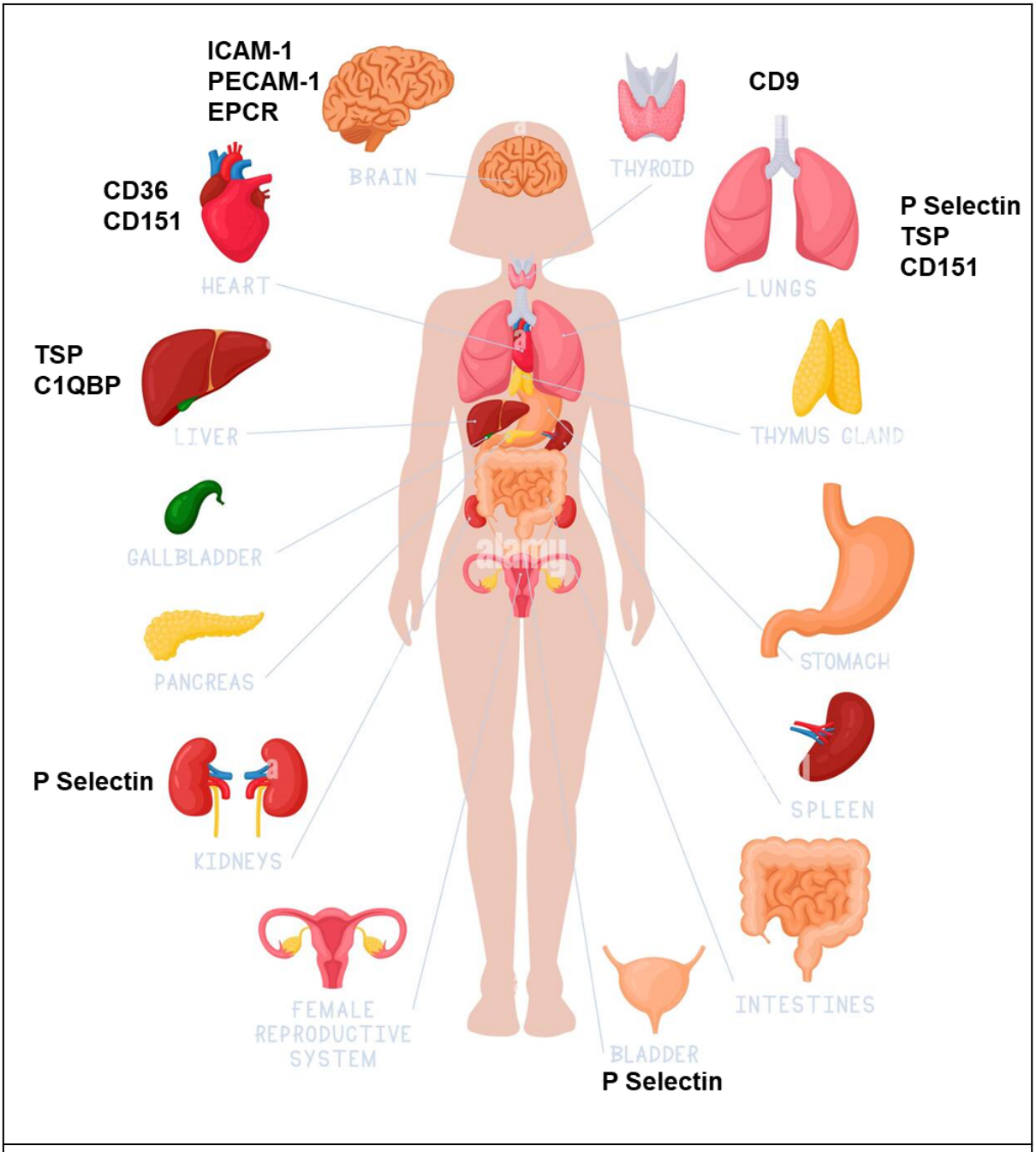


**Figure 6**. Tissue-wise distribution profile for PfEMP1 interacting major human endothelial receptors (image created from alamy.com).

### 3.2 Isolation and confirmation of plasmids obtained from Addgene

The plasmids harboring the genes for endothelial receptors were received in a bacterial stab. We grew the bacteria on Luria Bertani Agar medium and isolated the plasmids using Macherey Nagel mini prep kit. Single digestion was done to check the presence of the corresponding plasmids. We were able to see the bands at

expected sizes; CD36 (6139bp), CD9(5388bp), CD151(5476bp), ICAM-1(5831bp), PECAM(10,044bp), SELP(8951bp), THBS1(10,148bp), C1QBP(7484bp) (Figure 7). This gave us a confirmation of the presence of plasmids and allowed us to proceed further.

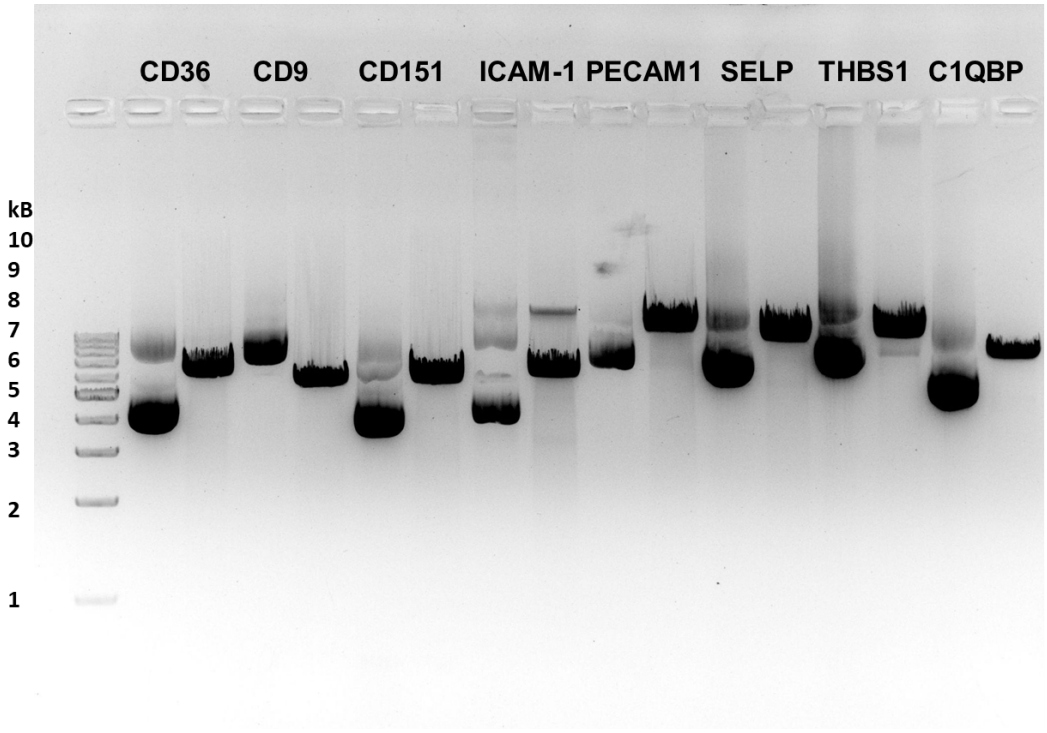


**Figure 7.** Agarose gel (1%) image representing bands of the plasmids CD36, CD9, CD151, ICAM1, PECAM1, SELP, THBS1, C1QBP

### 3.3 Subcloning of EPCR

In this study, we aimed to generate an overexpression line of EPCR in HEK293T cells. The commercially received plasmid contained EPCR in a bacterial expression system pGEMT, which was not suitable for our purpose. Therefore, we subcloned the EPCR gene into a pCMV9 vector, a well-known mammalian expression vector. To confirm the successful subcloning of EPCR into the pCMV9 vector, we performed PCR and double digestion with the restriction enzymes BamH1 and HindII. The PCR product was observed at the expected size of 717bp (Figure 8 (A), indicating the presence of EPCR in the vector. The double digestion resulted in the expected band

pattern (Figure 8 (B)), confirming the successful subcloning of EPCR into the pCMV9 vector.

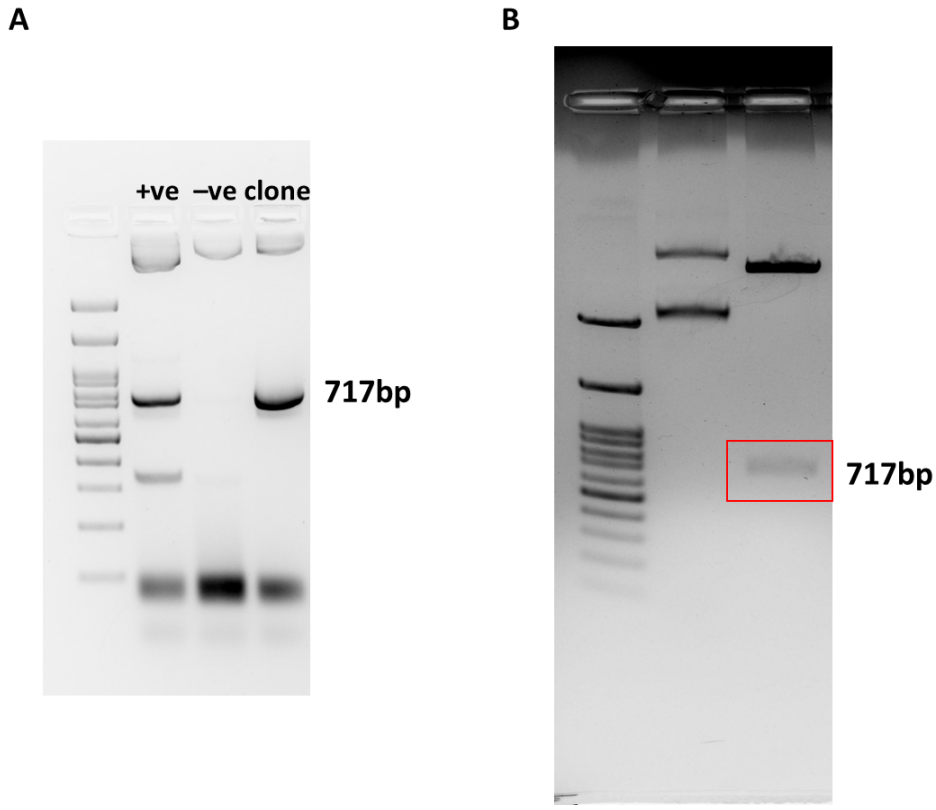


**Figure 8.** (A) PCR confirmation of EPCR clone (EPCR=717bp) (B) Digestion confirmation of clone

### 3.4 Static cytoadhesion assay with HBEC-5i

We wanted to validate the static cytoadherence assay with the HBEC-5i cell line, which predominantly shows the endogenous expression of ICAM-1 receptor, in our laboratory conditions. Western blot analysis using anti-ICAM-1 antibody confirmed the presence of ICAM-1 receptor(Figure 9 (A)). After conducting a static cytoadhesion assay with the primary cell line HBEC-5i, we found that red blood cells (RBCs) infected with parasites from the 3D7 parasite culture showed a significant amount of binding to the HBEC-5i cells. This was not observed with non-infected RBCs (Figure 9 (B)). This result confirmed the validation of the assay and assured

us to proceed with further laboratory investigations on the interaction between the PfEMP1 and endothelial cell receptors.

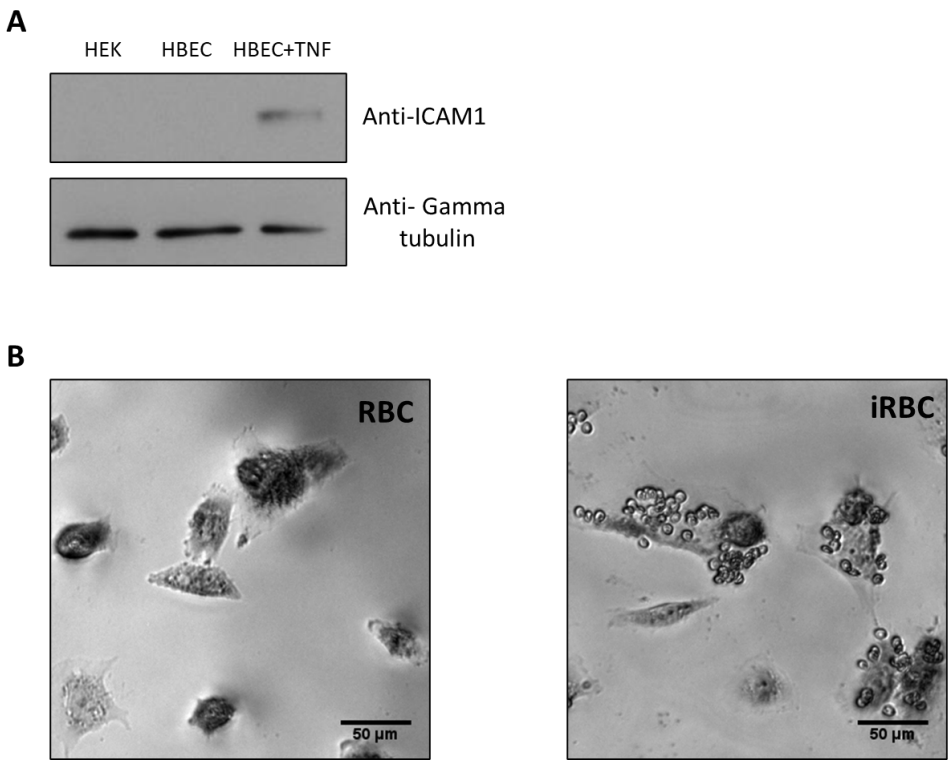


**Figure 9.** (A) Western Blot confirmation of ICAM1 receptor (B) Cytoadhesion of infected RBCs to HBEC-5i cells (Data credits: Bhagyashree Deshmukh)

### 3.5 Representative candidates from Var subgroups did not show any binding to HBEC-5i cells

Following validation, a cytoadherence assay was performed to check the PfEMP1 binding partners for ICAM-1 receptor using HBEC-5i cells and a 3D7 parasite culture. Subsequently, the Red Blood Cells (RBCs) that adhered to the HBEC-5i cells were collected and subjected to RNA isolation. The presence of RNA from the var gene family was then examined in these cells using an RT-qPCR reaction. Representative candidates from each var subgroup were selected based on their role in pathogenesis, which included PF3D7_0200100, PF3D7_0500100, PF3D7_1200600, PF3D7_1200100 (VarB); PF3D7_0420900 (VarC); and PF3D7_0809100 (Var B/C). Unfortunately, none of these candidates were found to

be expressed in the IEs adhered to the HBEC-5i cells. *P. falciparum* housekeeping gene tRNA synthetase was kept as control, which showed amplification, but at the 30th cycle of qPCR (Figure 10). This suggests two possibilities, one that the parasite RNA was very less in the sample, or the parasite-infected RBCs might not be getting attached to the endothelial cells.

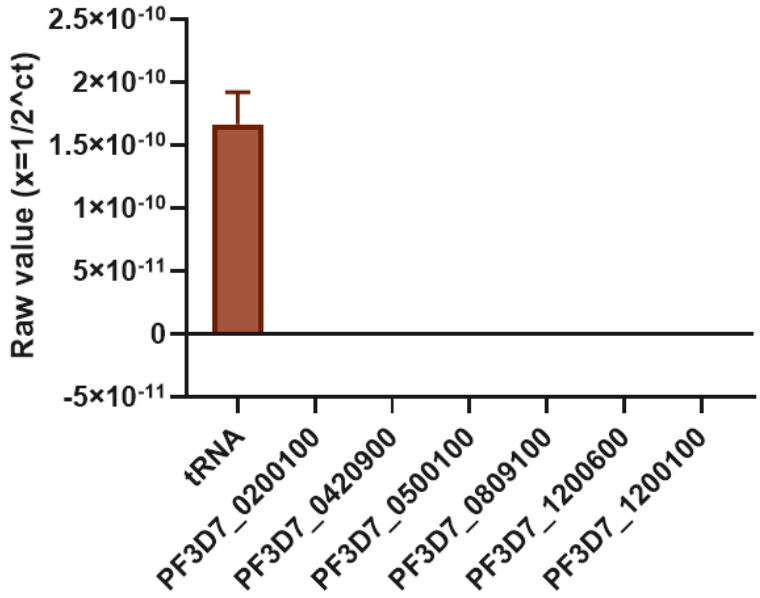


**Figure 10.** RT-qPCR analysis; raw value plotted. The representative candidates from selected var sub-groups were not present in the IEs adhered to HBEC-5i cells.

### 3.6 PF3D7_0420900 might be the interacting partner of CD151

We initially started the study with three receptors to standardize the transfection protocol. Three of the nine receptors selected for the interaction study were transfected into HEK293T cells. These receptors included CD36, CD9, and CD151 (Figure 11). These plasmids contain the fluorescent tag and hence were prioritized.

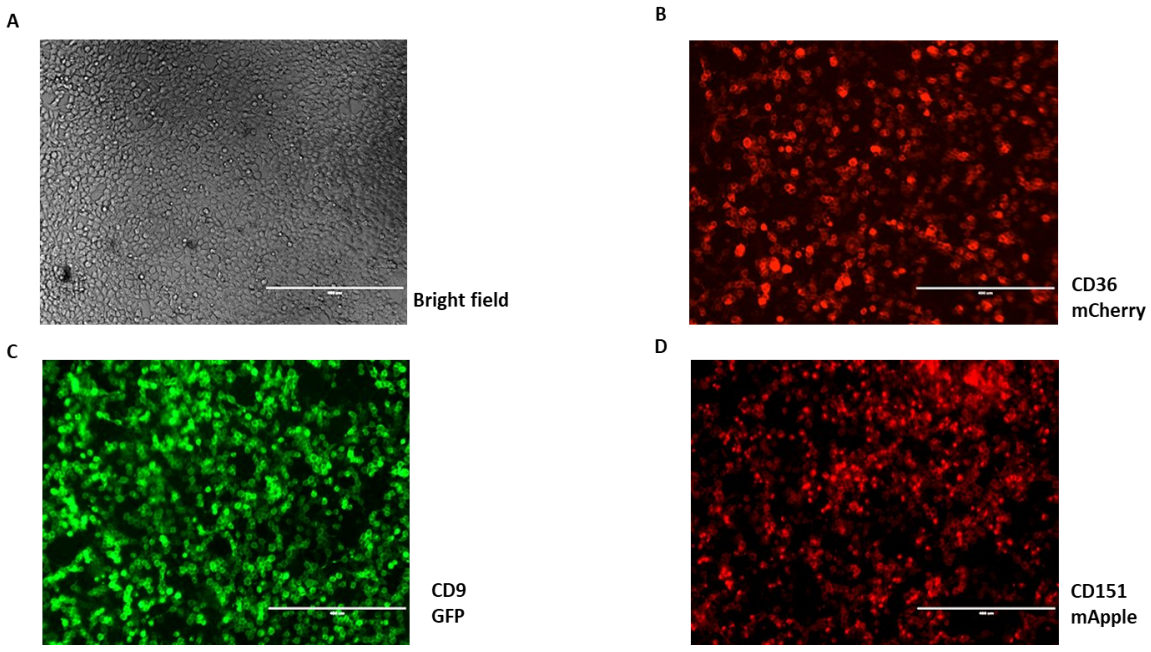


**Figure 11.** (A) Bright-field image of HEK293T transfected cells (B) Epifluorescence image for the expression of mCherry tagged CD36 plasmid in HEK293T (C) Epifluorescence image for the expression of mGFP tagged CD9 plasmid in HEK293T (D) Epifluorescence image for the expression of mApple tagged CD151 plasmid in HEK293T

A static cytoadhesion assay was conducted using a 3D7 parasite culture. The RNA sample generated was subjected to real-time qPCR. We checked the presence of var gene PF3D7_0420900. The housekeeping gene of *P. falciparum*, tRNA synthetase (PftRNA), was used as a positive control, and Human GAPDH was also used to check the contamination from HEK293T cells. We observed that PftRNA was present in CD9 and CD151 adhered samples and was not present in the control and CD36 sample (Figure 11 (A)). This indicates that the parasites did adhere to CD9 and CD151. The var gene PF3D7_0420900 showed the presence in the CD151 sample (Figure 11(B)), which suggests that PF3D7_0420900 might have an affinity for the CD151 receptor. It was noted that all the samples had very high human RNA contamination, confirmed by the presence of Human GAPDH in the samples (Figure 11(C)).

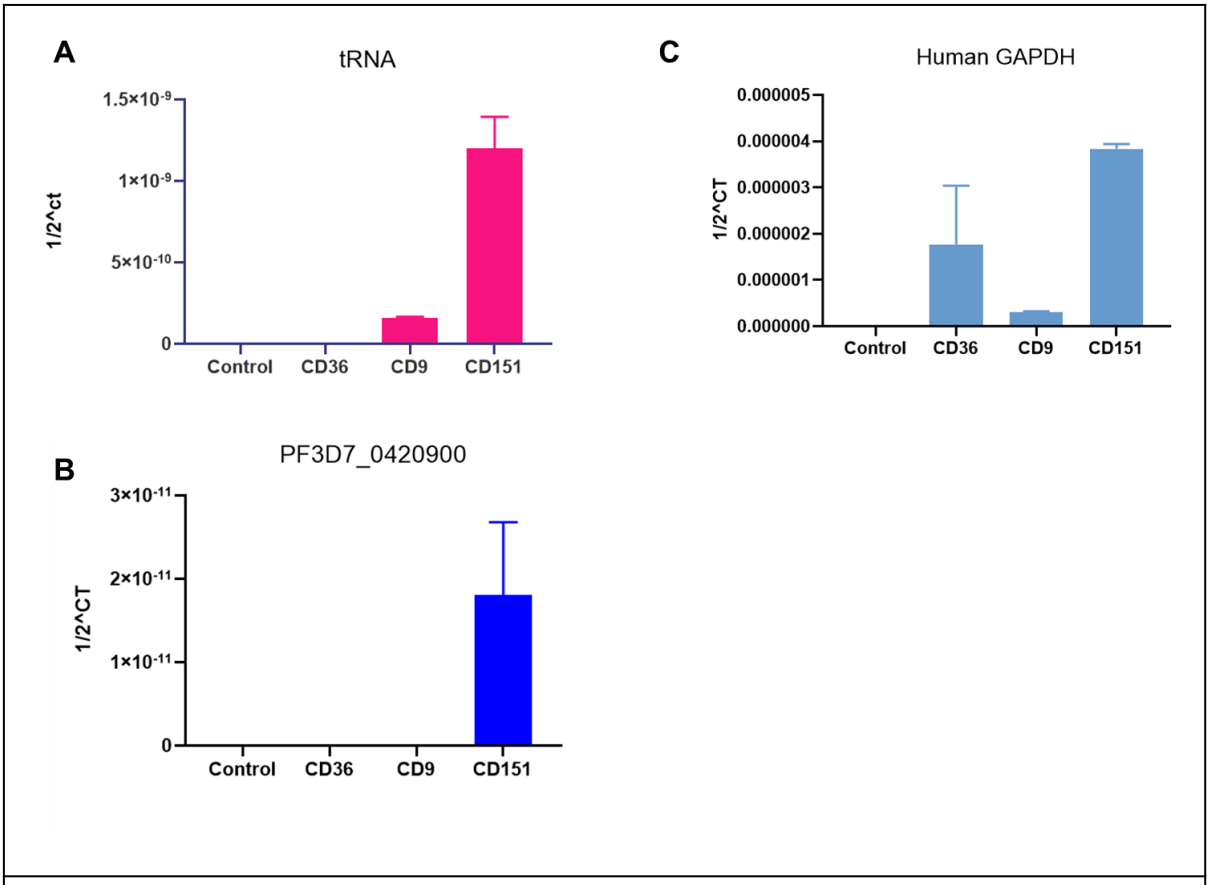


**Figure 12.** Real-time qPCR analysis raw values plotted (A) Comparison of Human GAPDH and *P. falciparum* tRNA synthetase present in the IEs adhered to the endothelial receptors. (B) PF3D7_0420900 present in the IEs adhered to the endothelial receptors

### 3.7 CF11 cellulose column can reduce the Human RNA contamination

As we detected the increased interference in human RNA, we were looking for methods to reduce this contamination. Therefore we employed the filtration technique using the CF11 cellulose column (Figure 13(A)). We checked for adequate enrichment of parasite RNA after filtering the samples using the CF11 cellulose column. We performed a cytoadhesion assay with CD36 transfected HEK293T cells. We scaled up our cultures to obtain more amount of RNA. The collected samples were split into two equal volumes. One of them was subjected to CF11 filtration, and the other was kept undisturbed. After filtration, both samples were subjected to RNA isolation and quantification using RT qPCR. The primers for Human GAPDH and *P. falciparum* tRNA synthetase were used. We observed that the CF11 filtration caused a depletion of human RNA by approximately 50 percent (Figure 13 (B), (C), (D)).

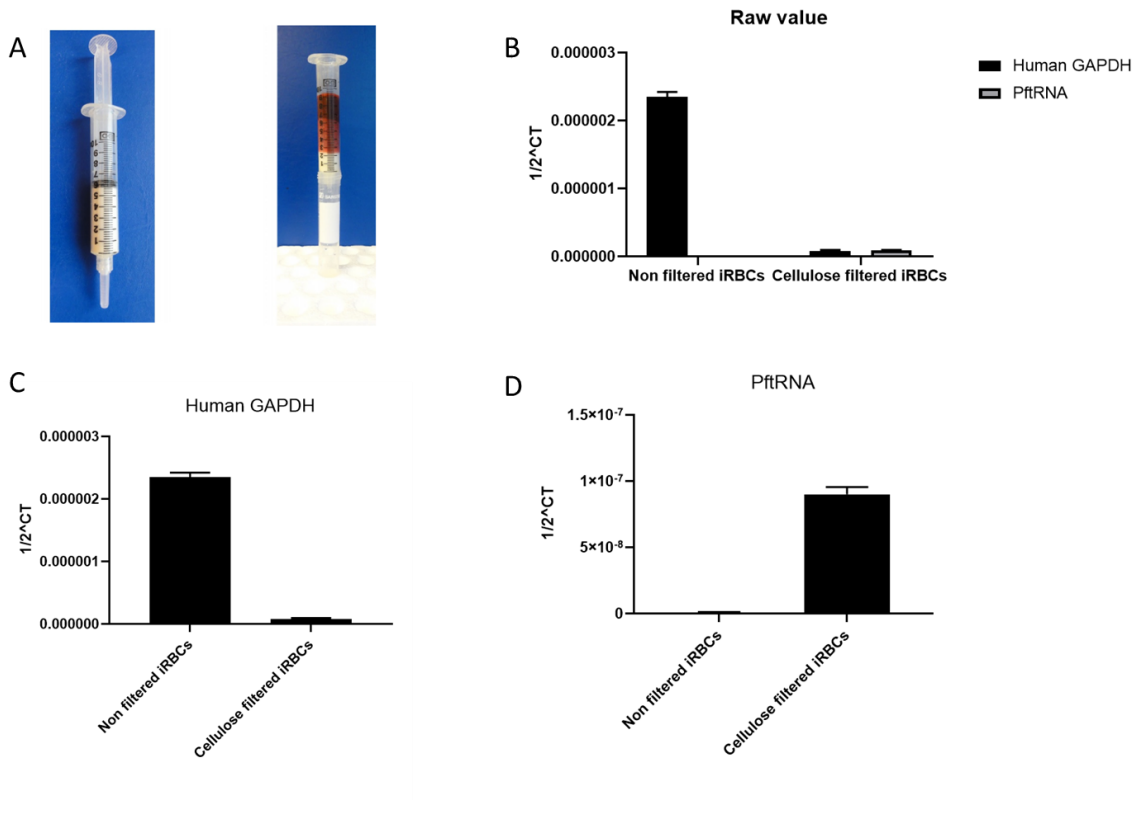


**Figure 13.** (A) A packed cellulose column and the sample padding through it (Venkatesan et al., 2012). RT-qPCR analysis; raw values plotted (B) Effect of cellulose filtration on the RNA amount from Human GAPDH and *P. falciparum* tRNA synthetase (C) Reduction in the amount of Human GAPDH RNA after cellulose filtration (D) Enrichment of *P. falciparum* tRNA synthetase after cellulose filtration

### 3.8 Sample preparation for RNA sequencing

We wanted to investigate the type of var gene interacting specifically with the human endothelial receptors. In order to proceed with our assumption, we have planned to perform an RNA sequencing of the IEs adhered to endothelial receptors. To begin with this, we have obtained the RNA from IEs adhered to the human endothelial receptors CD9 and CD151 overexpressed in HEK293T cells. The expression of the var gene in these samples would be compared with the RNA obtained from IEs adhered to normal HEK293T cells and HBEC-5i cells. We are expecting the upregulation of a particular var gene in the samples with overexpressed cell lines. The RNA has been obtained, and sequencing is in progress.

# Chapter 4: Discussion

Despite more than a century since its discovery, malaria remains a persistant cause of lethality globally. Unfortunately, we have yet to find a permanent cure or successfully eradicate the disease. According to the World Health Organization (WHO), it was thought that approximately 50% of the global population was susceptible to malaria in 2021. The causative agent *P. falciparum* has the ability to constantly evolve and adapt to various physiological stresses present in the human body (Hammam et al., 2021). It has developed strategies to evade immune recognition and disruption (Belachew et al., 2018). Therefore it is becoming troublesome to cope with this peculiar organism. In light of the significant impact malaria has had in recent years, it has become imperative to undertake a thorough investigation into the fundamental causes of parasite virulence in order to develop effective treatment strategies.

*P. falciparum*, during the intra-erythrocytic cycle, remodels the RBC surface by transporting a repertoire of adhesive proteins (Moxon et al., 2011). These proteins have an affinity for the diverse endothelial receptors of humans. It helps the parasite to adhere and sequester in deep microvasculature. Apart from adhesive phenotypes, these proteins have the capability to modulate inflammatory responses and endothelial activation (Sampaio et al., 2017). Cytoadhesion protects the parasite from immune recognition and is the primary cause of its persistent survival in the human body(Miller at al 2002). The host-parasite interaction involves two prime players, the PfEMP1 ligand and the endothelial receptor. The parasite produces a diverse repertoire of PfEMP1 proteins to interact with different endothelial receptors. These interactions are specific, and the specificity arises from the clonal antigenic variation exhibited by the var gene family, which encodes for PfEMP1 proteins in the parasite.

Cytoadhesion leads to multiple clinical manifestations, including severe forms of malaria, such as cerebral malaria and placental malaria. It has been observed that specific PFEMP1 proteins have been associated with these two lethal causes of malaria. Sequestration of parasites causes malfunctioning and multiple organ complications in severe malaria. Kidney failures, Acute Respiratory Distress Syndrome are common illnesses in severe malaria, though we have little knowledge of parasite tissue tropism. The specificity of PfEMP1 proteins for endothelial

receptors has been found in some cases, but a long hunt is still awaiting. There are pieces of evidence of parasite sequestration inside the post-capillary venules of multiple organs, but the interactions are poorly understood. Here we have looked more comprehensively at these interactions and are trying to characterize them as well. The study has focused on the endothelial receptors concerned with the *P. falciparum* IE cytoadhesion phenotypes and tried to understand which PfEMP1 protein interacts with them.

Tissue tropism is the ability of a pathogen to affect a particular organ. Multiple pathogens have been identified with this strategy to infect the host (Pereira et al., 2019). There is emerging evidence that tissue tropism also exhibits in *P. falciparum* malaria. The sequestration of *P. falciparum* in specific organs due to the presence of a huge repertoire of PfEMP1 proteins makes it unique. The characterization of these interactions would reveal the interacting partners at vascular endothelia and would serve as an important site to target for therapeutics.

The in vitro static adhesion assays with the primary HBEC-5i cell line revealed that the infected RBCs show an affinity for ICAM-1 receptor in laboratory conditions. Considering this as a proof of concept, we began our hunt to unleash the PfEMP1 interactions with other endothelial receptors. A comprehensive literature survey provided us with the known PfEMP1 receptors and their location where they are predominantly expressed in the human host. To clarify the interaction specificity, all the endothelial receptors, including ICAM-1, were overexpressed in HEK293T, an immortal cell line. The real-time qPCR reactions with the overexpression line of CD151 revealed that the IEs adhered had the expression of PF3D7_0420900, a gene from the varB subgroup. This suggests that PF3D7_0420900 could be the interacting partner of the CD151 receptor. But commenting on this interaction at this early stage would not be feasible, and further validation is required.

It was observed that during the collection of adhered IEs, the HEK293T cells were also getting detached along with the IEs from the cell plate. This was also reflected in the qPCR reactions. The total RNA isolated from the adhered IEs comprised more than 90 percent of human RNA. To troubleshoot this, we looked for methods to separate the human RNA from the parasite RNA present in the IEs. We used a CF11 cellulose filtration technique which has been used earlier to separate other blood components, especially the leukocytes, from the RBCs in clinical patient samples(Venkatesan et al 2012). The filtration occurs using a cellulose column with

size-exclusion chromatography at the cellular level. The CF11 filtration resulted in reducing human RNA contamination by almost 50 percent.

The real-time qPCR reactions were done as trials to validate the concept and have an idea of the RNA amount present in the collected sample. We aim to perform an RNA sequencing with the RNA isolated from the IEs adhered to all the endothelial receptors. In this regard, we have initiated with two well-known receptors; CD9 and CD151. The RNA sequencing is in progress. This would give us a clear picture of the interactions of PfEMP1 and endothelial receptors that we are interested in. We expect the upregulation of a particular var gene with the endothelial receptor of its affinity. We would also be looking for the reads in RNA sequencing that are mapped to all the surface proteins that are considered to be transported at the IE surface along with the PfEMP1 family. The RNA sequencing of the IEs adhered to various endothelial receptors is at the core of this project and is still in the process of being get done.

This study would help us understand the tissue specific sequestration pattern of the deadliest malaria parasite *P. falciparum.* These molecular insights will provide us the information of particular var genes associated with specific human endothelial receptors. It would gave us the idea of tissue tropism in *P. falciparum* malaria and open the avenue for further research.

# References


1. Antinori, S., Galimberti, L., Milazzo, L., & Corbellino, M. (2013). Plasmodium knowlesi: the emerging zoonotic malaria parasite. *Acta tropica*, *125*(2), 191–201. https://doi.org/10.1016/j.actatropica.2012.10.008
2. Aird, W. C., Mosnier, L. O., & Fairhurst, R. M. (2014). Plasmodium falciparum picks (on) EPCR. *Blood*, *123*(2), 163–167. https://doi.org/10.1182/blood-2013-09-521005
3. Balaji, S. N., Deshmukh, R., & Trivedi, V. (2020). Severe malaria: Biology, clinical manifestation, pathogenesis and consequences. *Journal of vector borne diseases*, *57*(1), 1–13. https://doi.org/10.4103/0972-9062.308793
4. Baruch, D. I., Pasloske, B. L., Singh, H. B., Bi, X., Ma, X. C., Feldman, M., Taraschi, T. F., & Howard, R. J. (1995). Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell*, *82*(1), 77–87. https://doi.org/10.1016/0092-8674(95)90054-3
5. Baruch, D. I., Pasloske, B. L., Singh, H. B., Bi, X., Ma, X. C., Feldman, M., Taraschi, T. F., & Howard, R. J. (1995). Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell*, *82*(1), 77–87. https://doi.org/10.1016/0092-8674(95)90054-3
6. Belachew E. B. (2018). Immune Response and Evasion Mechanisms of *Plasmodium falciparum* Parasites. *Journal of immunology research*, *2018*, 6529681. https://doi.org/10.1155/2018/6529681
7. Berendt, A. R., Simmons, D. L., Tansey, J., Newbold, C. I., & Marsh, K. (1989). Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for Plasmodium falciparum. *Nature*, *341*(6237), 57–59. https://doi.org/10.1038/341057a0
8. Brabin, B. J., Romagosa, C., Abdelgalil, S., Menéndez, C., Verhoeff, F. H., McGready, R., Fletcher, K. A., Owens, S., D'Alessandro, U., Nosten, F., Fischer, P. R., & Ordi, J. (2004). The sick placenta-the role of malaria. *Placenta*, *25*(5), 359–378. https://doi.org/10.1016/j.placenta.2003.10.019
9. Cunningham, D. A., Lin, J. W., Brugat, T., Jarra, W., Tumwine, I., Kushinga, G., Ramesar, J., Franke-Fayard, B., & Langhorne, J. (2017). ICAM-1 is a key receptor mediating cytoadherence and pathology in the Plasmodium chabaudi

malaria model. *Malaria journal*, *16*(1), 185. https://doi.org/10.1186/s12936-017-1834-8

10. Cooke, B. M., & Coppel, R. L. (1995). Cytoadhesion and falciparum malaria: going with the flow. *Parasitology today (Personal ed.)*, *11*(8), 282–287. https://doi.org/10.1016/0169-4758(95)80040-9
11. Cowman, A. F., & Crabb, B. S. (2006). Invasion of red blood cells by malaria parasites. *Cell*, *124*(4), 755–766. https://doi.org/10.1016/j.cell.2006.02.006
12. Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., Lwin, K. M., Ariey, F., Hanpithakpong, W., Lee, S. J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S. S., Yeung, S., Singhasivanon, P., Day, N. P., … White, N. J. (2009). Artemisinin resistance in Plasmodium falciparum malaria. *The New England journal of medicine*, *361*(5), 455–467. https://doi.org/10.1056/NEJMoa0808859
13. Flick, K., Ahuja, S., Chene, A., Bejarano, M. T., & Chen, Q. (2004). Optimized expression of Plasmodium falciparum erythrocyte membrane protein 1 domains in Escherichia coli. *Malaria journal*, *3*, 50. https://doi.org/10.1186/1475-2875-3-50
14. Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M. S., Nene, V., Shallom, S. J., … Barrell, B. (2002). Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature*, *419*(6906), 498–511. https://doi.org/10.1038/nature01097
15. Grobusch, M. P., & Kremsner, P. G. (2005). Uncomplicated malaria. *Current topics in microbiology and immunology*, *295*, 83–104.
16. Guizetti, J., & Scherf, A. (2013). Silence, activate, poise and switch! Mechanisms of antigenic variation in Plasmodium falciparum. *Cellular microbiology*, *15*(5), 718–726. https://doi.org/10.1111/cmi.12115
17. Hammam, E., Sinha, A., Baumgarten, S., Nardella, F., Liang, J., Miled, S., Bonhomme, F., Erdmann, D., Arcangioli, B., Arimondo, P. B., Dedon, P., Preiser, P., & Scherf, A. (2021). Malaria Parasite Stress Tolerance Is Regulated by DNMT2-Mediated tRNA Cytosine Methylation. *mBio*, *12*(6), e0255821. https://doi.org/10.1128/mBio.02558-21
18. Hviid, L., & Jensen, A. T. (2015). PfEMP1 - A Parasite Protein Family of Key Importance in Plasmodium falciparum Malaria Immunity and Pathogenesis.

*Advances in parasitology*, *88*, 51–84. https://doi.org/10.1016/bs.apar.2015.02.004

19. Jensen, A. R., Adams, Y., & Hviid, L. (2020). Cerebral Plasmodium falciparum malaria: The role of PfEMP1 in its pathogenesis and immunity, and PfEMP1-based vaccines to prevent it. *Immunological reviews*, *293*(1), 230–252. https://doi.org/10.1111/imr.12807
20. Lapp, Z., Obala, A. A., Abel, L., Rasmussen, D. A., Sumner, K. M., Freedman, E., Taylor, S. M., & Prudhomme-O'Meara, W. (2022). Plasmodium falciparum Genetic Diversity in Coincident Human and Mosquito Hosts. *mBio*, *13*(5), e0227722. https://doi.org/10.1128/mbio.02277-22
21. Lee, W. C., Russell, B., & Rénia, L. (2019). Sticking for a Cause: The Falciparum Malaria Parasites Cytoadherence Paradigm. *Frontiers in immunology*, *10*, 1444. https://doi.org/10.3389/fimmu.2019.01444
22. Loubens, M., Vincensini, L., Fernandes, P., Briquet, S., Marinach, C., & Silvie, O. (2021). Plasmodium sporozoites on the move: Switching from cell traversal to productive invasion of hepatocytes. *Molecular microbiology*, *115*(5), 870–881. https://doi.org/10.1111/mmi.14645
23. Luzolo, A. L., & Ngoyi, D. M. (2019). Cerebral malaria. *Brain research bulletin*, *145*, 53–58. https://doi.org/10.1016/j.brainresbull.2019.01.010
24. MacPherson, G. G., Warrell, M. J., White, N. J., Looareesuwan, S., & Warrell, D. A. (1985). Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *The American journal of pathology*, *119*(3), 385–401.
25. Miller, L. H., Baruch, D. I., Marsh, K., & Doumbo, O. K. (2002). The pathogenic basis of malaria. *Nature*, *415*(6872), 673–679. https://doi.org/10.1038/415673a
26. Milner, D. A., Jr, Lee, J. J., Frantzreb, C., Whitten, R. O., Kamiza, S., Carr, R. A., Pradham, A., Factor, R. E., Playforth, K., Liomba, G., Dzamalala, C., Seydel, K. B., Molyneux, M. E., & Taylor, T. E. (2015). Quantitative Assessment of Multiorgan Sequestration of Parasites in Fatal Pediatric Cerebral Malaria. *The Journal of infectious diseases*, *212*(8), 1317–1321. https://doi.org/10.1093/infdis/jiv205
27. Milner, D. A., Jr, Whitten, R. O., Kamiza, S., Carr, R., Liomba, G., Dzamalala, C., Seydel, K. B., Molyneux, M. E., & Taylor, T. E. (2014). The systemic

pathology of cerebral malaria in African children. *Frontiers in cellular and infection microbiology*, *4*, 104. https://doi.org/10.3389/fcimb.2014.00104

28. Moxon, C. A., Grau, G. E., & Craig, A. G. (2011). Malaria: modification of the red blood cell and consequences in the human host. *British journal of haematology*, *154*(6), 670–679. https://doi.org/10.1111/j.1365-2141.2011.08755.x
29. Ortolan, L. S., Avril, M., Xue, J., Seydel, K. B., Zheng, Y., & Smith, J. D. (2022). *Plasmodium falciparum* Parasite Lines Expressing DC8 and Group A PfEMP1 Bind to Brain, Intestinal, and Kidney Endothelial Cells. *Frontiers in cellular and infection microbiology*, *12*, 813011. https://doi.org/10.3389/fcimb.2022.813011
30. Rask, T. S., Hansen, D. A., Theander, T. G., Gorm Pedersen, A., & Lavstsen, T. (2010). Plasmodium falciparum erythrocyte membrane protein 1 diversity in seven genomes--divide and conquer. *PLoS computational biology*, *6*(9), e1000933. https://doi.org/10.1371/journal.pcbi.1000933
31. Salanti, A., Staalsoe, T., Lavstsen, T., Jensen, A. T., Sowa, M. P., Arnot, D. E., Hviid, L., & Theander, T. G. (2003). Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. *Molecular microbiology*, *49*(1), 179–191. https://doi.org/10.1046/j.1365-2958.2003.03570.x
32. Sampaio, N. G., Emery, S. J., Garnham, A. L., Tan, Q. Y., Sisquella, X., Pimentel, M. A., Jex, A. R., Regev-Rudzki, N., Schofield, L., & Eriksson, E. M. (2018). Extracellular vesicles from early stage Plasmodium falciparum-infected red blood cells contain PfEMP1 and induce transcriptional changes in human monocytes. *Cellular microbiology*, *20*(5), e12822. https://doi.org/10.1111/cmi.12822
33. Seydel, K. B., Fox, L. L., Glover, S. J., Reeves, M. J., Pensulo, P., Muiruri, A., Mpakiza, A., Molyneux, M. E., & Taylor, T. E. (2012). Plasma concentrations of parasite histidine-rich protein 2 distinguish between retinopathy-positive and retinopathy-negative cerebral malaria in Malawian children. *The Journal of infectious diseases*, *206*(3), 309–318. https://doi.org/10.1093/infdis/jis371
34. Seydel, K. B., Kampondeni, S. D., Valim, C., Potchen, M. J., Milner, D. A., Muwalo, F. W., Birbeck, G. L., Bradley, W. G., Fox, L. L., Glover, S. J., Hammond, C. A., Heyderman, R. S., Chilingulo, C. A., Molyneux, M. E., & Taylor, T. E. (2015). Brain swelling and death in children with cerebral malaria.

*The New England journal of medicine*, *372*(12), 1126–1137. https://doi.org/10.1056/NEJMoa1400116

35. Silva Pereira, S., Trindade, S., De Niz, M., & Figueiredo, L. M. (2019). Tissue tropism in parasitic diseases. *Open biology*, *9*(5), 190036. https://doi.org/10.1098/rsob.190036
36. Smith, D. L., Cohen, J. M., Chiyaka, C., Johnston, G., Gething, P. W., Gosling, R., Buckee, C. O., Laxminarayan, R., Hay, S. I., & Tatem, A. J. (2013). A sticky situation: the unexpected stability of malaria elimination. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *368*(1623), 20120145. https://doi.org/10.1098/rstb.2012.0145
37. Smith, J. D., Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S., Pinches, R., Newbold, C. I., & Miller, L. H. (1995). Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell*, *82*(1), 101–110. https://doi.org/10.1016/0092-8674(95)90056-x
38. SPITZ S. (1946). The pathology of acute falciparum malaria. *Military surgeon*, *99*(5), 555–572.
39. Su, X. Z., Heatwole, V. M., Wertheimer, S. P., Guinet, F., Herrfeldt, J. A., Peterson, D. S., Ravetch, J. A., & Wellems, T. E. (1995). The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. *Cell*, *82*(1), 89–100. https://doi.org/10.1016/0092-8674(95)90055-1
40. Talapko, J., Škrlec, I., Alebić, T., Jukić, M., & Včev, A. (2019). Malaria: The Past and the Present. *Microorganisms*, *7*(6), 179. https://doi.org/10.3390/microorganisms7060179
41. Tangpukdee, N., Duangdee, C., Wilairatana, P., & Krudsood, S. (2009). Malaria diagnosis: a brief review. *The Korean journal of parasitology*, *47*(2), 93–102. https://doi.org/10.3347/kjp.2009.47.2.93
42. Tavares, J., Formaglio, P., Thiberge, S., Mordelet, E., Van Rooijen, N., Medvinsky, A., Ménard, R., & Amino, R. (2013). Role of host cell traversal by the malaria sporozoite during liver infection. *The Journal of experimental medicine*, *210*(5), 905–915. https://doi.org/10.1084/jem.20121130
43. Taylor, S. M., Parobek, C. M., & Fairhurst, R. M. (2012). Haemoglobinopathies and the clinical epidemiology of malaria: a systematic review and

meta-analysis. *The Lancet. Infectious diseases*, *12*(6), 457–468. https://doi.org/10.1016/S1473-3099(12)70055-5

44. Trampuz, A., Jereb, M., Muzlovic, I., & Prabhu, R. M. (2003). Clinical review: Severe malaria. *Critical care (London, England)*, *7*(4), 315–323. https://doi.org/10.1186/cc2183
45. Turner, L., Lavstsen, T., Berger, S. S., Wang, C. W., Petersen, J. E., Avril, M., Brazier, A. J., Freeth, J., Jespersen, J. S., Nielsen, M. A., Magistrado, P., Lusingu, J., Smith, J. D., Higgins, M. K., & Theander, T. G. (2013). Severe malaria is associated with parasite binding to endothelial protein C receptor. *Nature*, *498*(7455), 502–505. https://doi.org/10.1038/nature12216
46. Venkatesan, M., Amaratunga, C., Campino, S., Auburn, S., Koch, O., Lim, P., Uk, S., Socheat, D., Kwiatkowski, D. P., Fairhurst, R. M., & Plowe, C. V. (2012). Using CF11 cellulose columns to inexpensively and effectively remove human DNA from Plasmodium falciparum-infected whole blood samples. *Malaria journal*, *11*, 41. https://doi.org/10.1186/1475-2875-11-41
47. White, N. J., Turner, G. D., Day, N. P., & Dondorp, A. M. (2013). Lethal malaria: Marchiafava and Bignami were right. *The Journal of infectious diseases*, *208*(2), 192–198. https://doi.org/10.1093/infdis/jit116
48. https://www.who.int/
49. William, T., Rahman, H. A., Jelip, J., Ibrahim, M. Y., Menon, J., Grigg, M. J., Yeo, T. W., Anstey, N. M., & Barber, B. E. (2013). Increasing incidence of Plasmodium knowlesi malaria following control of P. falciparum and P. vivax Malaria in Sabah, Malaysia. *PLoS neglected tropical diseases*, *7*(1), e2026. https://doi.org/10.1371/journal.pntd.0002026
50. Zibara, K., Malaud, E., & McGregor, J. L. (2002). CD36 mRNA and Protein Expression Levels Are Significantly Increased in the Heart and Testis of apoE Deficient Mice in Comparison to Wild Type (C57BL/6). *Journal of biomedicine & biotechnology*, *2*(1), 14–21. https://doi.org/10.1155/S1110724302000335