Adhesion Analysis of Different PfEMP1 Variants to CD36, ICAM-1 and Primary Endothelial Cells.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy.

By

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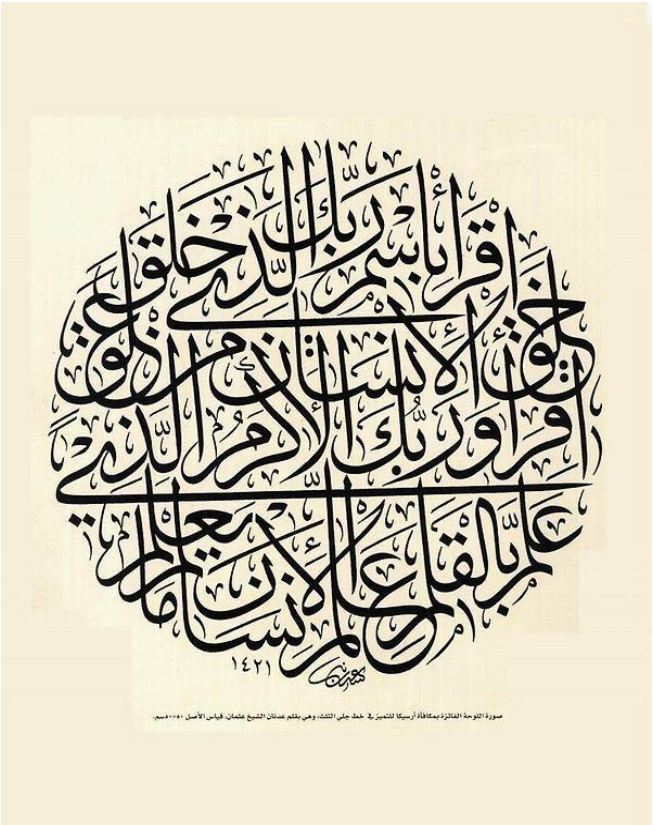
Abstract: Malaria is an ancient vector-borne disease that still has a huge impact on global health. Malaria pathogenesis is developed during the asexual intraerythrocytic stage where *P. falciparum* modifies the host erythrocytes by exporting repertoires of parasite proteins on to the surface of infected erythrocytes. PfEMP1 is one of these proteins that mediate different functions including the adhesion of IEs to the host receptors such as CD36, ICAM-1 and EPCR. The current study has characterised the adhesion of infected erythrocytes with different PfEMP1 variants to CD36, ICAM-1 and primary endothelial cells. The characterisation was carried using static protein and flow endothelial adhesion assays. First, the characterisation involved an analysis of the binding of recently selected ICAM-1 binding *P. falciparum* patient isolates on different ICAM-1 variants. The results showed that different isolates have variant-specific binding phenotypes suggesting that there might be variable contact residues on ICAM-1 being used by different parasite PfEMP1 variants. This observation was more emphasised by the adhesion of isogenic isolates that has been confirmed to express ICAM-1 binding domain from IT4 parasites. The second part of the study has characterised the adhesion of IEs with upsC PfEMP1 isolates from HB3 and IT4 isolates on CD36, ICAM-1 and endothelial cells. Three upsC IT4 isolates bound to CD36 and one of these isolates bound to ICAM-1 because it expresses DBLβ-ICAM-1 binding domain. In contrast, HB3 upsC isolates did not show preferential binding to CD36, ICAM-1 and the endothelial cells despite showing cross reactivity with adult hyperimmune sera. Finally, the adhesion of IEs with different length PfEMP1s was analysed. It was concluded that long PfEMP1 adapted to bind efficiently the short, but this might be due to the lack of variety of DBLs for adhesion in the short forms. Therefore, it is suggested that it is the domain constitution rather than size that seems to be important.

Dedication:

To my mother, who was kindly supporting me during PhD,
To my father, who kept encouraging me, especially, the unforgotten last call he made to me on the day he passed away while I was doing a flow assay.



Arabic calligraphy: the picture is written by Alshaikh Othman using Arabic hand writing called Al-Thuluth. The statement is quoted from (Holy Quran) and it is translated to: Read! in the name of your Lord Who has created (all that exists) (1). He created man from a clot (2). Read! and your Lord is Most Generous (3). Who has taught (the writing) by the pen (4). Taught man what he knew not (5). (Holy Quran Chapter 96, (the clot))

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Abbreviations:

AM: Asymptomatic malaria

CM: Cerebral malaria

CR1: Complement receptor 1

CSA: Chondroitin sulfate A

DC: Domain Cassettes

EBA: Erythrocyte Binding Antigen

ECs: Endothelial Cells

FACS: Fluorescence-activated cell sorting

HBMEC: Human Brain Microvascular Endothelial Cells

HDMEC: Human Dermal Microvascular Endothelial Cells

HBs: Homology blocks

HUVEC: Human Umbilical Vein Endothelial Cells

ICAM-1: Intracellular adhesion molecule-1

IE: Infected erythrocyte

IT4-ICAM-1: IT4 isolates expressing DBLβ domains that bind to ICAM-1

KSA: Kingdom of Saudi Arabia

LFA-1: Lymphocyte Function-Associated Antigen-1

MAHRP-1: Membrane-Associated Histidine-Rich Protein-1

MAHRP-2: Membrane-Associated Histidine-Rich Protein-2

MSPs: Merozoite Surface Proteins

PfEMP1: *Plasmodium falciparum* Erythrocytes Membrane Protein1

PfSBP1: *P. falciparum* skeleton binding protein 1

PNEPs : PTEX negative exported proteins

PTEX: *Plasmodium* translocon of exported proteins

PV: Parasitophorous vacuole

PVM: Parasitophorous vacuole membrane

RIEs: Ring infected erythrocytes

SM: Severe malaria

TNF: Tumour Necrosis Factor

UM: uncomplicated malaria

VSA: variant surface antigens

wRBCs: washed red blood cells

WHO: World Health Organisation

1. Malaria:

1.1 Disease overview:

Malaria is an ancient vector-borne disease that still has a huge impact on global health. It is one of the leading causes of death in countries with limited resources. It was described historically in a Chinese document from about 2700 BC. Also, there are other early documents describing malaria after that, but these need extra care when dealing with them (Cox, 2010). However, Hippocrates, in about 400 BC, was able to differentiate between “tertian” malaria fevers and “quartan” fevers on the basis of disease severity (Haldar et al., 2007).

Historically, the reason behind the word “malaria” derives from the Italian “mal ’ aria” meaning “bad air” because it used to be thought that the cause of malaria was miasmas arising from marshes. However, this was controversial until significant advancements in malaria research permitted the identification of the causative agent and the transmission vector at the end of 19th century. The identification of malaria parasites was by Charles Laveran, a French army surgeon, in 1880, and the identification of mosquitoes as transmission vectors was by Ronald Ross in 1897 (Cox, 2010). The evolutionary worldwide symbiotic relationship between humans and malaria parasites is estimated to have begun in Africa about ten thousand years ago (Hay et al., 2004).

There are six species that cause human malaria; *P. falciparum*, *P. vivax*, *P. malariae*, two distinct forms of *P. ovale* (Sutherland et al., 2010) and recently *P. knowlesi* has been found increasingly infecting humans especially in Malaysia (William et al., 2013). *P .falciparum* accounts for 90% of mortality caused by malaria worldwide.

World Health Organisation (WHO) estimated 3.2 billion people are at risk of malaria and 198 million cases of malaria occurred overall the world in 2013 (WHO, 2014). However, there is a discrepancy about malaria

mortality and morbidity reports most likely due to the measurement tools. In 2013, WHO estimated 584,000 deaths worldwide. In contrast, a systematic review in The Lancet reported deaths cases were about twice as much as stated by WHO in 2010 (Murray et al., 2012, Lozano et al., 2012). Most malaria deaths occur in children under five years old in Africa.

However, most cases were asymptomatic carriers that cause a major concern for malaria elimination programmes by facilitating continuous transmission by the mosquitoes. Along these lines, it has been noticed that malaria burden is shifting from the typical susceptible populations of young children and pregnant women to older children and adults (Cotter et al., 2013).

Considering the huge efforts towards malaria elimination programmes (Figure: 1.1), malaria mortality rate was reduced by about 47%, and incidence rate was decreased by 30% around the globe between 2000 and 2013 (WHO, 2014). Four countries have been certified as malaria free by the WHO since 2007 (WHO, 2013). Nowadays, many countries are trying to be certified as “malaria eliminated country” , including the Kingdom of Saudi Arabia (KSA) (Cotter et al., 2013). Saudi governmental efforts to control malaria started about 20 years after the foundation of the KSA in 1932. In 1963, KSA joined the WHO malaria eradication programme. KSA has succeeded to restrict malaria to its two main southern regions, Aseer and Jazan, which share borders with Yemen. Since 1990, Saudi government started to categorise the cases as indigenous or imported. There were outbreaks in the late 1990s, the worst in 1998. In 2004, the Kingdom re-established malaria control programme towards elimination status in 2015. Since then, the endogenous cases have continued decreasing to very low number, for instance, just 82 cases in 2012. However, this target is faced by many challenges, first of which is the imported cases, particularly with the current political disturbance taking place in Yemen, which is closed to the Saudi border. It is not surprising that local cases did increase in 2011 and

2012. The second problem is the rise of vivax malaria cases, which are less responsive to control than falciparum infections. It is believed that the economic development and improved health system have contributed to the current status of malaria in KSA (Coleman et al., 2014).

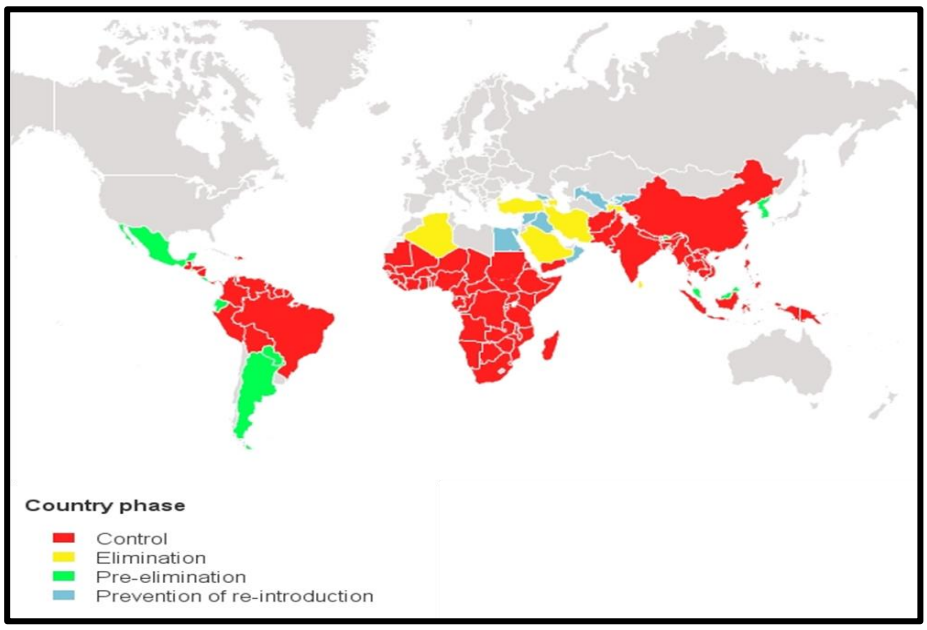

Figure 1.1: Classification of countries by stages of malaria elimination, as of December 2013. The map was created using WHO global malaria mapper. Lastly accessed on October 17, 2014 http://worldmalariareport.org/

1.2 Important clinical definitions:

Individuals infected by malaria experience different clinical outcomes. In early life, it usually starts with severe symptoms; approximately 10% of children develop symptoms of severe malaria with different pathological effects. Then, as individuals develop immunity to the severe manifestations they only suffer from mild disease or even can be as asymptomatic hosts, especially in highly endemic areas such as Sub-Saharan Africa.

1.2.1 Uncomplicated malaria (UM):

Generally, signs range from fever followed by headache, fatigue, muscle pains, and abdominal discomfort. Vomiting, nausea, and orthostatic

hypotension are also commonly associated with malaria (White et al., 2014).

1.2.2 Severe malaria (SM):

Generalised seizures in severe malaria caused by *P. falciparum* are associated with age dependent manifestations, especially in areas of high- transmission. It is worth clarifying that for concepts of SM there is unlikely to be a simple association between the clinical syndromes and the pathogenic mechanisms (Miller et al., 2002, White et al., 2014). For example, SM in children involves three overlapping syndromes: cerebral malaria, metabolic acidosis/ respiratory distress and severe anaemia. In adults, jaundice, acute renal injury and acute pulmonary oedema are more common and cerebral malaria and acidosis also occur. In the absence of early and proper clinical management death can result from severe manifestations and about 0.1% of the uncomplicated falciparum malaria cases die eventually (White et al., 2014, WHO, 2013).

1.2.2.1 Severe anaemia:

Malaria is the major cause of severe anaemia of in young children, particularly in high transmission areas. It is defined as haemoglobin concentration < 5 g/dl in the presence of *P. falciparum* parasitemia (Calis et al., 2008). Malarial anaemia results from the lysis of infected erythrocytes (IEs) to release new generations of merozoites, destruction of uninfected erythrocytes by unknown mechanism and reduced erythrocyte production and/or inadequate erythropoietic response. This is often followed by fever and chills, and the typical description of fever based on the cycle of the parasites may not be valid in the case of *P .falciparum,* which is usually asynchronous at least during the first days of illness (White et al., 2014).

1.2.2.2 Acidosis and hypoglycaemia:

Acidosis is one of the lethal manifestations associated with severe malaria. The acidotic breathing is considered as a bad prognosis

indicator in severe malaria patients. It is caused by the accumulation of organic acids, mainly lactic acid. Lactic acidosis is usually connected with hypoglycaemia, in children and pregnant women particularly (White et al., 2014).

1.2.2.3 Cerebral malaria:

Cerebral malaria (CM) is a complication of severe malaria. It is identified as the patient being in a coma, parasitaemia, Blantyre coma score ≤ 2 with the absence of other coma causes. Recent clinical observations have shown that infected humans with CM have marked retinopathy that has facilitated the diagnosis of this syndrome, reaching 95% sensitivity and 90% specificity in comparison with previous results using the gold standard examining autopsy samples (Beare et al., 2006). CM is often associated with a high mortality rate in malaria among other malaria outcomes (WHO, 2013).

1.3 Life Cycle:

1.3.1 Mosquito stage:

Female *Anopheles* mosquitoes transmit malaria during feeding on hosts' blood to use proteins for egg synthesis (Figure: 1.2). In malaria transmission regions individuals often carry the sexual forms of the parasites, gametocytes. During the blood feed, IEs with the gametocytes move into mosquitoes' gut where the erythrocytes are digested, and gametocytes released and developed into male and female gametes. Then, both gametes fuse to form diploid zygotes, which turn into motile forms called ookinetes that travel into the mosquito midgut wall and transform into oocysts. Inside the oocysts the human infectious forms, sporozoites, are formed. Once the oocysts rupture, sporozoites invade the body cavity of the mosquito and migrate to the salivary glands, whereby, they infect humans during another feed on a human blood meal. The life cycle inside the mosquito has been recently reviewed by (Smith et al., 2014).

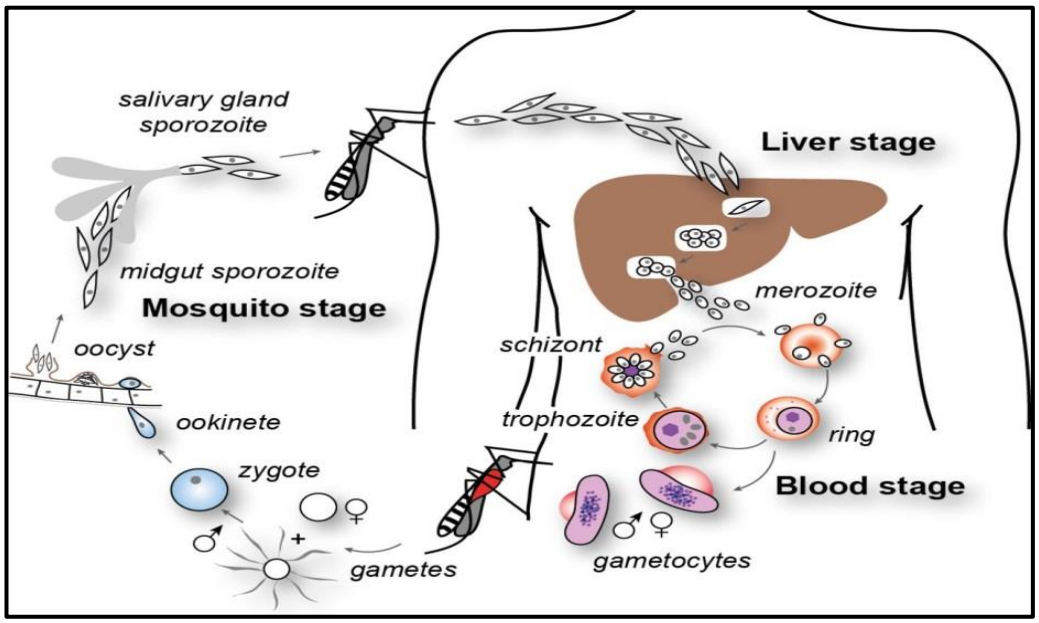

Figure 1.2: The life cycle of *P. falciparum* in human and mosquito.

1.3.2 Pre-erythrocytic stage:

In humans, injected sporozoites transform into merozoites, the erythrocyte invading form, in a growth phase known as the pre-erythrocytic stage. After a mosquito blood feed, sporozoites pass through the skin layer into either the blood stream, and thereby hepatocytes, where they multiply, or local lymph nodes where most of them probably die (Amino et al., 2006). In fact, recent studies showed that sporozoites in mice can stay at the bite sites for a few hours and can transform into merozoites in the epidermis and dermis and are able to invade erythrocytes (Gueirard et al., 2010). In *P. falciparum*, sporozoites invade hepatocytes and can produce thousands of daughter merozoites within a week in a hepatic schizont. However, non-falciparum species, such as *P. vivax* malaria parasites, can stay longer in the liver as a dormant form known as a hypnozoite (Wells et al., 2010). Once the hepatic schizonts rupture, released merozoites invade erythrocytes to start the erythrocytic cycle.

1.3.3 Erythrocytic stage:

The erythrocytic stage begins when the merozoites invade erythrocytes and develop into ring forms, mature trophozoites, and develop into schizonts before releasing a new generation of infective merozoites. Erythrocytic stage duration varies depending on the transmitted species; it takes 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale*, 72 hours for *P. malariae,* and 24 hours *for P. knowlesi*. The clinical signs and symptoms of malaria become detectable during this stage (White et al., 2014). However, some IE differentiate into gametocytes, the vector transmission stage. The signal for development to gametocytes is not understood (Liu et al., 2011) but may involve density dependent mechanisms. Indeed, preventing disease symptoms is one of the targets for malaria management programmes. Different studies have been extensively conducted to explore proper targets for alternative therapeutic interventions due to the spread of drug resistance to the available drugs in the field and lack of effective vaccines (Miller et al., 2013). Targeting the erythrocytic stage is essential not only to prevent the disease but possibly to inhibit transmission (or at least the production of gametocytes) as well. Therefore, the focus of the following sections is to highlight some alterations occuring to erythrocytes upon infection with *P. falciparum* and the contribution of these alterations to malaria pathogenesis.

1.3.3.1 Erythrocytes:

An erythrocyte is about 7-8 µm diameter, often considered as a simple cell that lacks organelles and transports oxygen and carbon dioxide by haemoglobin that occupies most of the cell (Fowler, 2013). In addition, it has a plasma membrane with uniform structure across the whole surface. The erythrocyte plasma membrane is strong and flexible to allow the movement smoothly through blood vessels (Mohandas and An, 2012, Fowler, 2013). The plasma membrane is supported by the membrane

skeleton, which is a network of long strands of α1 and β1 spectrin tetramers , interconnected by actin filaments forming a 2D hexagonal array (Fowler, 2013). Erythrocytes are regularly filtered in the spleen, thus, they are removed from the circulation often when they are over age of the life span of about 120 days, or the membrane loses its ability to move in the circulation (Mebius and Kraal, 2005).

1.3.3.2 Infected Erythrocytes (IEs):

Changes to the erythrocyte membrane are thought to take place soon after the initial contact between an erythrocyte and parasites (Hanssen et al., 2010b). The time estimated between the released merozoites to come in contact with erythrocytes is in the order of a few seconds. Merozoites have apical organelles that secrete products to promote host-cell invasion (Sam-Yellowe, 1996). Erythrocyte invasion by *Plasmodium* merozoites involves multi-step interactions between the parasite and erythrocytes by specific proteins. Initially, merozoites adhere to the erythrocyte plasma membrane in a reversible process. The adherence involves different receptors on erythrocytes and parasites; proteins on parasites at this stage are termed Merozoite Surface Proteins (MSPs) (Farrow et al., 2011). For example, Erythrocyte Binding Antigen (EBA) and Rhoptry (Rh) families are the most important families among MSP families. In two studies in 2010, EBA175 was found to bind sialic acid residues of Glycophorin A on the erythrocyte surface but sialic acid was not involved in binding between PfRh4 and complement receptor 1 (CR1) (Spadafora et al., 2010, Tham et al., 2010). After the initial binding, the merozoite reorients itself so that the apical tip points towards the erythrocyte. Then, the tight junction between merozoites and erythrocytes is formed and the attachment becomes irreversible at this stage. This process is facilitated by the interactions of two proteins MTRAP and AMA1. This stage is completed by a fully enveloped merozoite within a parasitophorous vacuole inside the erythrocyte, but it is still attached to the erythrocyte plasma membrane. The final step is the migration of the tight junction complex towards the erythrocyte. It is

assumed that the orthologous myosin, *Pf*MyoA, is responsible for entry of *P. falciparum* merozoite into the erythrocyte (Farrow et al., 2011). Then, the parasite develops within a parasitophorous vacuole (PV) and it is freely motile inside the vacuole. After 12 hours, the parasites differentiate to the ring form and can be seen in peripheral blood (Langreth et al., 1978, Bannister et al., 2000, Tilley et al., 2011).

1.3.4 Intraerythrocytic Developmental Cycle of *P. falciparum*:

During the cycle, the parasite needs to consume nutrients and then discard waste substances. This is not easily carried out inside erythrocytes that lack essential organelles. The parasite has to fulfil its growth requirements by exporting numerous proteins to the host cell cytoplasm and subsequently, in later stages, to the membrane (Bannister et al., 2004, Hanssen et al., 2010b). Several proteins are exported including kinases, lipases, proteases, chaperone-like proteins, and adhesins. This eventually remodels the IE and in turn reduces the IE membrane deformability and increases the permeability of the host cell membrane (Goldberg and Cowman, 2010, Glenister et al., 2002, Maier et al., 2009).

1.4 Host cell remodelling:

IEs remodelling is mediated by an array of diverse parasite-encoded export proteins that traffic within IE. These remodelling proteins extensively modify the cytoskeleton and membrane of IE and help in the formation of parasite-induced novel organelles such as 'Maurer's Cleft, tubulovesicular network, and parasitophorous vacuole membrane (PVM); these organelles have been called exomembrane system (Hanssen et al., 2010a). Of these organelles, Maurer’s Clefts have been described to have a role in the export of virulence proteins (Tilley and Hanssen, 2008).

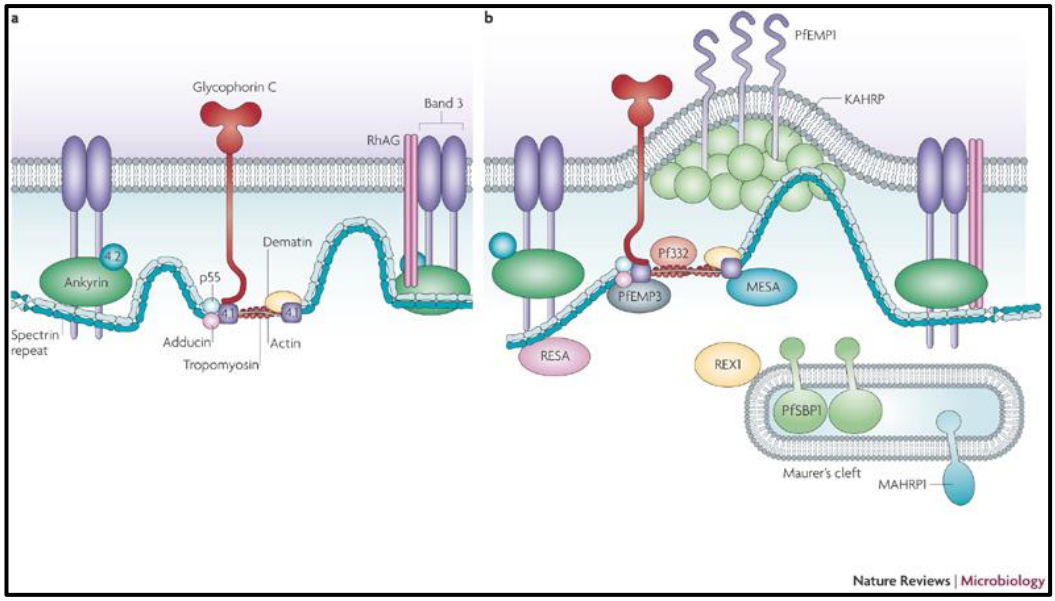

Figure 1.3: The membrane skeleton in uninfected and Plasmodium falciparum-infected red blood cells. a) Uninfected erythrocytes. Spectrin dimer repeat units form tetramers by joining actin and are stabilised by protein 4.1R and other molecules and anchored vertically by two junctions. b) Infected erythrocyte. In the early stage, RESA is linked with spectrin to stabilise the membrane skeleton. In later stage, KAHRP interacts with spectrin with other molecules that might be involved in PfEMP1 exportation to surface of IEs, which is demonstrated in the next figure. The figure is cited from (Maier et al., 2009).

1.4.1 Maurer's Clefts:

One of the the major components of the exomembrane system is Golgi-like organelles called Maurer's Clefts. The Maurer's Clefts were first characterised by Georg Maurer, using light microscopy in 1902 (Mundwiler-Pachlatko and Beck, 2013). Maurer's Clefts bud from PV membrane into the IE cytoplasm, and they attach to IE membrane by specialised tubular structures (Hanssen et al., 2010a). Although little is known about the function of Maurer's Clefts, they transiently localise some surface proteins prior to the arrival to their final (often membrane) destinations. There is an array of surface exported proteins forming protrusions that are called knobs that mediate significant roles for parasites development, survival and pathogenesis.

1.4.2 Knobs:

The remodelling proteins of *P. falciparum* specifically bind and modify natural interactions of the erythrocyte cytoskeleton proteins. The major remodelling proteins of *P. falciparum* are ring exported surface antigen (RESA), knob-associated histidine rich protein (KAHRP), *Plasmodium falciparum* erythrocyte membrane protein-3 (PfEMP3), mature-parasite-infected erythrocyte surface antigen (MESA) and *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1).

Once the merozoite occupies the erythrocyte, it produces immediately Pf155 protein also called ring-infected erythrocyte surface antigen (RESA). It interacts with spectrin to bind the erythrocyte membrane (Deloron et al., 1987, Aikawa et al., 1990). It has been found that RESA strengthens erythrocyte membrane (Silva et al., 2005, Pei et al., 2007). Several findings suggested different roles for RESA. It was suggested to have a role in enabling the flow of ring infected erythrocytes (RIEs) through capillaries, and can protect IEs from damage caused by fever (Pei et al., 2007). Also, it can protect RIEs from new invasion by other merozoites and some chemicals including antimalarial drugs (Orjih and Cherian, 2013). RESA can be detected by specific antibodies in infected individuals with *P. falciparum* (Kabilan et al., 1994, Genton et al., 2003).

The plasma membrane of IE is interrupted by protrusions known as knobs. One of the main components of the knobs is knob-associated histidine-rich protein (KAHRP). KAHRP interacts with the RBC membrane skeleton to form the knobs (Aikawa et al., 1985, Waller et al., 1999). KARHP alters IE cytoskeleton by interacting with spectrin and actin (Kilejian et al., 1991, Chishti et al., 1992) and also with ankyrin. KAHRP binds to repeat 4 of α-spectrin. Although KAHRP–α-spectrin association has no effect on the membrane mechanical properties, interaction of KAHRP with spectrin is required for the proper assembly of KAHRP into the knob complex found at the erythrocyte membrane (Pei et al., 2005). Recently, the interaction with ankyrin R was found to be necessary for the attachment of KAHRP to the host cell membrane

(Weng et al., 2014). KAHRP accumulates on the cytoplasmic face of the membrane. KAHRP is required for knob formation and thus for efficient IE cytoadherence (Crabb et al., 1997).

1.4.3 PfEMP3:

It is a protein linked to the cytoplasmic face of the RBC membrane in the trophozoite stage of the IE. PfEMP3 interacts with spectrin adjacent to the actin–protein 4.1R junction, disturbing the spectrin–actin–4.1R complex, and it may well contribute to deformability loss in mature IE. It was shown that PfEMP3 and KAHRP account for major rigidity of IE with 51% of lost deformability dependent on these proteins (Glenister et al., 2002).

There are other proteins included in IE remodelling and several reviews have updated the recent findings about IE remodelling (Maier et al., 2009). Although PfEMP3 has no impact for PfEMP1 surface expression, higher expression of a truncated form of PfEMP3 changes the form of Maurer's cleft and subsequently prevents PfEMP1 trafficking (Maier et al., 2009).

1.5 PfEMP1:

The uniqueness of *P. falciparum* virulence among other *Plasmodium* species that infect humans is thought to be due to the expression of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). It was identified in 1984, through the use of radioiodination and immunoprecipitation with isolate specific immune sera (Leech et al., 1984). PfEMP1 is a variable, high molecular weight and trypsin sensitive protein. PfEMP1 was immunoprecipitated by strain specific sera from a homologous strain but not from others. Moreover, the binding of homologous IE to endothelial cells was blocked by strain specific sera as reviewed by (Sherman et al., 2003, Kraemer and Smith, 2006). From these observations, it was concluded that PfEMP1 is an immunogenic

molecule that mediates antigenic variation and sequestration (details about these PfEMP1 roles found in section 1.7, 1.8 and 1.9).

PfEMP1 features are summarised in box 1. It should be noted that there are several proteins exported to IE surface including RIFINs and STEVORs, but their roles in malaria pathogenesis are less understood (a brief description about them can be found in later sections).

PfEMP1:

- The most extensively studied of variant surface antigen (VSA).
- A high-MW protein (200-350 kDa)
- Sensitive to trypsin treatment.
- Immunogenic, (immunoprecipitated with immune sera).
- Encoded by highly diverse *var* gene family members.
- Expressed as a single member as commonly known on the IE surface at a given time through mutually exclusive transcription.
- Mediate sequestration in the vital organs.

Box 1: Summary of PfEMP1 key features.

1.5.1 PfEMP1 export to the IE membrane:

There are several alterations that occur in the IE including changes in ion channel behaviour (Decherf et al., 2004, Bouyer et al., 2006), and the development of new channels to enable nutrient delivery to the parasite (Saliba et al., 1998, Biagini et al., 2004), however, these alterations are beyond the theme of this thesis. Changes of interest include cell deformability due to the membrane rigidity (Glenister et al., 2002, Cooke et al., 2014) and the effect on the microcirculation (Diez-Silva et al., 2012, Boddey and Cowman, 2013).

The parasite's proteins have many membranes to cross to reach the surface of IE (Marti and Spielmann, 2013). Two export pathways have been suggested: one is channel mediated and the other vesicle mediated, with the latter thought to mediate PfEMP1 export although the

evidence for this is incomplete. Protein export starts in the parasite endoplasmic reticulum (ER), crossing the parasitophorous vacuole membrane (PVM) approaching two destinations; IE cytosol and cytoskeleton or to membranous structures such as Maurer's Clefts and its outer surface (Prajapati et al., 2014).

Recently, one potential trafficking pathway to the cytosol of IE and its components has been identified and called *Plasmodium* translocon of exported proteins (PTEX) (de Koning-Ward et al., 2009) (Elsworth et al., 2014b). It is responsible for the export of many proteins, although there are other proteins that are transported independently of PTEX and this group is known as PTEX negative exported proteins (PNEPs), among which is PfEMP1 (Boddey and Cowman, 2013).

The export processes are not fully understood but we know several details. It initiates at about 16 h post-invasion. Vesicular budding from Maurer's clefts followed by PfEMP1 fusion with the IE membrane are proposed mechanisms (Boddey and Cowman, 2013). The Maurer's Clefts are generated at an early stage in the erythrocytic cycle and are highly mobile before attaching under the IE membrane by 16 - 20 h after invasion (Gruring et al., 2011, McMillan et al., 2013). It is thought that electron dense filaments called tethers lead to this attachment between the Maurer's Clefts and the IE membrane (Hanssen et al., 2010a, Elsworth et al., 2014a). Also, as mentioned above, alteration of host actin contributes to Maurer's Clefts immobilization. One tether protein is called MAHRP2 and there may be some other unidentified proteins contributing to this process (McMillan et al., 2013). The trafficking of PfEMP1, KAHRP and PfEMP3 come through the Maurer's Clefts to the IE adhesive knobs (Wickham et al., 2001). Some reports have found some Maurer's Clefts proteins including PfEMP1 are soluble before the membrane localizations. It was supported by the time course between the Maurer's Clefts synthesis and the expression of PfEMP1. The solubility of the exported proteins to Maurer's Clefts suggests a role for chaperones to keep them in this state (Papakrivos et al., 2005, Gruring et al., 2011), for

instance, Hsp40 proteins and Hsp70-x which is only found in *P. falciparum* and the closely related *P. reichenowi* (Kulzer et al., 2012, Elsworth et al., 2014a).

Other Maurer's Clefts proteins have been proposed as essential for correct PfEMP1 transport including PfSBP1 and MAHRP1; two integral membrane proteins (Gruring et al., 2011, McMillan et al., 2013). MAHRP1 is required for correct PfEMP1 entrance to the Maurer's Clefts and also shows a structural function for the Maurer's Clefts (Spycher et al., 2008). Parasites with a SBP knockout mutation failed to traffic PfEMP1 to the IE surface and it is not clear yet whether this caused PfEMP1 not to enter the Maurer's Clefts or disturbed the delivery from Maurer's Clefts to the surface (Cooke et al., 2006, Maier et al., 2007). REX1 and Pf332 are Maurer's Clefts proteins and likewise they are crucial for appropriate localization of Maurer's Clefts and the transport of PfEMP1. Upon their deletion, it has been noticed that Maurer's Clefts were stacked and PfEMP1 transport was decreased (Elsworth et al., 2014a).

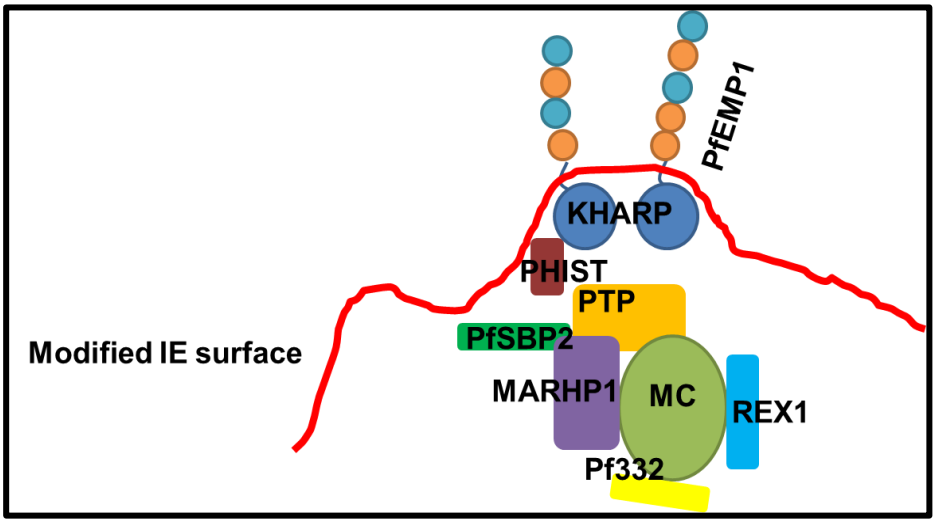

Figure 1.4 PfEMP1 exportation to the surface of infected erythrocytes. The figure demonstrates the structural organelle called Maurer's Clefts (MC), and several proposed proteins that play a role in PfEMP1 exportation to the IE surface; *P. falciparum* skeleton binding protein 1 (PfSBP1), Membrane-Associated Histidine-Rich Protein-1 (MAHRP-1), Ring exported protein 1 (REX1), Pf332, knob-associated histidine rich protein (KAHRP), PfEMP1-trafficking proteins (PTP), Proteins encode *Plasmodium* helical interspersed subtelomeric (PHIST) domains and others.

Proteins encoding *Plasmodium* helical interspersed subtelomeric (PHIST) domains were also involved in PfEMP1 trafficking and IE membrane rigidity (Maier et al., 2008). Mayer et al (2012) showed that a single PHIST protein interacts with the ATS domain of PfEMP1 (Mayer et al., 2012), and it was thought that PHIST proteins were involved in delivery of parasite proteins to the IE membrane (Prajapati et al., 2014). Six PEXEL proteins were also recognized as important for PfEMP1 trafficking called PfEMP1-trafficking proteins 1–6 (PTP1–6). Disruption and inactivation of some PTPs disrupted PfEMP1 export (Rug et al., 2014).

To conclude, it has been shown that a minimal structure is needed for PfEMP1 export and display on IE. This involves; semiconserved head region, a transmembrane domain and cytoplasmic tail (Melcher et al., 2010). Rask *et al.* (2010) predicted that the N-terminus of PfEMP1 is myristoylated in the cytoplasm of the parasite; therefore, this might assist protein trafficking and anchoring the protein in the IE membrane (Rask et al., 2010).

1.6 *Var* genes:

After almost a decade from the molecular identification of PfEMP1, the genes encoding this protein were identified in 1995, and called *var* (for variant), by three research groups (Baruch et al., 1995, Su et al., 1995, Smith et al., 1995). Each parasite genome encodes about 50-60 different *var* genes, that are expressed in a (mainly) mutually exclusive fashion (Gardner et al., 2002, Scherf et al., 1998, Chen et al., 1998, Guizetti and Scherf, 2013). The *var* genes are large (6–13 kb), with extreme sequence diversity so that in theory they could encode proteins of around 200–500 kDa. Most of the *var* genes are located in subtelomeric regions of all 14 chromosomes where the other variant antigen-encoding genes are located, such as the *rif* and *stevor* gene families, with a smaller group located in central regions of the chromosomes (Gardner et al., 2002). Figure 1.5 shows that *var* genes encode structurally typical PfEMP1 that have intracellular exon of one domain, and

multi-domains extracellular exon composed of DBL-α and CIDRα (known as the head structure) and followed by different classification and numbers of DBL and CIDR domains (Smith, 2014, Gardner et al., 2002).

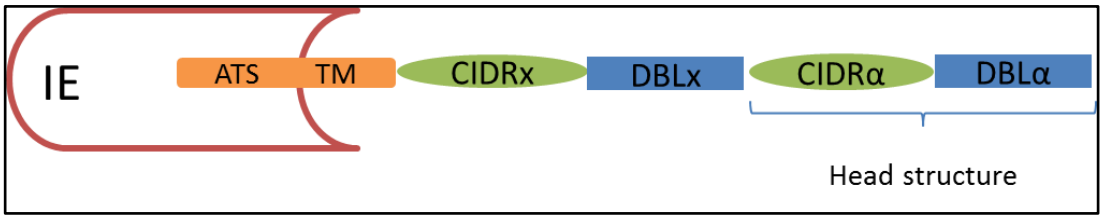

Figure 1.5: PfEMP1 structure: Typical PfEMP1 showing the intracellular exon of one domain followed by the extracellular exon composed of multi-domains: DBL-α and CIDRα known as the head structure followed by different classification and numbers of DBL and CIDR domains. The figure modified from (Smith, 2014).

The most important feature describing *var* genes is the significant sequence diversity. *Var* gene repertoires are virtually unlimited due to extreme levels of *var* gene polymorphism observed over the entire globe (Chookajorn et al., 2007a). *Var* gene evolution has been described as extremely rapid. It was proposed that they do not show stable positions in the genome (Kraemer et al., 2007). Thus, *var* genes sequences are extremely diverse (Barry et al., 2007). The great diversity is due to the ability of individual *var* genes to recombine with other repertoires during the sexual stage in the mosquito abdomen (Babiker et al., 1994, Paul et al., 1995, Su et al., 1999). In addition, there are reports suggesting ectopic recombination events between *var* genes of the same genome during both meiosis and mitosis (Freitas-Junior et al., 2000, Taylor et al., 2000b).

It has been well established that the most highly conserved domain is DBL-α, and it is found in nearly all PfEMP1s. Based on DBL-α, primers were designed (Kyes et al., 1997) and optimized (Taylor et al., 2000a) to explore the diversity of *var* genes. It was generally found that PfEMP1s are highly diverse in parasites locally as well as from overall the world, thus, underlining the repertoire complexity of the *var* genes (Kyes et al., 1997, Taylor et al., 2000a). More recent work in Indonesia has emphasised this diversity (Sulistyaningsih et al., 2013).

Many reports showed several sequences appeared more frequently than others within individual patients (Kyriacou et al., 2006, Kirchgatter and Portillo Hdel, 2002, Fowler et al., 2002, Conway et al., 1999, Taylor et al., 2000b, Mugasa et al., 2012). In Tanzania, for example, isolates from children with asymptomatic infections and severe malaria were examined to study the *var* gene expression profiles. The findings showed that there is a dominant expression of one particular *var* gene for each isolate with unique sequences. Nevertheless, these dominant sequences were different between isolates. Therefore, Mugasa *et al* (2012) suggested that each parasite retains its individual *var* gene variants and this explains the reason of having multi-exposure does not always protect from disease in subsequent infections (Fowler et al., 2002, Trimnell et al., 2006). Overall various reports emphasised minimal overlaps in the *var* gene repertoires in Africa (Chen et al., 2011) and Mali (Kyriacou et al., 2006).

Despite the tremendous diversity, the majority of *var* genes can be categorized based on their upstream sequence (Ups), chromosomal location, and direction of transcription into three major groups ups A, B and C and two intermediate groups (B/A and B/C). UpsA and ups B genes are subtelomeric genes that are oriented tail to tail, whereas ups C genes are found in the centre of the chromosomes and are oriented head to tail in a tandem repeat manner (Kraemer and Smith, 2003, Lavstsen et al., 2003) (Figure 1.6).

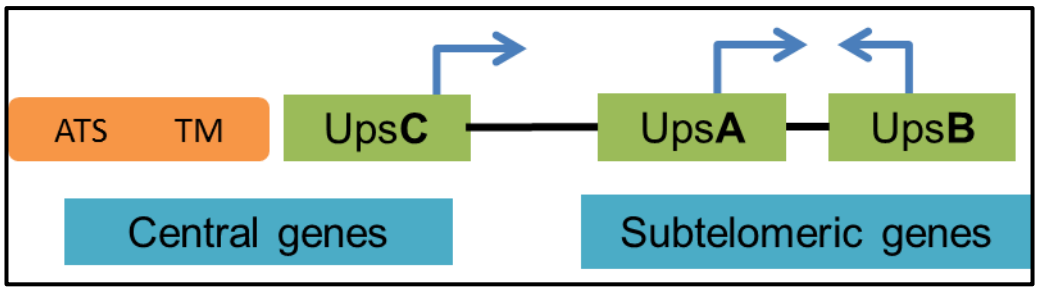

Figure 1.6 *Var* genes classifications. *Var* genes can be classified by their chromosomal location and upstream promoter (Ups) type into subtelomeric ups A and B and the central ups C.

Rask *et al* (2010) redefined the homology blocks (HBs) concept that was initially described by Smith *el al* (2000) to establish other classifications of PfEMP1 based on domain cassettes (DC). The analysis was performed using data from seven genomes of *P. falciparum* including 3D7, IT4 and HB3. They defined DCs as the presence of at least two consecutive domains belonging to specific subclasses and found in at least three examined genomes. Subsequently, very encouraging studies have related some of the DCs to SM including DC8 and DC13 (Avril et al., 2012, Claessens et al., 2012, Lavstsen et al., 2012), DC4 (Bengtsson et al., 2013) and DC5 (Berger et al., 2013).

Another proposed classification of PfEMP1 repertoires divided them into long and conserved (mainly Ups group A) and short and diverse (mainly Ups group B and C) (Buckee and Recker, 2012). This latter classification was suggested after a significant, non-random link between the domains composing *var* genes and the extent of their sequence conservation. Most of the PfEMP1s have a tandem DBL-CIDR domain at the N-terminus, known as the semi-conserved head structure. Short PfEMP1s have an extra DBL and CIDR to form four domain extracellular units. In contrast, long PfEMP1s have some other domains. Understanding the *var* gene diversity and classifications can be significant for designing vaccine and chemotherapies that target clinical malaria outcomes.

Highly diverse PfEMP1s, mainly group C and some of group B, are more associated with mild disease or asymptomatic infection (AM). Field studies have attempted to find a proper association between *var* gene expression and disease manifestation in different parts of the world (Jensen et al., 2004, Kyriacou et al., 2006, Rottmann et al., 2006, Bertin et al., 2013). It was shown that the diversity was higher in isolates from AM patients. This finding was suggested due to the enormous *var* genes repertoire associated with asymptomatic infection as reported by occurrence of more singletons in isolates from AM.

On the other hand, isolates from severe malaria children mostly transcribe *var* group A that has DBL-1α domain with reduced number of cysteine residues (2-cys) in Brazil (Kirchgatter and Portillo Hdel, 2002), Kenya (Bull et al., 2005), Mali (Kyriacou et al., 2006) and Tanzania (Rottmann et al., 2006). Consequently, severe malaria is thought to be restricted to a subset of *var* genes (Jensen et al., 2004, Rottmann et al., 2006, Avril et al., 2012, Claessens et al., 2012) not only that but limited subsets could be specifically accumulated in vital organs such as brain (Montgomery et al., 2007, Tembo et al., 2014). *In vitro*, 3D7 selected on pooled plasma from semi-immune children from Ghana and Tanzania (Jensen et al., 2004) showed up-regulated *var* genes were among the Group A *var* group that encode high molecular weight PfEMP1. Also, there was one Group B/A *var* was up-regulated as well, which also encodes a relatively large PfEMP1 with a complex domain structure. On the other hand, group C members were two-thirds of the down-regulated genes, and they encode relatively small PfEMP1 proteins. In Tanzania, for example, children with cerebral malaria had group A *var* genes up-regulated (Mugasa et al., 2012). Recent studies have linked the expression of PfEMP1 encoded by ups A and ups B/A with children with CM.

The diversity of the *var* gene family has challenged the identification of particular PfEMP1 variants involved in the sequestration during CM. However, the expression of a restricted subset of PfEMP1 has been proposed for causing severe malaria based on DCs as previously mentioned. It has been shown that *var* genes encoding DC8 and DC13 were highly transcribed in children who had CM in Tanzania (Lavstsen et al., 2012). two other joint studies found that *P. falciparum* isolates expressing DC8 PfEMP1 had significant binding to brain endothelial cells compared to other PfEMP1 (Avril et al., 2012, Claessens et al., 2012). Also, Bengtsson *et al.* (2013) found that antibodies to DC4 were cross-reactive with group A PfEMP1 (Bengtsson et al., 2013). In addition, DC5 containing var genes were implicated with severe malaria children in Tanzania (Berger et al., 2013).

1.6.1 Var gene evolution:

Although the *var* gene repertoire is highly diverse, in the sequenced genomes to date, they share three conserved variants (*var1*, *var2csa*, and *type3 var*). The occurrence of frequent recombination has contributed to the fact that it is difficult to examine *var* gene evolution. However, it was thought that mosaic recombination could have had a vital (negative) role in the evolution of the *var2csa* gene, a special member which has been associated with pregnancy-associated malaria (PAM) (Trimnell et al., 2006, Bockhorst et al., 2007). *Var2csa* led to this finding because its uniqueness in the genome and it recombines homologously only, although, its basic composition is still relatively similar to other *var* gene family members (Ferreira et al., 2007, Zilversmit et al., 2013).

The origin of *P. falciparum* itself is still an area of doubt (Prugnolle et al., 2011). Recent findings go against earlier thoughts proposed in 1991 that *P. falciparum* acquired its virulence due to recent adaption in humans from avian malaria species (Waters et al., 1991). The former thought was challenged by the finding that *P. falciparum* diverged about 6-10 million years ago from the chimpanzee malaria *P. reichenowi* (Escalante and Ayala, 1995).

P. reichenowi, which also encodes *var* genes (Rask et al., 2010), was included in genomic analysis together with *P. falciparum* isolates by Trimnell *et al.* (2006). They found that despite the conserved *var2csa* ortholog in all *P. falciparum* isolates, *var2csa* is highly polymorphic in comparison with other non-*var* genes. However, the unpredicted finding was the sequence similarity of the *var2csa* ortholog in the chimpanzee malaria *P. reichenowi*. Thus, it was suggested that *var* genes have co-evolved for a long time with primates from an ancient origin about 6-10 million years ago. In contrast, the same study analysed *Type 3 var* genes, the most highly conserved gene among upsA *var* genes, and confirmed *Type 3 var* orthologs in all *P. falciparum* isolates with one exception, which were not found in *P. reichenowi* (Trimnell et al., 2006).

Up to date, there are at least six divergent *falciparum*-like species that infect African Great Apes (chimpanzees and gorillas) (Prugnolle et al., 2011) but the knowledge about PfEMP1 protein functions in non-human primate is very limited. However, Smith *et al.* (2013) have highlighted the importance of the co-evolution of *var* genes with primates so that it could improve our understanding of malaria pathogenesis in terms of the adaption of cytoadhesion traits. Experts believe that the investigation of ancestral PfEMP1 binding properties might be based on advantages for parasite growth and transmission (Smith et al., 2013).

1.6.2 The structure and the shape:

Currently, there are only two available PfEMP1-ligand structures. Recent studies have shown that full-length PfEMP1 ectodomains have different shapes. For example, ITvar13, an ICAM-1 binder, has a rigid extended structure and using a single domain to bind to ICAM-1 (Brown et al., 2013). In contrast, VAR2CSA forms a compact structure by folding back on itself using a single domain to bind with high affinity to its ligand CSA (Srivastava et al., 2010). VAR2CSA is composed of six DBL domains and a single CIDRpam domain, whereas IT4VAR13 follows a more typical structure of PfEMP1. It was suggested that PfEMP1s have at least two different shapes. Indeed, the development of therapeutic interventions targeting appropriate antigens needs more details about the molecular mechanisms of PfEMP1 recognition of host receptors to mediate sequestration, immune responses and possibly to limit the variation that needs to be incorporated.

1.7 Antigenic variation:

The parasitic microorganism faces several challenges before it reaches a 'secure' position in the host. It starts by avoiding mechanical clearance, immune recognition and destruction after immune responses. Thus, some pathogens have had to evolve sophisticated strategies to ensure possible lifelong existence in hosts and high infectivity for species continuity. Among the mechanisms that contribute to this is antigenic variation, which is defined as "the changes of the molecules of the parasites exposed to the immune system over the course of an infection". (Deitsch et al., 2009). Therefore, it challenges the host to target such populations and importantly the host might not even be able to eliminate the organism, as seen in most of the *P. falciparum* infections (Pasternak and Dzikowski, 2009). As mentioned above, perhaps unusually for an intracellular parasite, many proteins are transported to the surface of IE during the development inside erythrocytes. However, only a small subset of their encoding genes is expressed at a given time. In the case of *var* genes, only a single gene among the whole repertoire is transcribed whereas all the others are silenced. This is known as mutually exclusive expression (Roberts et al., 1992, Smith et al., 1995), also called monoallelic expression, and it the most extreme form of clonal variation (Scherf et al., 1998). Single *var* gene transcription begins at the ring stage soon after merozoite invasion of the erythrocytes (Figure 1.7). Then, after about 16 hrs, it becomes in the poised state during trophozoite and schizont stages ready for activation in the following cycle. Then, *var* gene expression can switch to another variant over time which causes alterations in immune responses and adhesion phenotypes. Mutually exclusive expression is controlled by genetic and epigenetic control factors. In a recent review by Guizetti and Scherf (2013), they outlined *var* gene expression in *P. falciparum* in four stages; default silencing, activation, poised state and switching (Guizetti and Scherf, 2013).

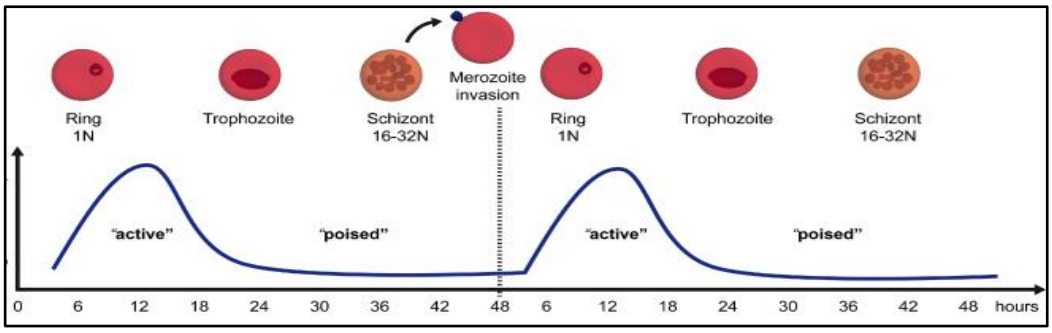

Figure 1.7: *Var* gene activation and silencing throughout asexual blood stage development of *P. falciparum*. A. single *var* gene transcription begins at the ring stage soon post merozoites invasion of the erythrocytes reaching the peak at 12-16 hrs post inasion. Then, it becomes in the poised state during trophozoite and schizont stages ready for activation in the following cycle. The figure is cited from (Guizetti and Scherf, 2013).

Var gene silencing involves three factors; epigenetic elements, nuclear arrangement and intron role. First, studies have indicated that plasmodial *Sir2* genes have an effect on the chromatin of clonally variant gene families (Duraisingh et al., 2005, Tonkin et al., 2009). Histone deacetylation by *Sir2* might allow the formation of Histone 3 lysine 9 trimethylation (H3K9me3), the silencing heterochromatin mark which is heavily found in promoter regions of repressed *var* genes (Chookajorn et al., 2007b). H3K9me3 histone modification stimulates heterochromatin formation through *P. falciparum* Heterochromatin Protein 1 (PfHP1) to silent but not active *var* genes (Perez-Toledo et al., 2009). Second, a striking feature of *P. falciparum* virulence is the nuclear arrangement (Scherf et al., 2008). *Var* genes tether at the nuclear periphery by an unknown mechanism (Lopez-Rubio et al., 2009, Ralph et al., 2005). The attachment of *var* genes at the periphery is suggested to be fundamental for default silencing (Guizetti and Scherf, 2013). This was explained by impairing perinuclear localization of intron-carrying *var* genes, which led to partial de-repression. Third, the intron and the *var* promoter mediate a significant role in control of silencing. The intron silences expression by one-to-one pairing with *var* promoter while unpaired intron activates the respective promoter (Deitsch et al., 2001, Frank et al., 2006).

The activation of a *var* gene, likewise, involves different factors. The activation starts by replacing H3K9me3, the suppression marker, by Histone 3 lysine 4 bi- and tri-methylation (H3K4me2/3) and Histone 3 lysine 9 acetylation (H3K9ac) (Lopez-Rubio et al., 2007). Dependently, PfHP1 loses the association with the 5′ ups in the active gene (Perez-Toledo et al., 2009). Another modification includes Histone 4 lysine acetylation which has a role in transcriptional activation, and it is regulated by a histone acetyltransferase MYST member called *Plasmodium falciparum* MYST (PfMYST) (Miao et al., 2010). In addition, there is an association between the enhanced transcriptional activities with the histone variant H2A.Z around the transcription start site during ring stages, while it is reduced in later stages and frequent removal might lead to different *var* gene activation in the subsequent cycle (Guizetti and Scherf, 2013). Moreover**,** in a recent study, it has been found that most of the *var* genes contain an important motif for mutual exclusive expression. It is an eight-base pair sequence motif found in the 5′ UTR known as mutually exclusive element (MEE). This was suggested to be the limited activating factor binding site (Guizetti and Scherf, 2013).

It was suggested that a single *var* is transcribed early at ring stage and stops at about 12-16 hrs but remains in a state called poised state to be activated on the following erythrocytic cycle (Figure 1.7). The mechanism of the movement from active to the poised state is not yet understood. It has been described that a putative methyltransferase, PfSet10, has been specifically linked with the poised *var* gene (Guizetti and Scherf, 2013).

Our understanding of the molecular mechanisms of *var* gene switching is still limited. There is no specific point known for the control of switching events in cell cycle. Variable *var* gene switching rates of isolates *in vitro* (Roberts et al., 1992) differ from those seen in human volunteers (Peters et al., 2002). Horrocks *et al*. (2004) showed that each *var* gene owns its own rate of switching (Horrocks et al., 2004).

Recently, mathematical modelling alongside experimental data concluded that *var* switching is non-random and involved highly

structured switching patterns, and this might adjust the length of infection (Recker et al., 2011). Interestingly, the pattern of *ups A var* gene expression in *P. falciparum*-infected patients changed in *in vitro* cultured parasites to low activation rates and being random in ups B and C parasites. It was suggested that the intrinsic switch rate might be affected by host factors (Guizetti and Scherf, 2013).

There was no preferential expression observed in the Horrocks *et al*. (2004) study. However, later reports proposed that central *var* genes switch off slower than sub-telomeric *var* genes, and this could explain the quick drop in the sub-telomeric gene transcription during culture adaptation (Frank et al., 2007, Peters et al., 2007, Zhang et al., 2011). Recent analysis to characterise the antigenic switching network in HB3 *P. falciparum* has shown that *var* gene activation followed a global hierarchy favouring towards highly diverse genes located in the central chromosomes (Noble et al., 2013).

1.8 Immunity:

Although, it has been more than 50 years since Cohen et al. (1960) showed the significant role of antibodies to mediate immunity to intra-erythrocytic stage malaria (Cohen et al., 1961), the immunity to this stage is not yet fully understood. However, among the variant surface antigens (VSA) of *P. falciparum*, PfEMP1 is a prime target of protective antibodies, reviewed with other VSA in (Chan et al., 2014). The transmission intensity has been shown to play a major role in acquiring immunity (Nielsen et al., 2004). On the one hand, individuals in endemic areas acquired resistance to severe malaria manifestations by about the age of five years. However, they keep suffering from mild malaria due to exposure to repeated infections before achieving almost protective immunity to the clinical disease by adulthood. On the other hand, in low-transmission areas all individuals at different ages are susceptible to malaria manifestations (Doolan et al., 2009). However, no clear

understanding of the mechanism of antibody-mediated protection is available (Crompton et al., 2014).

The gradual acquisition of protective antibodies could be due to the high diversity of VSA as it is been shown above for PfEMP1 and antigenic variation. Thus, the role of PfEMP1 in immunity will be briefly highlighted here, while considering that other VSA are involved in the intra-erythrocytic stage immunity, which can be seen in a recent review (Chan et al., 2014). Recent evidence showed that PfEMP1 is a major target of malaria humoral immunity in endemic regions (Chan et al., 2012). This supports an earlier observation by Bull *et al.* (1998) that frequent *P. falciparum* infections might provoke the production of specific antibodies to PfEMP1 (Bull et al., 1998). But, the issue is that *P. falciparum*–specific antibodies, in general, disappear or almost become undetectable for the recorded infections by about 3–9 months in young children (Akpogheneta et al., 2008, Portugal et al., 2013).

Plasmodium plays a role in dysregulation of $CD4^+$ T cell and B cell functions to evade humoral immunity. It was suggested that the response in children is mediated by short-lived plasma cells rather than long-lived plasma cells (LLPCs). The repeated infections that build gradual protection could be due to the role of LLPCs. The role of memory B cells (MBCs) is doubtful but recent studies support the ability of *P. falciparum* to produce long-lived MBCs, though they are relatively inefficient compared with other pathogen responses (Crompton et al., 2014).

It is possible that Pattern Recognition Receptors on B cells and DCs enhance B cell responses due to the chronic exposure to PAMPs of *P. falciparum*. For example, TLR9 agonist CpG enhances the IgG and MBC response in naïve adults (Alcais et al., 2010), but not frequently exposed adults (Jallow et al., 2009). Also, TLR2 through GPI mediates signals to activate NF-KB and the production of inflammatory cytokines, including TNF, and adhesion molecules such as ICAM-1 which contribute to malaria pathogenesis through a vital process known as sequestration (Krishnegowda et al., 2005, Gowda, 2007).

1.9 Sequestration:

Sequestration is another virulence mechanism besides antigenic variation that protects *P. falciparum* from immune detection and destruction and ensures prolonged survival for the species. It occurs at the mature stages of the asexual intra-erythrocytic cycle of *P. falciparum* and this explains their disappearance from the circulation due to their ability to localise to different organs such as brain. Craig *et al* (2012) highlighted several hypotheses that have associated the sequestration with the disease manifestations (Craig et al., 2012b). One reason may be that abnormal physiological changes occur to IEs, such as their rigidity (Dondorp et al., 1999, Dondorp et al., 2002), the other is the expression of adhesion-receptors in response to the pro-inflammatory mediators (Armah et al., 2005, Clark et al., 2008), also, toxin production (Schofield et al., 1996), and endothelial activation (Chakravorty et al., 2008, Hollestelle et al., 2006, Moxon et al., 2014). In addition, the impact of coagulation pathway in mediating IEs binding to ECs (Francischetti, 2008, Moxon et al., 2013, Turner et al., 2013) and binding of IEs to specific adhesion receptors on endothelial cells (Ochola et al., 2011, Newbold et al., 1997).

Sequestration was initially described when the Italian malariologists Marchiafava and Bignami saw high parasites density in malignant malaria patients compared with benign malaria patients. They described that in post-mortem there were high accumulations of parasites with predominant parasite pigment occupying the tissue microvascular in comparison with circulating parasites in the peripheral circulation. They also reported cerebral endothelium dysfunction (Craig et al., 2012b). Since then, there has been some advance in our understanding of some of the key events that facilitate sequestration and importantly could provide possible targets to develop either inhibitors or vaccines (Rowe et al., 2009). PfEMP1 is generally accepted as the key parasite surface molecule that mediates sequestration with multiple human receptors. Figure 1.8 illustrates the binding of IEs that occurs with several receptors

of endothelial cells, uninfected erythrocytes or platelets, which are variably associated with the severe malaria in phenomena known as cytoadhesion, rosetting and clumping respectively (Rowe et al., 2009). The binding of IE also occurs on receptors on other cells including dendritic cells, monocytes and macrophages to modulate host immune functions (Chua et al., 2013). The most common receptors that mediate the binding are CD36 (Barnwell et al., 1989, Oquendo et al., 1989), ICAM-1 (Berendt et al., 1989), and EPCR (Turner et al., 2013). In addition, more than ten other receptors have been identified as being involved in the cytoadhesion.

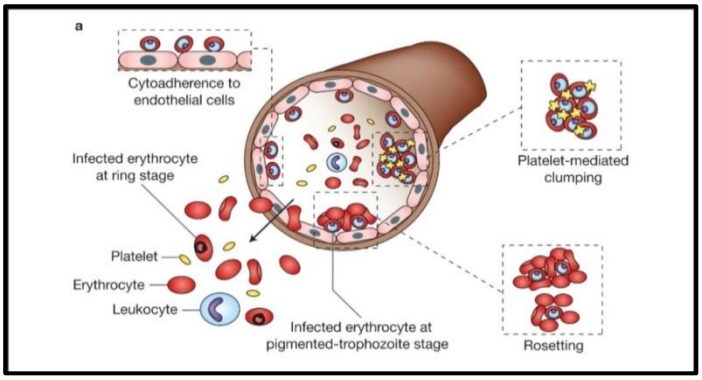

Figure 1.8: Adhesion of *Plasmodium falciparum*-infected erythrocytes to human cells. The diagram demonstrates the adhesion properties of *P. falciparum*-infected erythrocytes to different host cells. IEs with trophozoites and schizonts of *P. falciparum* parasites have the ability to bind to a range of host cells, such as endothelium (cytoadherence), uninfected erythrocytes (rosetting) and platelets (platelet-mediated clumping). The figure has been cited from (Rowe et al., 2009).

1.9.1 CD36:

CD36 is an integral membrane protein expressed on a variety of host cells including endothelium and platelets (Rowe et al., 2009). CD36 is involved in immune responses in humans (Febbraio et al., 2001, Greenwalt et al., 1992), platelet adhesion (McGilvray et al., 2000) and the regulation of membrane transport systems. CD36, as stated before, is a common receptor for almost all *P. falciparum* isolates in field studies, though there are important exceptions such as *var2csa* expressing isolates do not bind to CD36 (Fried and Duffy, 1996, Beeson et al., 2000).

In terms of clinical association, there is no certain role for CD36 in malaria pathogenesis (Rowe et al., 2009). CD36 adhesion phenotype is seen for IE from severe or uncomplicated malaria patients (Newbold et al., 1997, Rogerson et al., 1999). Other evidence showed it is more linked to uncomplicated malaria isolates (Ochola et al., 2011). In contrast, a very recent study conducted on patients from Benin found that isolates from CM patients bind to CD36 more than isolates from UM (Almelli et al., 2014). In another study, CD36 adhesion enabled protection from CM in South East Asia (Cortes et al., 2005). Also, it has been proposed that CD36 might provide protection against anaemia caused by malaria (Chilongola et al., 2009). Group B and C PfEMP1 variants are more associated with binding to CD36 (Robinson et al., 2003, Kraemer and Smith, 2006, Cabrera et al., 2014), which might support a link to UM. The binding site for *P. falciparum* was mapped to amino acids 139-184 of CD36 using blocking studies by monoclonal antibodies and peptides (Rowe et al., 2009). The binding to CD36 is reduced in CD36-deficient malaria patients, nevertheless, CD36 deficiency does not protect against severe malaria (Fry et al., 2009).

In addition, the role of CD36 as a receptor for rosetting has been restricted to laboratory isolates of *P. falciparum*, and this role is probably not clinically important based on field isolates because the group A PfEMP1 variants that are more associated with rosetting do not bind to CD36. In addition, a CD36 dependent clumping mediated phenotype was proven by inhibition with anti-CD36 antibodies, and by the absence of clumping by CD36-deficient platelets. However, not all CD36 binders can form clumps, which might be explained by either more receptors required for clumping or CD36 having distinct epitopes that can differentiate CD36-mediated clumping (Rowe et al., 2009).

Some CIDR1 domains in the PfEMP1 head structure have been shown to bind CD36 (Baruch et al., 1997, Smith et al., 1998), and the majority of PfEMP1 variants encodes CIDR1 domains (Robinson et al., 2003).

1.9.2 ICAM-1:

Another commonly used receptor is ICAM-1. It has a cytoplasmic tail, a transmembrane domain and five extracellular Ig-like domains (van de Stolpe and van der Saag, 1996, Chakravorty and Craig, 2005). The central role of ICAM-1 on endothelial cells is allowing leukocyte transmigration from the circulation to the tissues in inflammatory sites induced by cytokine stimulation. This is mediated by the binding to leukocyte receptors such as leukocyte function-associated antigen (LFA-1) or macrophage-1 antigen (Mac-1). ICAM-1 also mediates binding to pathogenic organisms, such as human rhinoviruses (HRVs) (Greve et al., 1989, Staunton et al., 1989) and *P. falciparum* infected erythrocytes (Berendt et al., 1989). Early studies found that ICAM-1 binding sites for IE, rhinoviruses, LFA-1 and fibrinogen are overlapping, but also have distinct regions (Berendt et al., 1992, Ockenhouse et al., 1992).

Similar to CD36, the involvement of ICAM-1 in malaria pathogenesis is not clear. However, there are various evidences supporting the involvement of ICAM-1 in SM, including CM. Initially, in 1994, it was found that post-mortem samples taken from people diagnosed with CM showed accumulation of IE co-localised with ICAM-1 in brain vessels (Turner et al., 1994). Additional lines of evidence of the involvement of ICAM-1 in CM can be found in chapter (3.1) (Madkhali et al., 2014). The interaction between IE and ICAM-1 involves the BED face of ICAM-1, including the DE loop (Tse et al., 2004) and DBLβ domains of PfEMP1 (Smith et al., 2000a, Howell et al., 2008). The results were observed from ICAM-1 binders Group A PfEMP1s; including PFD1235w, Dd2var32 (Jensen et al., 2004) and PF11_0521 (Oleinikov et al., 2009, Gullingsrud et al., 2013).

1.9.3 Endothelial protein C receptor (EPCR):

EPCR is expressed at relatively low levels in microvascular endothelium of many organs including brain (Moxon et al., 2013). It is encoded by the endothelial protein C receptor gene (PROCR). It acts as a receptor for the zymogen, protein C in the process of protein C activation regulating coagulopathy protection. It is also involved in anti-inflammatory responses via the signalling receptor PAR1. It also binds to Mac-1 (CD11b/CD18) leading to monocyte adhesion to ECs. As mentioned above ICAM-1 is able to bind to Mac-1, thus, both can bind to Mac-1 and PfEMP1. It was suggested that architectural similarities may exist between the two surface of Mac-1 and PfEMP1 (Aird et al., 2014).

Recently, EPCR has been shown to be involved in CM by two different studies. First, Moxon *et al* (2013) have addressed the role of EPCR post-mortem in children that had died of cerebral malaria. The study showed that IE at endothelial sites co-localized with loss of EPCR (Moxon et al., 2013). They also showed that children with CM had higher levels of soluble EPCR in CSF, however, the plasma level was not altered compared to other malaria syndromes, which suggests that EPCR local loss associated with sequestration mediates cerebral coagulation disruption and inflammation. Another study examined the binding of EPCR (Turner et al., 2013) to specific PfEMP1 variants that have been recently associated with SM (Avril et al., 2012, Claessens et al., 2012, Lavstsen et al., 2012). The study examined the binding of PfEMP1 containing DC8 to EPCR on endothelial cells. It was found that DC8 containing IE were adherent to the endothelial cells of many organs including the brain and that this was mediated by EPCR. This finding was confirmed by significantly higher binding to EPCR by parasites isolated from severe malaria patients when compared to the parasites isolated from mild or uncomplicated malaria patients. DC8 is a tandem of four domains DBLα2- CIDRα1.1- DBLβ12- and DBLγ4/6- domains, and DC13 (also associated with SM) have the tandem of DBLα1.7-CIDRα1.4. Analysis using surface plasmon resonance showed that EPCR pre-

incubation with either DC8 or DC13 CIDR domains blocks EPCR-APC interaction, showing that DC8 and DC13 domains use the APC binding site. The EPCR binding site has been mapped to CIDRα1. A mutation of PROCR called rs867186-G allele has been linked to the elevated sEPCR in plasma. In this study, the association of PROCR rs867186 genotype was shown to protect from severe malaria examined in a Thai population (Naka et al., 2014).

1.9.4 PECAM-1:

Platelet endothelial cell adhesion molecule 1 (PECAM1 or CD31) is widely expressed on platelets, endothelial cells, monocytes and granulocytes. The PECAM1 binding site for IEs has been mapped to the first four immunoglobulin-like domains of PECAM1 (Rowe et al., 2009). Two PfEMP1 domains were previously shown to bind to PECAM-1; CIDR1α and DBL2δ (Chen et al., 2000). It was reported that about 50% of field isolates from Kenya bound to PECAM1 but there was no significant association between PECAM1 binding phenotype and severe malaria (Heddini et al., 2001). In addition, polymorphism studies showed no protection role for PECAM1 against severe malaria in Africa. On the other hand it increased the risk of cerebral malaria in Thailand (Rowe et al., 2009). Recently, PECAM1 binding was assigned to PfEMP1 containing DC5, but other PfEMP1 variants were not excluded. DC5-PECAM1 binding was associated with severe malaria in Tanzanian children, although the numbers examined were limited. Thus, it was suggested that DC5-PfEMP1 expressing parasites can mediate severe malaria. It was also shown that protection from malaria fever and anaemia was associated with anti-DC5 antibody levels (Berger et al., 2013).

1.9.5 CSA:

Pregnancy associated malaria (PAM) is characterised by considerable accumulation of IEs and monocytes in the placenta (Walter et al., 1982).

Despite the fact that adults develop clinical immunity in endemic areas, women become at risk to SM again their primigravida. The adhesion of IEs to the placenta is mediated by CSA and VAR2CSA PfEMP1 members. Then, antibodies are acquired against VAR2CSA and women acquire protection against PAM in the following pregnancies. In fact CSA is the best representative for specific usage by distinct PfEMP1 members leading to specific malaria outcome, PAM. This example has opened the opportunity to identify certain members for other malaria syndromes such as CM.

1.9.6 Other characterised receptors:

1.9.6.1 Thrombospondin:

Thrombospondin (TSP) is an adhesive glycoprotein found in plasma once thrombin activates platelets. Although it was the first molecule identified as a receptor for *P. falciparum* cytoadherence, little is known about it in malaria pathogenesis. Three different ligands have been identified binding TSP; PfEMP1, altered Band 3 protein, and red-cell-derived phosphatidylserine (a membrane phospholipid). TSP binding was not associated with severe malaria from Kenyan isolates (Rowe et al., 2009).

1.9.6.2 Heparan sulphate:

Evidence has shown heparin binding is involved in SM in Kenya (Rowe et al., 2009). The glycosaminoglycan heparan sulphate was described as a dual receptor responsible for rosetting and subsequently the binding of rosetting forms on ECs (Vogt et al., 2003). It was recently shown that rosetting and cytoadherence are mediated by different receptors for one isolate. Heparin cytoadhesion was strongly mediated by NTS-DBLα and DBL2γ domains, whereas, rosetting was mainly mediated by NTS-DBL1α domains with complement receptor 1, a rosetting mediating receptor. This finding demonstrated that an individual isolate can use different

receptors to mediate IE binding to ECs and uninfected erythrocytes (Adams et al., 2014).

1.9.6.3 VCAM-1, E-selectin and P-selectin:

The binding of field isolates to E-selectin and VCAM1 was very low and no link with disease severity was found. The parasite ligand has not been identified and the binding site on E-selectin is unknown (Schofield et al., 1996). P-selectin binding was found in field isolates. Little is known about the binding sites on both IE and P-selectin. It seems that the binding site is distinct from the site of interactions between P-selectin and leukocytes because antibodies that block this interaction did not show any effect on *P. falciparum* binding. Purified PfEMP1 binding to P-selectin *in vitro* suggests PfEMP1 may be a ligand on IE (Rowe et al., 2009).

1.9.6.4 Other receptors:

Very recently, a group of EC receptors were tested for their binding to a pooled sample of Ghanaian patients isolates (Esser et al., 2014). They used Chinese Hamster ovary (CHO-745) cells transfected with tetraspanins members (CD9 and CD151), multidrug-resistance protein 1 (MDR1), multidrug resistance-associated protein 2 (MRP2), truncated forms of tumour necrosis factor receptors 1 (TNFR1) and 2 (TNFR2) and the erythropoietin receptor (EPOR). The pooled sample bound to all these receptors higher than the lab strain FCR3. Other endothelial receptors have also been identified including fibronectin, integrin αvβ3, NCAM and others, summarised in (Rowe et al., 2009). However, no ligands on IE have been linked to these ECs receptors. Their roles in malaria pathogenesis are also unknown. The involvement of the whole set of these unknown roles in sequestration make it a more challenging task to tackle to inhibit malaria syndromes via adhesion, and could open the question about how the sequestration phenomenon is linked to severe malaria.

1.10 Gametocyte sequestration:

Gametocytogenesis occurs through 5 different stages (I–V) in about two weeks. It was reported early on that developing gametocyte stages (I- IV) are not found in the peripheral circulation whereas the mature stage (V) is circulating for transmission. This sequestration is mainly seen in the spleen and bone marrow unlike asexual stages that bind in the microvasculature (Baker, 2010). Early work suggested that developmental gametocytes (I-IIa) binding to ECs was inhibited by anti-CD36 and anti-ICAM-1 (Rogers et al., 1996). Interestingly, PfEMP1 was proposed to be responsible for hiding the first stages of gametocytes. Thus, it might be a valuable vaccine target for blocking transmission. It has been reported that the transcriptional switching in gametocytes prefers particular type C *var* genes *in vitro,* independent of the expressed PfEMP1 in the asexual forms (Sharp et al., 2006). This could exclude the role of PfEMP1 in the sequestration of stage IIb–IV gametocytes. Other work showed that the binding of stage III–IV gametocytes might be mediated ICAM-1, CD49c, CD166 and CD164 (Rogers et al., 2000). However, the binding was described as lower avidity. This confusion has been addressed in very recent data that has concluded that the gametocytes binding process is different from the asexual binding to ECs (Silvestrini et al., 2012), with gametocyte sequestration not being dependent on adhesion but instead on variable rigidity induced in different gametocyte stages. This is interesting because it shows interaction between parasite ligands and human receptors is not always required to mediate sequestration, but this could be important mechanism for mature gametocytes to restore their deformability and circulate in the blood stream for transmission (Aingaran et al., 2012).

1.11 RIFIN proteins:

Repetitive interspersed family proteins (RIFIN) are encoded by the largest multigene family identified in *P. falciparum* called *rif* genes (Cheng et al., 1998). There about 150–200 genes per haploid genome occupying the subtelomeric regions close to the *var* genes. RIFINs differ

from PfEMP1s by the presence of multiple RIFIN variants on the IE surface on different stages such as sporozoites, merozoites and gametocytes (Chan et al., 2014). RIFINS were classified into two groups; A-type and B-type RIFINs. Little information about their biological functions is available. Early studies implicated RIFINs in mediating rosetting phenotypes, but later studies showed that PfEMP1 is the main ligand for rosetting (Rowe et al., 2009). In addition, it was shown that one B-type *rif* was highly upregulated in mature gametocytes, calling into doubt the role of RIFINs in gametocytes sequestration (Liu et al., 2011).

1.12 STEVOR proteins:

The third largest protein family identified in *P. falciparum* is subtelomeric variable open reading frame proteins (STEVOR). It is encoded by the *stevor* multigene family. There are about 30–40 copies of stevor genes found in the genome. Also, they are found near to the *var* and *rif* genes (Cheng et al., 1998). Unlike *var*, many *stevor* copies were expressed in a single parasite at the same time. They are transcribed in all developmental stages (Chan et al., 2014). *Stevor* variants expression profile in IEs showed the same variants expressed during asexual and sexual forms (Sharp et al., 2006), which in turn could possibly exclude its roles in gametocyte adhesion. However, recent data have suggested some of STEVOR's biological roles. Increased IE rigidity was caused by the excessive expression of *stevor* which may improve PfEMP1 mediated sequestration (Sanyal et al., 2012). Also, STEVOR was proposed to have a role in parasite invasion (Garcia et al., 2005). Supportively, in a very recent study about STEVOR, Niang, *et al* (2014) have reported a dual role for STEVOR in mediating rosetting phenotypes and enhancing merozoite invasion. STEVOR recognises Glycophorin C (GPC) on the erythrocyte surface and the binding was related to the GPC levels on the erythrocytes. Interestingly, the binding was PfEMP1-independent (Niang et al., 2014).

1.13 SURFIN proteins:

Surfin proteins are high-molecular-weight antigens encoded by 10 *surf* genes (Winter et al., 2005). The pattern of *surf* genes expression is different based on the intraerythrocytic stage (Mphande et al., 2008). It is thought that one member expressed at the mature stage was found at knobs of IEs, this might indicate that it co-localises with PfEMP1 (Winter et al., 2005). However, another variant was shown localising to the PV in immature intraerythrocytic stage. No distinct role for these proteins has been identified (Mphande et al., 2008).

1.14 PfMC-2TM:

A novel gene family is found at many of *P. falciparum's* subtelomeric regions of the chromosomes. This family encodes protein called *Plasmodium falciparum* Maurer's clefts two-transmembrane protein (PfMC-2TM). Little is known about PfMC-2TM, but it was shown that domains of this protein localized in the PV and PVM (Tsarukyanova et al., 2009).

1.15 Modified erythrocyte band 3:

Modified erythrocyte band 3 has been proposed to mediate binding of IE to Thrombospondin (Lucas and Sherman, 1998, Eda et al., 1999) and CD36 (Winograd et al., 2004). CD36 adhesion was reduced upon the induction of chemical modifications of band 3 but this was not observed for Thrombospondin (Winograd et al., 2004). A recent report has shown that binding to CD36 was significant even if parasites had little or no detectable PfEMP1 expression, but this was not observed for ICAM-1 (Chan et al., 2012).

1.16 PfEMP1-based Vaccine development:

It seems logical that the major antigen expressed on the IE surface should be a target for vaccine development. However this can be countered by the arguments that PfEMP1 variants are highly polymorphic

and can rapidly switch upon the effect of immune responses. Therefore, PfEMP1 may not be the appropriate choice for vaccine inventors. Nevertheless, as explained above, accumulating evidence suggest that the PfEMP1-IE interactions could possibly be targets for therapeutic interventions to control malaria. The acquisition of natural antibodies that reduce the effects of the disease is obvious evidence. The recent classification of PfEMP1 based on the conserved DCs and their associations with SM is also a significant advancement in malaria pathogenesis and should lead to better understanding for targeting malaria. The good example is VAR2CSA which is an exceptional member of the PfEMP1 family that mediates binding to CSA in PAM, affecting especially a primagravidae and her child. However, multigravidae are at less risk due to antibodies developed against VAR2CSA, which are cross-reactive due to the unusual conserved DBL binding domains composition. This PfEMP1 has attracted researchers' hope to develop a vaccine to PAM targeting VAR2CSA and as a tool in disease control (Hviid, 2010, Badaut et al., 2010). Also, Buckee and Recker (2012) in their recent evolutionary study of PfEMP1 domains have thought that the domains that mediate other adhesion phenotypes may have conserved features because of the functional constraints that mediate high affinity binding. Consequently, it was suggested that a large proportion of the *var* gene reservoir worldwide share the proposed conserved domains. This could lead to novel vaccine opportunities based on PfEMP1 even if it only aims to protect from severe disease outcomes (Buckee and Recker, 2012).

The intensive focus on studying IE adhesion phenotypes and their contribution to malaria pathogenesis may well be explained by the significant outcomes of these studies in the past two decades which in turn might lead to the development of treatments targeting sequestration. The huge variation between the adhesion phenotypes and clinical outcomes usually bring doubt about the mechanisms that lead to the severe malaria. However, recent findings about the role of EPCR in CM are good evidence for the need of studying IE adhesion.

1.17 Aims:

- Characterisation of the binding of a set of new ICAM-1 binding isolates to provide further information about the interaction between ICAM-1 and PfEMP-1 (Chapters; 3.1 and 3.2).
- Comparison between the adhesion of ICAM-1 binding isolates to HUVEC and HBMEC (Chapter 3.3).
- Characterisation of the adhesion phenotypes of upsC PfEMP1 variants to CD36, ICAM-1 and primary endothelial cells (Chapter 4.1).
- An analysis of the adhesion phenotypes of PfEMP1 variants based on their length (Chapter 4.2).

This was mainly achieved by static protein adhesion assay and flow endothelial cells adhesion assay.

2. Methods:

2.1 Parasite culture:

Information about parasite isolates is provided in the method section of each chapter. Parasites were cultured under standard culturing conditions. It was cultured in 1% haematocrit in O+ human erythrocytes, using complete medium (RPMI 1640 medium supplemented with 37.5 mM HEPES, 7 mM D-glucose, 6 mM NaOH, 25 mg/ml of gentamicin sulphate, 2 mM L-glutamine, and 10% human serum) at a pH of 7.2, in a gas mixture of 96% nitrogen, 3% carbon dioxide, and 1% oxygen. The quantities of medium components to achieve required concentrations are given in the section below.

2.1.1 Growth and washing media preparation:

The materials below were mixed with the indicated quantities and filtered for parasite washing and growth medium under aseptic conditions in a laminar flow hood.

2.1.1.1 Washing medium:

- 500 ml RPMI 1640 (R0883, Sigma, USA) stored at 4°C.
- 5 ml L- Glutamine solution (200 mM, G7513, Sigma) stored at -20°C.
- 18.75 ml of HEPES Buffer (1 M, H0887, Sigma) kept at -20°C and stored at 4°C once thawed.
- 5 ml 20% Glucose solution (7 mM, Sigma).
- 3 ml 1 M Sodium Hydroxide solution.
- 1.25 ml Gentamicin Sulphate solution to give 25 ng/µl (10 mg, G1272, Sigma) stored at 4°C.

2.1.1.2 Growth medium:

100 ml of the mixture was taken and used as washing medium; it was stored at 4°C. Then, 45 ml pooled human serum was added to the remaining mixture and then filtered and stored at 4°C. All solutions used in parasite culture were warmed in 37°C water bath or incubator prior to use.

2.1.1.3 Human pooled serum:

Blood was collected from the Royal Hospital (Liverpool, UK) in non-anticoagulant blood collection bags and stored overnight at 4°C. Then, it was centrifuged at 3000 rpm for 20 minutes. After this, the serum was carefully removed, mixed and stored in 45 ml aliquot in -20°C.

2.1.2 Red Blood Cells separation:

Non-erythrocyte components were removed from the whole blood before using it in parasite culture. First, 12.5 ml of Histopaque (10771, Sigma) was put in 50 ml Falcon tubes. 25 ml of washing medium was added and mixed with 25 ml of blood cells in a 50 ml Falcon tube. Then, 12.5 ml of the blood mixture was added to Histopaque dropwise. The solution was then centrifuged at 3000 rpm for 15 minutes after which the supernatant was removed before adding approximately triple the volume of washing medium. The mixture was mixed and centrifuged at 3000 rpm for 5 minutes. The supernatant was aspirated and the RBC pellet was resuspended in an equal volume of washing media and stored at 4°C. The RBCs are known as washed RBC (wRBC) and have a haematocrit of approximately 50%.

2.1.3 Parasites thawing:

Appropriate volumes of 12% NaCl, 1.8% NaCl, 0.9% NaCl with 0.2% glucose, washing media and growth media were warmed at 37°C before used. The stabilate was carefully removed from liquid nitrogen following the code of practice according to LSTM policy. The parasites were warmed rapidly at 37°C before transferring them into a 50 ml falcon tube. Depending on the volume of the pellet, one-fifth of its volume of 12% NaCl was added dropwise and mixed with the pellet. In other words, if the pellet was 1000 µl, then, 200 µl of 12% NaCl was added. It was incubated at room temperature for five minutes. After that, five volumes of 1.8% NaCl to the original pellet volume was added dropwise and incubated for five minutes. This is followed by the addition of five volumes of 0.9% NaCl containing 0.2 % glucose and incubated for five

minutes at room temperature. The mixture was centrifuged at 1800 rpm in a bench top centrifuge for five minutes. The pellet was washed with washing medium and centrifuged at 1800 rpm for five minutes. Then it was resuspended in an appropriate volume of complete medium, transferred to a small culture flask and gassed for 30 seconds. The flask was incubated in a 37°C incubator.

2.1.4 Evaluating parasite growth and continuous culture maintenance:

The parasitemia was assessed using Giemsa thin smear and accordingly appropriate volume of wRBC was added. Parasitemia was assessed by making a Giemsa thin smear and examination under a light microscope. Five hundred (500) RBCs were counted, the number of IEs and their stages were recorded. The smears of cultures were acquired by taking a drop from culture and smoothly spread on a labelled glass slide and air-dried at room temperature. The smear was fixed with absolute methanol for few seconds before staining with 10X diluted Giemsa for about 20 minutes at room temperature. Giemsa stain stock solution was diluted with 10 % phosphate-buffered water (20 mM Na_2HPO_4 and 4 mM KH_2PO_4 at pH 7.2). Then, the stain was washed using tap water and air-dried, before examining the smear under a binocular light microscope using an oil immersion (100×) objective lens.

Parasites culture was mostly adjusted at 1% parasitemia and 1% haematocrit. The calculations for adjusting the parasitemia for continuous culturing were carried using the formula: $P_1\ V_1 = P_2\ V_2$, where P_1 is the counted parasitemia for the assessed culture, V_1 is the volume of the assessed culture, P_2 is the required parasitemia to continue culturing the parasites, which is usually 1- 1.5 %, and V_2 is the required volume of required to maintain the growth at given the parasitemia. To adjust the haematocrit at 1%, 10 µl of 100% packed RBCs was required for 1 ml of culture. But, because the stock of wRBC diluted to 50%, the volume must be doubled. Haematocrits of parasite culture and wRBC were assessed using a Coulter counter.

2.1.5 Parasite synchronisation:

2.1.5.1 Plasmion flotation:

The culture was transferred to a 50 ml Falcon tube and centrifuged at 1800 rpm for five minutes and the supernatant was discarded. The pellet was resuspended in incomplete medium in 1.5X of pellet volume. Then, the mixture was transferred into a 15 ml Falcon tube. After this, the suspension was mixed with an equal volume of Plasmion and allowed to settle for 20 - 30 mins at 37^{o}C. Trophozoite stage knobby IEs could be seen in the top layer of the suspension. This was carefully transferred to another 15 ml Falcon tube. The tube was centrifuged at 1500 rpm for 5 minutes and the supernatant discarded. Then, the pellet was gently resuspended in 10 ml incomplete media and centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated and a thin smear of it was prepared before adding the appropriate volume of complete medium and fresh wRBC, and gassed as described earlier. Smears taken after this procedure usually showed more than 50% IEs at mature stages. This selection was routinely performed to ensure that knobby populations are kept for adhesion assays as described earlier by (Jensen, 1978).

2.1.5.2 Sorbitol:

In some occasions, culture was synchronised using 5% of sorbitol (S3889 ,Sigma), which is a selective lysis for the IEs with trophozoites (Lambros and Vanderberg, 1979). It is used if the culture is at high rings parasitemia. The culture was pelleted and treated by 10X of 5% of sorbitol for 20 minutes 37^{o}C. Then, it was centrifuged at 1800 rpm for five minutes. The supernatant was discarded and the pellet washed with 10X of washing medium. Then, the pellet was resuspended in an appropriate volume of growth medium, transferred to a new flask, gassed and incubated at 37^{o}C. To prepare 5% of sorbitol, 25 g of sorbitol (Sigma Co, UK) was dissolved in 500 ml of distilled water, filtered and kept at 4^{o}C.

2.1.6 ICAM-1 Selection:

50 µl Protein A Dynabeads (10001D, Invitrogen) were washed 3 times with 200 µl 1% BSA/PBS using a magnet to retain the beads each time, and then resuspended in 200 µl 1% BSA/PBS (A8327, 30% Bovine serum albumin and D8537, Dulbecco's phosphate buffer saline, Sigma). 2.5 µg/ml ICAM-1^{Ref} protein was added to the bead suspension. The mixture was rotated at 15 rpm at room temperature for 60 minutes. ICAM-1 labelled Dynabeads were purified on the magnet, washed three times in 1% BSA/PBS, and resuspended in 200 µl 1% BSA/PBS. Parasite culture was enriched for mature stages using Plasmion as described above. The enriched IE were incubated with ICAM-1 labelled Dynabeads and rotated for 45 minutes at room temperature. Unbound parasites were removed by three gentle washes with 1% BSA/PBS. IE-bound beads were resuspended in complete media with fresh washed red blood cells and cultured as standard.

2.1.7 Selection of IT4var14 on BC6 antibody:

The selection for IT4var14 was carried out using BC6 mAb (Oxford University) that recognises IT4var14 specifically (Smith et al., 1995). It followed the same protocol for ICAM-1 selection, except protein G Dynabeads (10003D, Invitrogen) was used instead of Protein A Dynabeads.

Batches of parasite stabilates were selected on recombinant ICAM-1 and BC6 were cryopreserved for providing enough materials for adhesion assays at relatively similar populations. This was made to minimize the effect of mixed populations and antigenic switching. The parasites were used in binding assays for only three weeks post-selection.

2.1.8 Cryopreservation of parasites:

The cultures of about 5-8% at ring stage parasitemia were pelleted and cryopreserved by re-suspending in a glycerolyte freezing medium. Cryopreservation was carried out after the following calculations:

Three volumes of the pellet resuspended in five volumes of the cryopreservation media. The addition of cryopreservation media was carried in two steps. First, the pellet resuspended with one-fifth volume of the required cryopreservation solution. Then, it was ensured that the pellet was completely resuspended in the media and allowed to stand for five minutes at room temperature. Second, the remaining volume was added gently but completely mixed before transferring to properly labelled cryovials. The cryovials were kept in a rack, covered with tissue to allow slow freezing at -80^{o}C for overnight. On the following day, vials were transferred to cryostore. Example for the calculations of cryopreservation is given below: If the pellet was 1000 µl, then the cryosolution required was 1666 µl. (1666 µl / 5) = 333 µl, this volume was first suspended with the pellet. Then, (1666-333) = 1333 µl, this remaining volume for second resuspension.

2.2 ECs culturing:

HDMEC (C-12210) and HUVEC (C-12200) were obtained from Promocell. The original vials contain 1 ml of cryopreserved cells at passage 1 (P1). HBMEC (ACBRI 376) (P3) were obtained from Cell system.

2.2.1 Thawing ECs:

Cryopreserved cells were warmed briefly at 37°C and properly sprayed with disinfectant prior to transferring the cells into T25 flask contains 5 ml of warmed medium. The cells were allowed to attach for 2-3 hours at 37°C in a CO_2 incubator. Then, the medium was replaced by the same volume of warm medium and incubated for 48 hours. Cells were checked for confluence and culture media replaced. Once cells were confluent, sub-culturing was carried out following the standard protocol and manufacturer's instructions.

2.2.2 ECs sub-culturing:

All the used media were recommended by the manufacturers. HDMEC and HBMEC medium were cultured in Endothelial Cell Growth Medium MV (Ready-to-use) (C-22020) which was supplemented with Endothelial Cell Growth Medium MV SupplementMix (C-39225). HUVEC media Endothelial Cell Growth Medium (Ready-to-use) (C-22010) was supplemented with Endothelial Cell Growth Medium SupplementMix (C-39215). Media were kept at 4°C and aliquots were warmed before use.

Sub-culturing was done using Promocell detach kit (C-41220). The kit contains HEPES-buffered Balanced Salt Solution (HEPES-BSS), Trypsin/EDTA Solution and Trypsin Neutralization Solution (TNS). All solutions were aliquoted and warmed at 37 °C prior to use. Culture medium in the flask was aspirated; then, 1.5 ml of HEPES-BSS was added to wash the remaining medium and it was aspirated before adding 1.5 ml of trypsin to detach the cells. Cell detachment was observed under an inverted microscope. Then, similar volume of TNS was added immediately as soon as the cells have detached in order to reduce possible effect of trypsin. The mixture was transferred into 15 ml Falcon tube and pelleted at 1200 rpm for 3 minutes. Then, the cells were resuspended gently into warm fresh media and distributed into gelatine coated T-75 flask. Cells at this stage considered at P2, cells were expanded until P3 and then cryopreserved for adhesion assays at P4, P5 and P6.

2.2.3 ECs cryopreservation:

Cells were detached for cryopreservation as described for sub-culturing. But, instead of suspension in media, they were resuspended in Promocell Cryo-SFM (C-29910) at 5-7.5 $X10^5$ cells/ml. The vials were frozen gradually (-20°C/few minutes) to -70°C overnight before storing them in liquid nitrogen.

Recombinant proteins:

Many thanks for Mr Tadge Szestak, laboratory manager, for providing proteins stocks of ICAM-1-Fc reference (ICAM-1^{Ref} (Gray and Craig, 2002)). Mutant ICAM-1 variants (ICAM-1 Kilifi (ICAM-1^{Kilifi}), ICAM-1 S22/A (ICAM-$1^{S22/A}$) , ICAM-1 L42/A (ICAM-$1^{L42/A}$) and ICAM-1 L44/A (ICAM-$1^{L44/A}$)) were prepared as described previously (Tse et al., 2004).

2.3 Static adhesion assays:

Purified recombinant proteins were spotted in triplicate in a radial pattern using 2 µl spots on 60mm plastic petri dishes (Falcon 1007; Becton Dickinson, Oxford, UK) at concentrations of 50 µg/ml for ICAM-1, two plates were prepared for each parasite isolate. The dishes were incubated in a humidified chamber for 2hrs at 37^{o}C to allow the proteins to adsorb to the surface, after that the spots were aspirated off and the plastic petri-dishes were filled with 1% BSA/PBS, blocking buffer, and incubated overnight at 4^{o}C. The plates were warmed at 37^{o}C for one hour prior the assay. IE were suspended in binding buffer (RPMI 1640 R4130 (Sigma, Dorset, UK) in 2% glucose at pH 7.2) at 3% parasitemia and 1% haematocrit. The blocking buffer was removed from the dish prior to adding 1.25 ml of the IE suspension. The plates were incubated at 37^{o}C for one hour with gentle resuspension every 10 minutes. Then, the IE suspension was removed by gentle manual washing (4–6 washes) with binding buffer medium. The bound IE were fixed with 1% glutaraldehyde in PBS for 1 hour and stained with 10% Giemsa for 20 minutes. Six pictures were captured for each spot under x20 magnification using software HC Image (Sewickley, USA). The pictures were analysed by Image-Pro version 7 (Rockville MD, USA). The results were expressed as the mean number of IE bound per mm^2 of surface area.

2.4 Static inhibition assays:

The same static binding technique described above was applied with the addition of mAbs at 5µg/ml to the IE suspension prior adding it to the plates. All the anti-ICAM-1 mAbs were commercially available; 15.2 (AbD serotec), My13 (Invitrogen), 8.4A6 (Sigma), BBIG-I1 (R&D systems). Anti-CD36 IVC7 was kindly provided from Dr van der Schoot.

2.5 Flow cell adhesion assay:

Sub-culturing followed the standard protocol following manufacturer's instructions. Cells were washed with HEPES-buffered Balanced Salt Solution), trypsinised and neutralised. However, for the flow assay Accutase (Sigma) was used as an alternative detaching reagent instead of trypsin.

Details about the system can be found on the Cellix website via: http://www.cellixltd.com/. TheVenaFlux is a semi-automated microfluidic system able to perform cell adhesion studies under shear flow mimicking *in vivo* flow rates. It is designed to facilitate the study of cell adhesion, and to be more physiologically relevant than static assays. Its construction makes it easier to use than previous systems used to mimic physiological rates of flow in vessels for *P. falciparum* adhesion studies. The system includes VenaEC 8-channels designed for growth of human endothelial cells with continuous feeding during the experiment and parameters that can be adjusted and monitored during the experiment through the VenaFlux software.

VenaEC 8-channels (Cellix - Dublin, Ireland) were coated with 12 µl of 100 µg/ml fibronectin and incubated in a humidified petri-dish at 4°C overnight. Cells were activated with 10 ng/ml TNF 16–24 hours before the day of the assay. On the following day, VenaEC 8-channels were warmed at 37°C for 30 minutes. The endothelial cells (EC) were treated with Accutase, detached and then neutralised with medium. The EC were pelleted at 1200 rpm for 3 minutes and resuspended in an appropriate

volume EC medium to achieve 1.5×10^6 cells/ml. Then, 5 µl of this suspension were seeded onto each channel and incubated at 37°C. Once the cells attached to the channels, they were fed every 30 minutes until the EC become confluent, usually within 2–3 hours. The IE suspension for both binding and inhibition assays was prepared as described in static assays except the haematocrit was adjusted to 2%.

The assay was run following the Cellix protocol using the VenaFlux software. VenaEC 8-channels were connected to Cellix system in a microscope stage enclosed within a plastic chamber to keep the temperature at 37°C. The flow through the channels was adjusted to run 0.04 Pa and the IE suspension was drawn through the channel for five minutes. After that, binding buffer was passed through the cell at the same rate to wash for two minutes. The bound IE were counted in six fields and converted to the number of IE/mm^2. For binding inhibition, all mAbs were used at 5 µg/ml. The IVC7 anti-CD36 mAb was kindly provided by Prof. Ellen van der Schoot (Sanquin, Amsterdam).

2.6 Flow protein adhesion assay:

Flow protein adhesion assay was carried out using the same system that was mentioned above with the following changes; The proteins chip was called Vena8 Fluoro+™ biochip (Cellix) and it was coated with 5 µl of 50 µg/ml ICAM-1 or CD36, and incubated in a humidified petri-dish at 37°C for two hours before blocking with 1% BSA/PBS and kept at 4°C overnight. On the following day, the channels were warmed at 37°C for 30 minutes while the IE suspension was prepared as described in static assay protocol except that the haematocrit was adjusted to 2%.

2.7 Detection of IT4-ICAM-1 gene expression:

2.7.1 RNA extraction:

Synchronized ring stage parasites 16–18 h pelleted infected erythrocytes were completely dissolved in 10× volumes of TRIZOL reagent

(Invitrogen) and stored at −80 °C until RNA purification. RNA extraction was performed as previously described (Kyes et al., 2000). Appropriate precautions were taken to avoid RNase contamination when preparing and handling RNA.

Frozen samples were allowed to warm at room temperature for 10-15 minutes. All the following volumes were added based on 1 ml of dissolved parasites in Trizol. 200 µl of chloroform which was added until the sample homogenized. The sample was vigorously shaken by hand for 15 seconds and incubated for 2–3 minutes at the room temperature. The sample was centrifuged at 12,000 × *g* for 30 minutes at 4°C. The sample was very carefully removed from the centrifuge, because RNA remains in an upper colourless aqueous phase which should be nearly half of the whole mixture. The tube was then angled at 45° and the aqueous phase was carefully transferred into a new tube. Thereafter, RNA was precipitated by adding 500 µl of 100% isopropanol and mixed gently by inverting the tube for a few times and incubated on ice for 120 minutes or alternatively at 4°C overnight. The sample was quickly vortexed before centrifugation at 12,000 × *g* for 30 minutes at 4°C. After precipitation, RNA pellets as a gel-like form and the pellet is usually unseen at this step, thus, extra care is required while pipetting the supernatant out. RNA was washed with of 500 µl 75% ethanol (75 ethanol: 25 DEPC-H_2O) and then centrifuged at 7500 × *g* for 5 minutes at 4°C. The supernatant was discarded and the RNA pellet was allowed for air-drying at room temperature for no more than 5 minutes. Then, the pellet resuspended by DEPC-H_2O by just dropping the solvent onto it and incubated at 55–65°C for 10 minutes. It was then placed on ice and mixed by pipetting before measuring the concentration using Nanodrop. The sample was immediately treated with DNase I or alternatively stored at –80°C if not proceeding to cDNA synthesis.

2.7.2 cDNA synthesis:

DNase I treatment was carried out according to the manufacturer's instructions. Following the DNase I treatment, cDNA synthesis was performed using SuperScript™ III RT (Invitrogen). It was carried out in 20-µl reaction volume which is suitable for 10 pg–5 µg of total RNA. The reaction included 1 µl of 250 ng of random primers, 250 ng of the RNA, 1 µl 10 mM dNTPs, 4 µl 5X First-Strand Buffer, 1 µl 0.1 M DTT, 1 µl RNaseOUT™ (Recombinant RNase Inhibitor) (Invitrogen), 1 µl of SuperScript™ III RT, 1 µl of *E. coli* RNase H (Invitrogen) and distilled water.

The reaction was initiated by adding random primers, RNA, dNTPs, water and then, heat incubated at 65°C for 5 minutes, followed by at least 1 minute incubation on ice. The tube was briefly centrifuged before adding 5X First-Strand Buffer, DTT, RNaseOUT and SuperScript™ III RT and gently mixed by pipetting up and down. Then, it was incubated at 25°C for 5 minutes, 50°C for 45 minutes and 70°C for 15 minutes in PCR machine. There was need to do another reaction that was not treated by SuperScript RT to give RT negative control for the q-RT-PCR.

2.7.3 Quantitative PCR:

The q-RT-PCR was run using Stratagene mx3005p (Agilent). PCR cycling conditions were 95°C for 3 mins followed by 40 cycles of 95°C for 10 s, 60°C for 10 s with a final cycle at 95°C for 1 min, 55°C for 30 s, 95°C for 30 s. The reaction contained nuclease-free PCR-grade H_2O, 10 µl 2× SYBR green QPCR master mix, 1 µM of primers, and 50 ng cDNA. The internal controls were genes encoding adenylosuccinate lyase and seryl-tRNA synthetase. Data were acquired using the formula:

$$2^{-\Delta CT} = 2^{-}$$ (the mean of three readings CT of IT4varX – the mean of CT of housekeeping genes).

Ct values were obtained by exporting data sheets from the MxPro QPCR Software (version 4.10).

The primers of IT4-ICAM-1 isolates were published by (Wang et al., 2012). The sequences are given in the following table:

Target IT-ICAM1 gene	Forward primer	Reverse primer
IT4var01	**TGCAATGTAACACACTCACG**	**CACTATACCACAGGCATCTTC**
IT4var13	**TCGGGCAACAACACTATCAA**	**CCCCATTCAATAAACCATCG**
IT4var14	**AAACCGACACAACAACCGACGACGAC**	**ACTATTTCGCACGCATCTGGTGGC**
IT4var16	**ACCGGAAGCACCACAAGAAC**	**GCACCACTTATGCATTTCCATCC**
IT4var31	**CAAGATGGCAGCATTGAAAA**	**CGCCTCCTTCTGCATCTTAC**
IT4var41	**GGACATGTCAGGTCATCACG**	**ACCATTCTGCCCATTCAGTC**

Table 2.1.1: The primers of IT4-ICAM-1 isolates were published by (Wang et al. 2012).

2.8 Adult Hyperimmune sera (HIS) reactivity:

Fluorescence-activated cell sorting (FACS) was used to assess the ability of adult HIS to recognise the surface of IEs with upsC HB3 *P. falciparum* isolates that were not able to bind to recombinant proteins such as CD36 and also did not bind to ECs. More than 50% of mature stages were enriched using Plasmion as previously described. Then, 3 µl of the washed pellet was resuspended in 1 ml of PBS with 1% bovine serum albumin (1%BSA/PBS) to prepare the IE suspension. 100 µl which contains about 8-10 × 10^6 cells were transferred to 1.5 ml Eppendorf tubes. The cells were washed and the pellet of all isolates including A4 was resuspended with 1:10 of 30 mg adult HIS sera (unknown reactivity) and 1:10 human sera (negative control, serum was collected from the Royal Hospital (Liverpool)). The positive control for PfEMP1 labelling was IT4var14 (A4) incubated with 20 µg/ml BC6 for an hour at 37°C followed by the appropriate secondary antibody.

After incubation, cells were pelleted and washed twice with 1% BSA/PBS. After that, bound IEs were resuspended in 1:100 dilution of APC-conjugated mouse anti-human IgG secondary antibody (Molecular probes) mixed with 10 µg/ml ethidium bromide for staining the IE nuclei. For A4-BC6 labelling, APC conjugated goat anti-mouse IgG (Thermo

Scientific) was used. Cells were incubated covered from light for 60 minutes at 37°C. Then, cells were washed twice with 1% BSA/PBS and resuspended with 400 µl of Cell Wash (FACS solution). The analysis was performed using Becton-Dickinson FACSCalibur flow cytometer (BD LSR11). Data were acquired using FACSCalibur flow cytometer, and 50,000 events were collected and analyzed with FlowJo software (Tri Star, San Carlos, CA, USA).

2.9 FACS for Endothelial Cells receptors detection:

Cells were grown in 12 well-plates until they become confluent at 37°C in a 5% CO2 incubator. The cells in some wells were stimulated with 10 ng/ml TNF overnight. The cells were washed with HEPES, trypsinized and neutralised as described above. Cells were centrifuged at 5000 rpm for 5 minutes and the pellets were washed with 1% BSA/PBS prior to adding conjugated monoclonal antibody APC- mouse anti human ICAM-1, APC- mouse anti human-EPCR and FITC mouse anti human CD31 for both stimulated and non-stimulated cells (BD Biosciences Ltd). It was incubated at 37°C for 60 minutes in dark conditions. In parallel, isotype matched antibody were used to ensure the absence of non-specific labelling (BD Biosciences Ltd). The cells were then washed with 1% BSA/PBS before transferring to FACS solution to measure the receptor expression by FACS.

3.1 An Analysis of the Binding Characteristics of a Panel of Recently Selected ICAM-1 Binding *P. falciparum* Patient Isolates.

3.1.1 Introduction:

The later stages of the asexual intraerythrocytic cycle of *P. falciparum* are not seen in the circulation because of their ability to localise to different organs in a phenomenon called sequestration. Understanding some of the key events that facilitate sequestration is important to identify possible targets to develop either inhibitors or vaccines (Rowe et al., 2009). *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) (Baruch et al., 1995, Smith et al., 1995, Su et al., 1995) mediates sequestration with various human receptors (Rowe et al., 2009) including ICAM-1 (Berendt et al., 1989), CD36 (Barnwell et al., 1989, Oquendo et al., 1989) and EPCR (Turner et al., 2013).

ICAM-1 binds to LFA-1 or Mac-1 and also mediates binding to pathogens, such as human rhinoviruses (HRVs) (Greve et al., 1989, Staunton et al., 1989), and *P. falciparum* infected erythrocytes (Berendt et al., 1989). The ICAM-1 binding sites for IEs, rhinoviruses, LFA-1 and fibrinogen are overlapping, but also have distinct regions (Berendt et al., 1992, Ockenhouse et al., 1992).

Several lines of evidences support the involvement of ICAM-1 in malaria pathology. First, a study conducted on post-mortem samples obtained from people diagnosed with CM showed accumulation of IEs in brain vessels that co-localised with ICAM-1 (Turner et al., 1994). In addition, ICAM-1 was found up-regulated in brain accompanied with *P. falciparum* infection (Turner et al., 1998). Isolates from SM patients and particularly from CM revealed higher binding to ICAM-1 than isolates from AM (Newbold et al., 1997, Ochola et al., 2011, Turner et al., 2013), although this is not observed in all studies. Additional line of evidence is the association between CM and a homozygous mutation in ICAM-1 in Kilifi, Kenya, named ICAM-1^{Kilifi} (Fernandez-Reyes et al., 1997),

although it should be noted that other observations such as those in the Gambia and Thailand did not show an association between ICAM-1^{Kilifi} and severe malaria (Bellamy et al., 1998, Ohashi et al., 2001). In contrast, ICAM-1^{Kilifi} was suggested to have a protective role in Gabon (Kun et al., 1999).

The binding site of ICAM-1 to PfEMP1 has been studied using alanine replacement mutagenesis and ICAM-1-specific mAbs. The binding region on ICAM-1 for *P. falciparum* IEs was revealed to involve the BED face of ICAM-1 which is three β-strands of ICAM-1 named B, D, and E, including the DE loop (Figure 3.1.1) (Tse et al., 2004). Earlier studies have investigated ICAM-1 binding phenotypes under both flow and static conditions on endothelial cells and purified proteins. These studies have shown that IE have subtle differences in binding to ICAM-1 with variable affinities and avidities ranging from 2.8 nM to 144 nM for a number of PfEMP1 variants from the IT4 lineage (Brown et al., 2013).

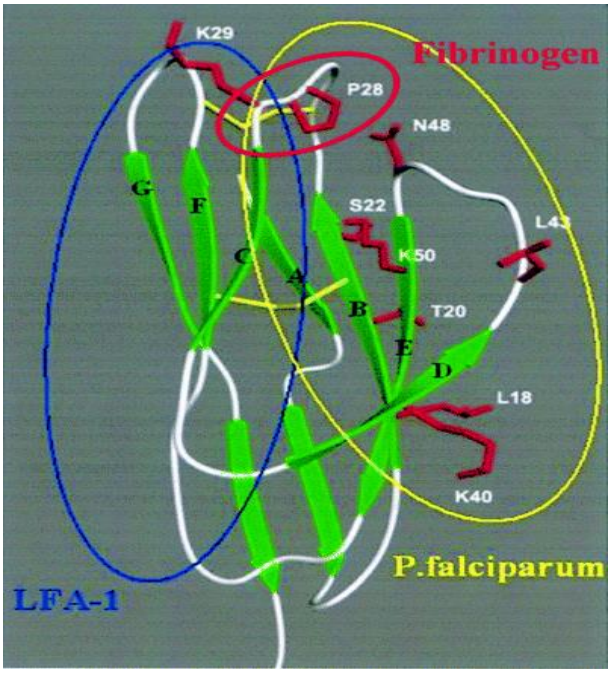

Figure 3.1.1: The crystal structure of the N-terminal domain of human ICAM-1, showing the binding sites for IEs with P. falciparum, LFA-1, fibrinogen. The strands of the β-barrel are labelled A to G. The figure shows the BED side. It is cited from (Tse et al., 2004)

PfEMP-1 proteins are encoded by 50-60 extremely variable *var* genes per parasite genome (Gardner et al., 2002). Inspite of the variation of the *var* genes, they can be categorised into three major groups; A, B, and C based on their chromosomal locations and promoter sequence (Kraemer and Smith, 2003, Lavstsen et al., 2003). Group A *var* genes are more conserved than the others and have been associated with severe malaria (Jensen et al., 2004). A typical PfEMP-1 structure contains two to seven Duffy-binding–like (DBL) domains and one to two cysteine-rich interdomain region (CIDR) domains (Gardner et al., 2002). Specific domains have been implicated in binding to certain host receptors (Smith et al., 2000a). A very recent sub-classification for DBL and CIDR domains from seven parasites genomes has identified number of shared combinations of short tandem domain cassettes (DCs) in several different parasite strains (Rask et al., 2010). Among the Group A PfEMP-1s, there are several ICAM-1– binding DBL domains isolates identified so far, including PFD1235w, Dd2var32 (Jensen et al., 2004) and PF11_0521 (Oleinikov et al., 2009, Gullingsrud et al., 2013). Additionally, from these isolates, it was possible to classify a novel tandem three-domain of PfEMP1 region named DC4, and antibodies to this region have been described to be cross-reactive with group A PfEMP1 proteins that bind to ICAM-1 (Bengtsson et al., 2013). However, although DC8 and DC13 cassettes found in Group A PfEMP1 proteins were associated with SM (Avril et al., 2012, Claessens et al., 2012, Lavstsen et al., 2012), IEs expressing DC8 and DC13 did not to bind to ICAM-1 (Avril et al., 2012, Lavstsen et al., 2012).

In the current chapter, the binding phenotypes for ICAM-1-selected, recently lab-adapted patient isolates was investigated under static and flow adhesion assays. The analysis included four mutant ICAM-1 variants that have previously shown different effects on laboratory isolates (Tse et al., 2004) and the effect of four anti-ICAM-1 mAb using static assays, increasing the number of the isolates in comparison with earlier studies. Understanding crucial events in cytoadherence is important in identifying possible targets in order to develop either effective inhibitors or vaccines.

3.1.2 Methods:

3.1.2.1 Parasites isolates:

Laboratory isolates, A4 (Ockenhouse et al., 1992) and ItG (Roberts et al., 1992) and lab-adapted patient isolates 8146, 8206, 8131, 6392, PO-69, (from Kenya) J1, PCM-7, BC-12 and GL-6 (from Thailand (Poyomtip et al., 2012)) were cultured as described in general methods using standard culturing techniques at 1% haematocrit in O+ human erythrocytes (Trager and Jensen, 1976). A batch of parasite stabilates selected on recombinant ICAM-1 was made to reduce the effect of antigenic switching and mixed populations. The parasites were used in binding assays for only about 10 cycles after ICAM-1 selection.

All patient isolates were collected with consent as part of clinical studies in Thailand and Kenya, and all patient material have been removed during culture, replaced with blood sourced commercially from the UK Blood Transfusion Service.

3.1.2.2 ICAM-1 selection:

Described in the general methods.

3.1.2.3 Adhesion assays:

Static and flow protein adhesion assays were used to assess the binding of parasites to ICAM-1. Also, flow endothelial cells were performed on HDMEC. The binding to CD36 and ICAM-1 on ECs was inhibited by 5 µg/ml anti-CD36 IV-C7 and anti-ICAM-1 15.2 mAbs. All these procedures were explained in the general methods including culturing ECs.

3.1.3 Results:

3.1.3.1 Static adhesion of new ICAM-1 binding isolates on ICAM-1^{Ref}:

Based on the level of binding to ICAM-1^{Ref}, all the isolates were categorised into high and low-avidity parasites. ItG was defined as a high-avidity ICAM-1 binder whereas, A4 was characterised as low-avidity ICAM-1 binder from previous studies (Gray et al., 2003). Only two of the lab-adapted isolates were high-avidity ICAM-1 binders; 8146 and 8206. The rest of the isolates were assigned as low-avidity binders (Figure 3.1.2).

Parasites	Mean IE binding/mm^2	N	SE
ItG	4746	24	470
A4	1939	9	477
8146	5147	9	1150
P069	1515	11	250
PCM7	689	6	269
6392	1584	8	655
8131	424	10	93
BC12	1362	10	188
8206	5901	11	942
J1	2391	8	622
GL6	1968	9	531

Table 3.1.1: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

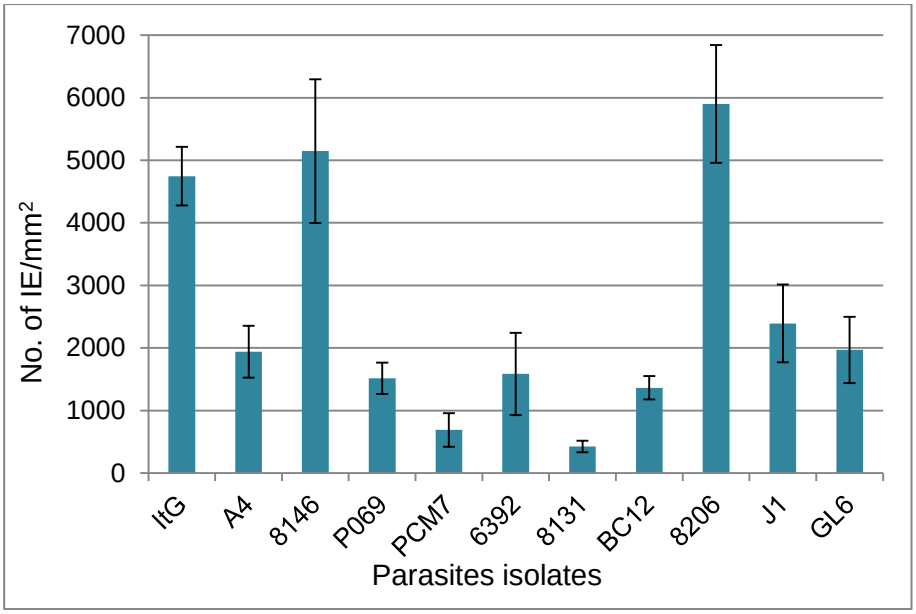

Figure 3.1.2: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}, 2 µl spots of 50 µg/ml ICAM-1^{Ref} were placed onto 6 cm dishes and standard protein static binding assays conducted with IE suspended in binding buffer at a parasitaemia of 3% and a haematocrit of 1%. The results show the mean of binding and the bars represents SE (n can be found in table 3.1.1).

3.1.3.2 Static adhesion of new ICAM-1 binding isolates to mutant ICAM-1 variants:

The current study showed that there were considerable differences in IE binding to mutant ICAM-1 proteins (Figures 3.1.3 - 3.1.6). The binding of isolates was variably disturbed by the ICAM-1^{Kilifi} mutation. ICAM-1^{Kilifi} affected the binding of four isolates by approximately 50%. Furthermore, there were three isolates whose binding was decrased by more than 75%, 6392, PCM7 and 8131. Whereas the ItG and PO69 parasites was only reduced by 20% (Figure 3.1.3 and Table 3.1.2). The S22/A mutation considerably reduced the binding of the high-avidity isolates and some of the low-avidity isolates; GL6 and 6392, by around 80%. In addition, there was a moderate effect on PO69, BC12 and J1 (Figure 3.1.4 and Table 3.1.3). On the other hand, the effect of L42/A mutation revealed critical effect on all of the isolates, decreasing the binding by at least 50% incomparison with ICAM-1^{Ref}, with the binding almost entirely inhibited for most isolates, including the high-avidity isolates (Figure 3.1.5 and Table 3.1.4). By contrast, L44/A mutation increased the binding for some isolates, and reduced the binding of GL6 only (Figure 3.1.6 and Table 3.1.5).

A) Static adhesion of new ICAM-1 binding isolates on ICAM-1^{Kilifi}:

Parasites	Mean IE binding/mm^2	N	SE
ItG	3807	20	489
A4	897	9	236
8146	3315	6	1461
P069	1219	8	408
PCM7	103	5	40
6392	126	5	42
8131	103	7	22
BC12	609	8	86
8206	3984	7	1116
J1	1386	5	557
GL6	1031	8	378

Table 3.1.2: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1^{Kilifi}. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

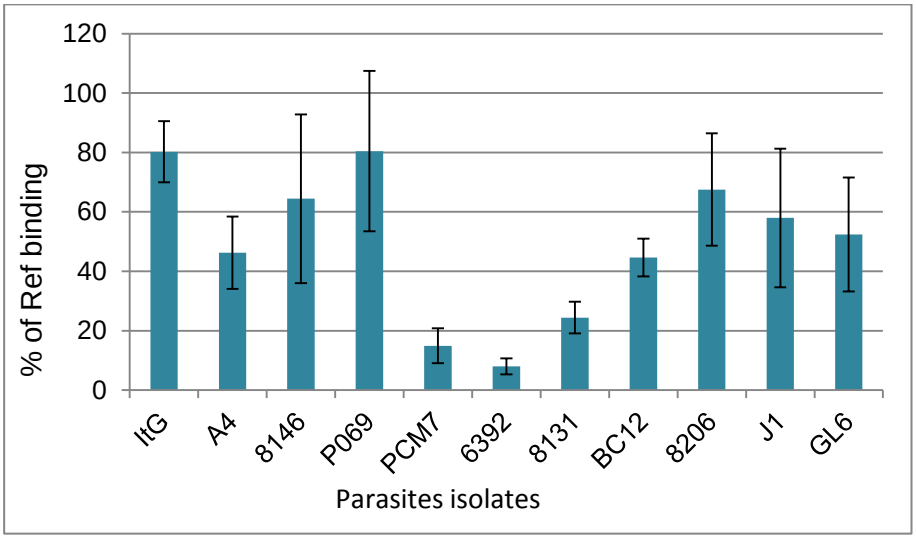

Figure 3.1.3: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1^{Kilifi}. The results show the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.1.2).

B) Static adhesion of new ICAM-1 binding isolates on ICAM-1$^{S22/A}$:

Parasites	Mean IE binding/mm^2	N	SE
ItG	336	20	67
A4	1221	9	285
8146	663	6	315
P069	1084	8	347
PCM7	819	5	306
6392	359	5	120
8131	693	7	228
BC12	895	8	121
8206	111	7	26
J1	1020	5	542
GL6	109	8	37

Table 3.1.3: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1$^{S22/A}$. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

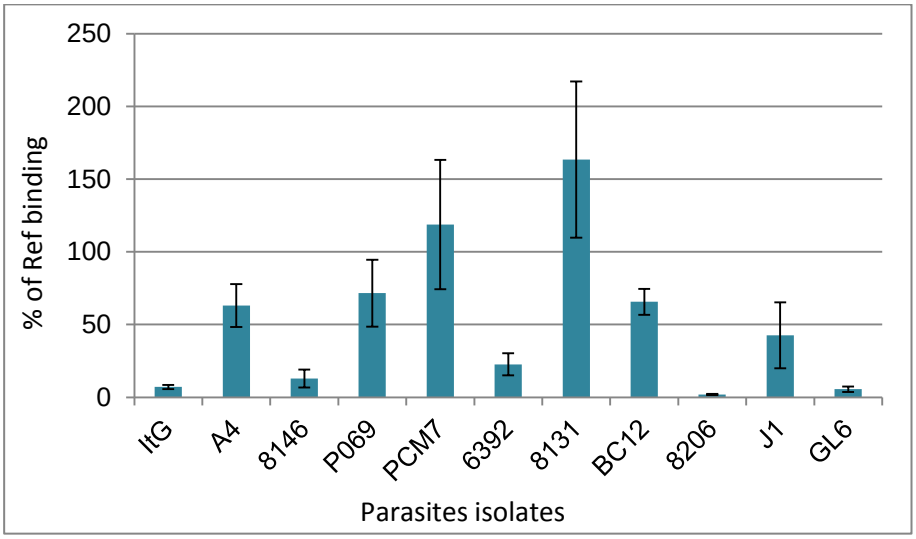

Figure 3.1.4: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1$^{S22/A}$. The results show the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.1.3).

C) Static adhesion of new ICAM binding isolates on ICAM-1$^{L42/A}$:

Parasites	Mean IE binding/mm^2	N	SE
ItG	103	4	13
A4	281	3	187
8146	67	3	20
P069	163	3	38
PCM7	35	3	6
6392	471	3	119
8131	204	3	27
BC12	583	3	29
8206	90	4	23
J1	107	3	73
GL6	45	3	8

Table 3.1.4: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1$^{L42/A}$. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

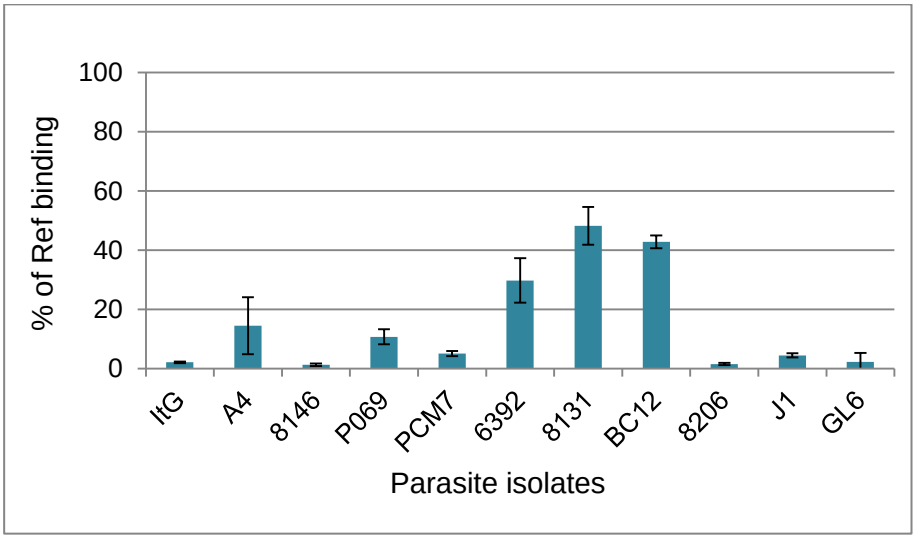

Figure 3.1.5: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1$^{L42/A}$. The results show the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.1.4).

D) Static adhesion of new ICAM binding isolates on ICAM-1$^{L44/A}$:

Parasites	Mean IE binding/mm^2	N	SE
ItG	4920	4	648
A4	2767	3	850
8146	8458	3	1123
P069	1724	3	284
PCM7	761	3	47
6392	4257	3	681
8131	1069	3	347
BC12	2000	2	293
8206	10385	4	248
J1	2058	3	579
GL6	821	4	18

Table 3.1.5: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1$^{L44/A}$. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

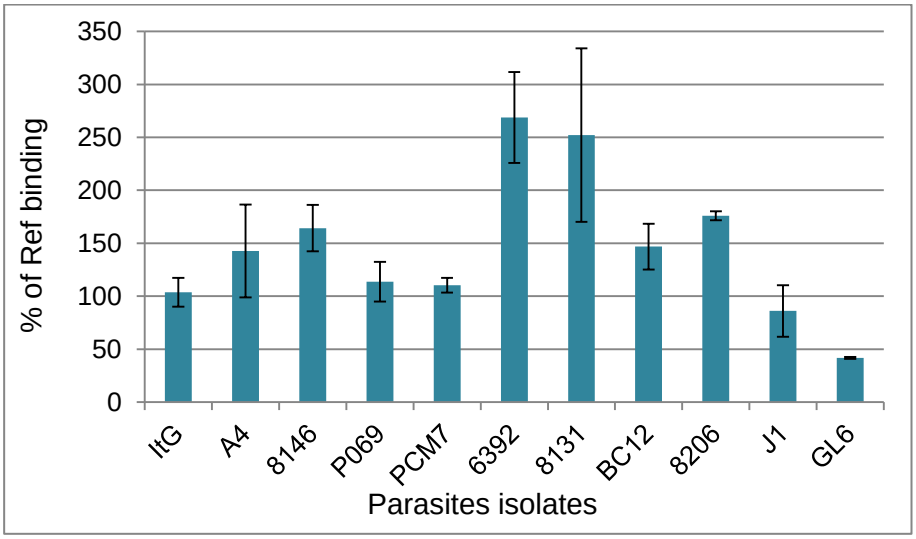

Figure 3.1.6: Static adhesion of IEs with selected ICAM-1 binding isolates to ICAM-1$^{L44/A}$. The results show the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.1.5).

3.1.3.3 Static inhibition of new ICAM binding isolates using anti-ICAM-1 mAbs:

The effect of anti-ICAM-1 mAbs on the binding of IE to purified ICAM-1 under static conditions has been investigated using specific mAbs reacting with epitopes on Ig-like domains one and two. MAbs 15.2, BBIG-I1 and My13 mapping to domain one, and 8.4A6 mAb mapping to domain two were used in a study that differentiated between the binding sites on ICAM-1 for IE and LFA-1 (Berendt et al., 1992). Different mAbs have shown different inhibitory effects on the isolates (Figures 3.1.7 - 3.1.10). The results are shown as the percentage of the binding of each isolate against the binding to ICAM-1^{Ref}. The binding to ICAM-1 of most of the isolates was reduced by about 75% by two mAbs My13 and 15.2. Nevertheless, there was only 40% inhibition caused by 15.2 and My13 to PO69 (Figure 3.1.7 and 3.1.8 and Tables 3.1.6 and 3.1.7). The range of inhibition of BBIG-I1 was between 25%–75% for nearly all isolates except there was almost no effect on 8206 (Figure 3.1.9 and Table 3.1.8). There was different inhibition caused by 8.4A6; the effect varied between 25–50% for most of the isolates, although there was no effect by 8.4A6 on the ItG and 8206 isolates (Figure 3.1.10 and Table 3.1.9) These variations again suggest the use of variable contact residues between ICAM-1 and variant PfEMP-1 proteins.

A) Static inhibition of new ICAM binding isolates using 15.2 mAb:

Parasites	Mean IE binding/mm^2	N	SE
ItG	158	4	46
A4	649	4	249
8146	355	4	93
P069	1021	4	135
PCM7	55	4	9
6392	105	4	17
8131	15	4	7
BC12	248	4	60
8206	1434	4	395
J1	233	4	70
GL6	428	4	329

Table 3.1.6: 15.2 mAb static inhibition of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

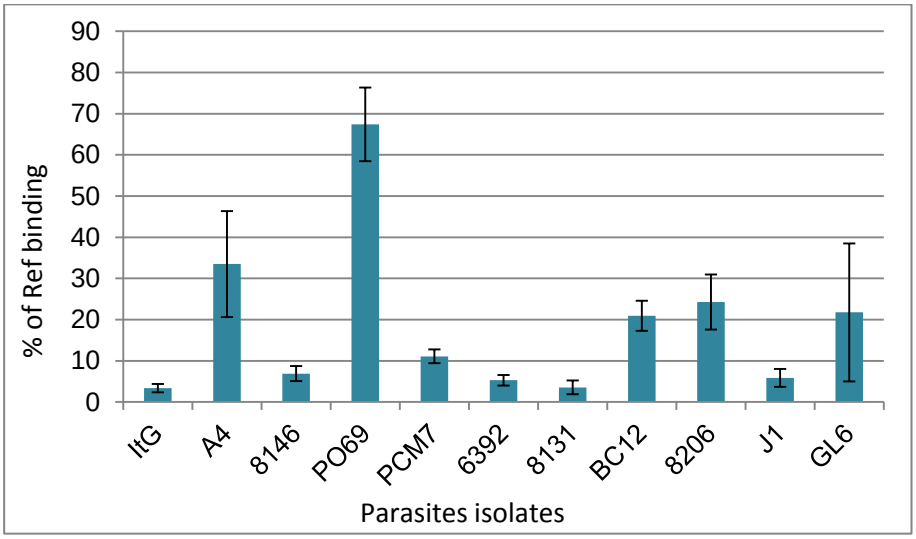

Figure 3.1.7: 15.2 mAb static inhibition of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}. The results show the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.1.6).

B) Static inhibition of new ICAM binding isolates using My13 mAb:

Parasites	Mean IE binding/mm^2	N	SE
ItG	685	4	184
A4	300	4	80
8146	795	4	233
P069	1106	4	245
PCM7	54	4	10
6392	270	4	72
8131	45	4	9
BC12	1075	4	327
8206	1818	4	527
J1	432	4	70
GL6	269	4	134

Table 3.1.7: My13 mAb static inhibition of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

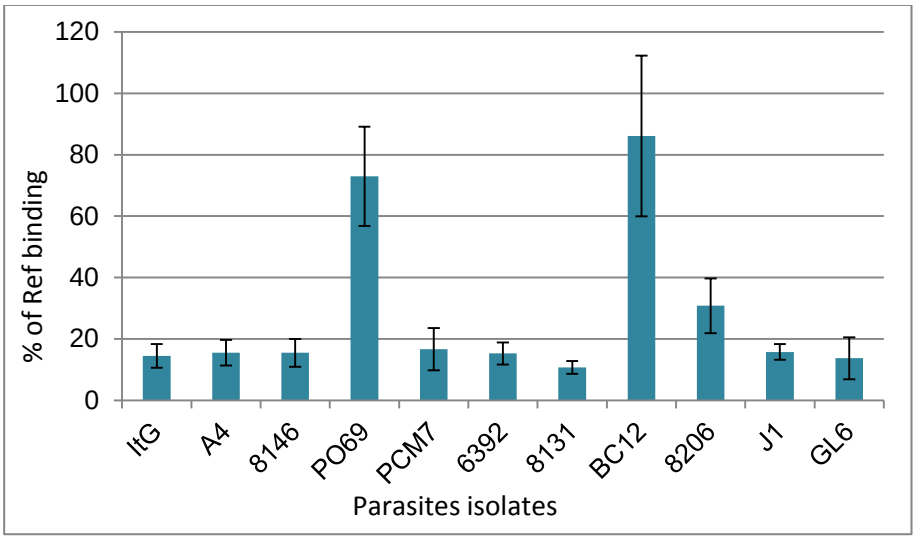

Figure 3.1.8: My13 mAb static inhibition of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}. The results show the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.1.7).

C) Static inhibition of new ICAM binding isolates using BBIG-I1mAb:

Parasites	Mean IE binding/mm^2	N	SE
ItG	984	4	182
A4	503	4	129
8146	2372	4	1088
P069	1106	4	342
PCM7	69	4	10
6392	353	4	114
8131	105	4	36
BC12	979	4	401
8206	4407	4	788
J1	1573	4	678
GL6	747	4	610

Table 3.1.8: BBIG-I1 mAb static inhibition of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

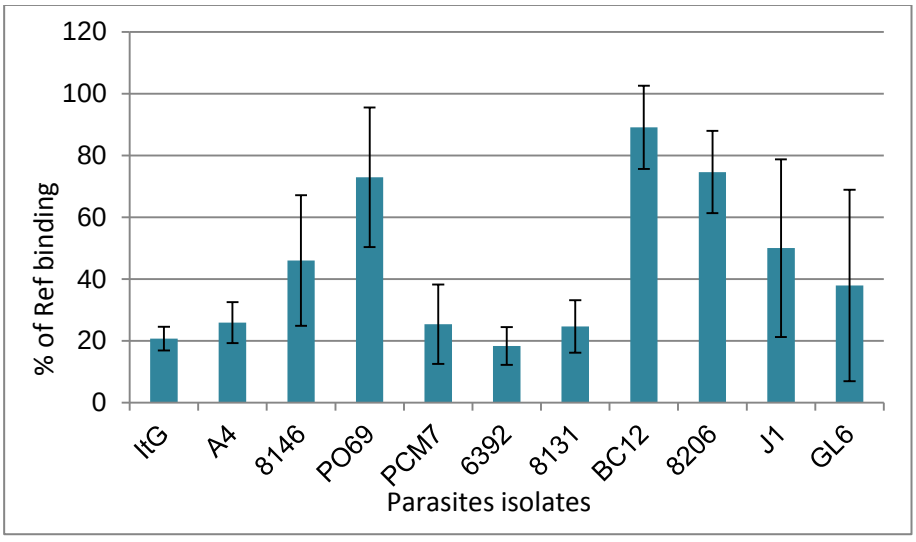

Figure 3.1.9: BBIG-I1 mAb static inhibition of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}. The results show the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.1.8).

D) Static inhibition of new ICAM binding isolates using 8.4A6 mAb:

Parasites	Mean IE binding/mm^2	N	SE
ItG	5494	4	1597
A4	885	4	366
8146	2173	4	280
P069	740	4	174
PCM7	183	4	39
6392	578	4	182
8131	101	4	28
BC12	659	4	145
8206	6769	4	1820
J1	760	4	97
GL6	144	4	32

Table 3.1.9: 8.4A6 mAb static inhibition of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

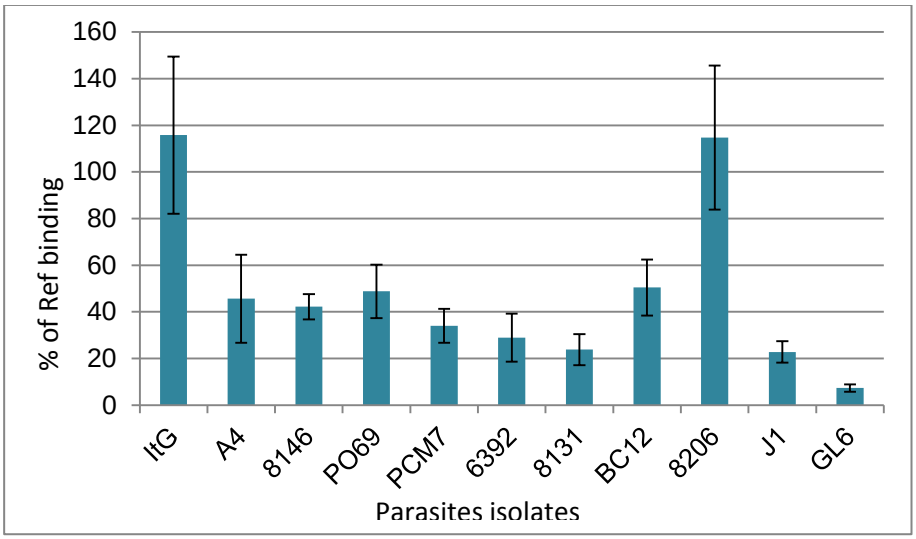

Figure 3.1.10: 8.4A6 mAb static inhibition of IEs with selected ICAM-1 binding isolates to ICAM-1^{Ref}. The results show the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.1.9).

3.1.3.4 Characterisation of new ICAM-1 binding isolates to HDMEC under flow conditions:

A) Flow adhesion of new ICAM-1 binding isolates to HDMEC

The binding level on HDMEC under flow conditions was similar for seven out of eleven isolates examined within the range 200–300 IE/mm^2 (Figure 3.1.11). In contrast, two isolates, J1 and 8146, bound within the range 600–700 IE/mm^2 to HDMEC although J1 being assigned as low-avidity ICAM-1 binder on purified ICAM-1. Another two isolates, 8131 and 8206, revealed relatively less binding to HDMEC although 8206 was considered as a high-avidity ICAM-1 binder on ICAM-1 protein. Additional analysis using an anti-ICAM-1 mAb revealed similar activity for 15.2 mAb on almost all isolates, reducing binding by approximately 50% (Figure 3.1.12 and Table 3.1.10). However, the binding was reduced by approximately 80% for eight isolates in the presence of the IV-C7, anti-CD36, mAb and for the other three was reduced by about 60% (Figure 3.1.13 and Table 3.1.11).

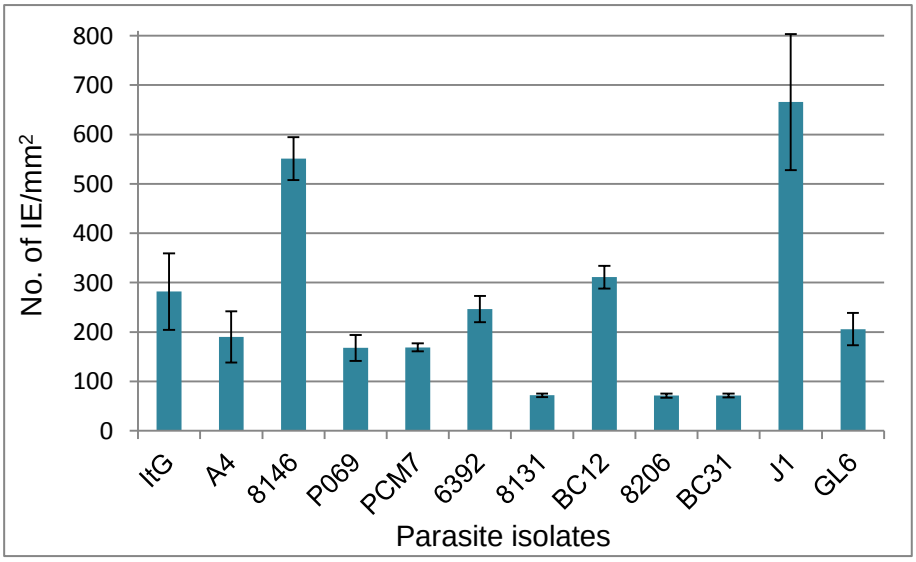

Figure 3.1.11: HDMEC flow endothelial cell adhesion assay of IEs with selected ICAM-1 binding isolates: HDMEC seeded on channels pre-coated with fibronectin; IE were passed on confluent cells for five minutes followed by washing by binding buffer for two minutes before counting 6 fields. The parasitaemia was 3% and a haematocrit of 2%. The results show the mean of binding and the bars represents SE (n >3).

B) ICAM-1 inhibition of new ICAM binding isolates using 15.2 mAb under flow condition:

Parasites	Mean IE binding/mm^2	N	SE
ItG	115	15	18
A4	91	3	10
8146	279	3	39
P069	68	3	13
PCM7	118	3	1
6392	123	3	27
8131	29	3	5
BC12	228	3	4
8206	42	3	4
J1	336	3	31
GL6	171	3	40

Table 3.1.10: Flow ICAM-1 inhibition on HDMEC of IEs with selected ICAM-1 binding isolates, anti-ICAM-1 (15.2) was used at 5 µg/ml. The table shows the mean of the IEs binding in the presence of 15.2 mAb, N: number of experiments and the SE.

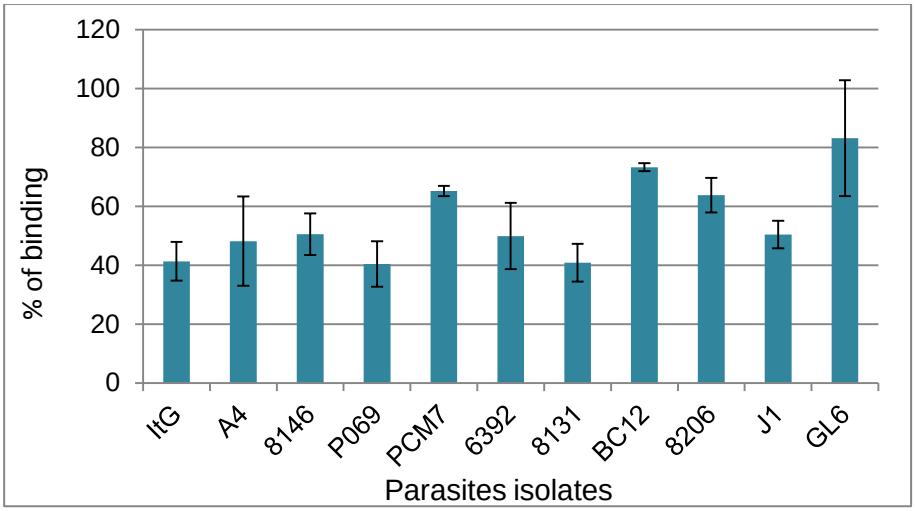

Figure 3.1.12: Flow ICAM-1 inhibition on HDMEC of IEs with selected ICAM-1 binding isolates using anti-ICAM-1 (15.2) mAb at 5 µg/ml. The figure shows the % binding against no inhibitory effect and the bars represents SE (n can be found in table 3.1.10).

C) CD36 inhibition of new ICAM binding isolates using IVC7 mAb under flow condition:

Parasites	Mean IE binding/mm^2	N	SE
ItG	26	8	5
A4	22	3	3
8146	42	3	11
P069	64	3	2
PCM7	15	3	2
6392	37	3	4
8131	18	3	2
BC12	114	3	13
8206	13	3	2
J1	42	3	17
GL6	27	3	8

Table 3.1.11: Flow CD36 inhibition on HDMEC of IEs with selected ICAM-1 binding isolates, anti-CD36 (IVC7) mAb was used at 5 µg/ml. The table shows the mean of the IEs binding in the presence of IVC7 mAb, N: number of experiments and the SE.

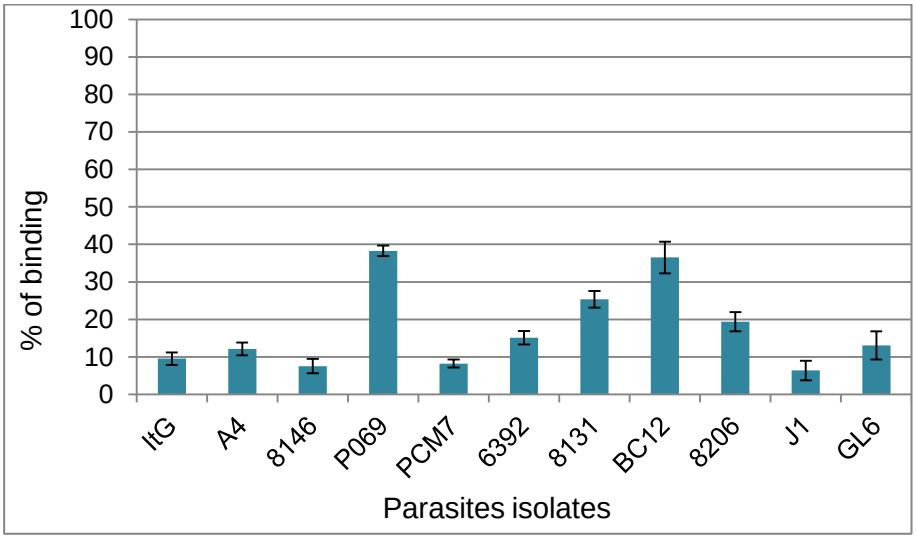

Figure 3.1.13: Flow CD36 inhibition on HDMEC of IEs with selected ICAM-1 binding isolates using anti-CD36 (IVC7) mAb at 5 µg/ml. The figure shows the % binding against no inhibitory effect and the bars represents SE (n can be found in table 3.1.11).

3.1.4 Discussion:

The aim of this part was to establish the binding characteristics of a set of new ICAM-1 binding isolates to provide additional information about the interaction between ICAM-1 and the parasites. The purpose of using field isolates is usually to examine the association between clinical outcomes and binding phenotypes. The selection of ICAM-1 binding PfEMP-1 populations in this study introduces bias by potentially increasing small sub-populations from the original sample and so cannot be used to derive associations between the binding phenotypes and clinical outcomes. Our original study used three genetically distinctive ICAM-1-binding laboratory isolates (two of which (A4 and ItG) are included in this study for comparison), screened against 25 mutant ICAM-1 proteins using static and flow adhesion systems (Tse et al., 2004).

Based on this earlier work, binding and inhibition assays were carried on a larger number of recently lab-adapted isolates using the ICAM-1 mutations previously revealed to disrupt the binding and discriminating between laboratory isolates, using static assays only. Binding to endothelial cells was examined using a flow adhesion system.

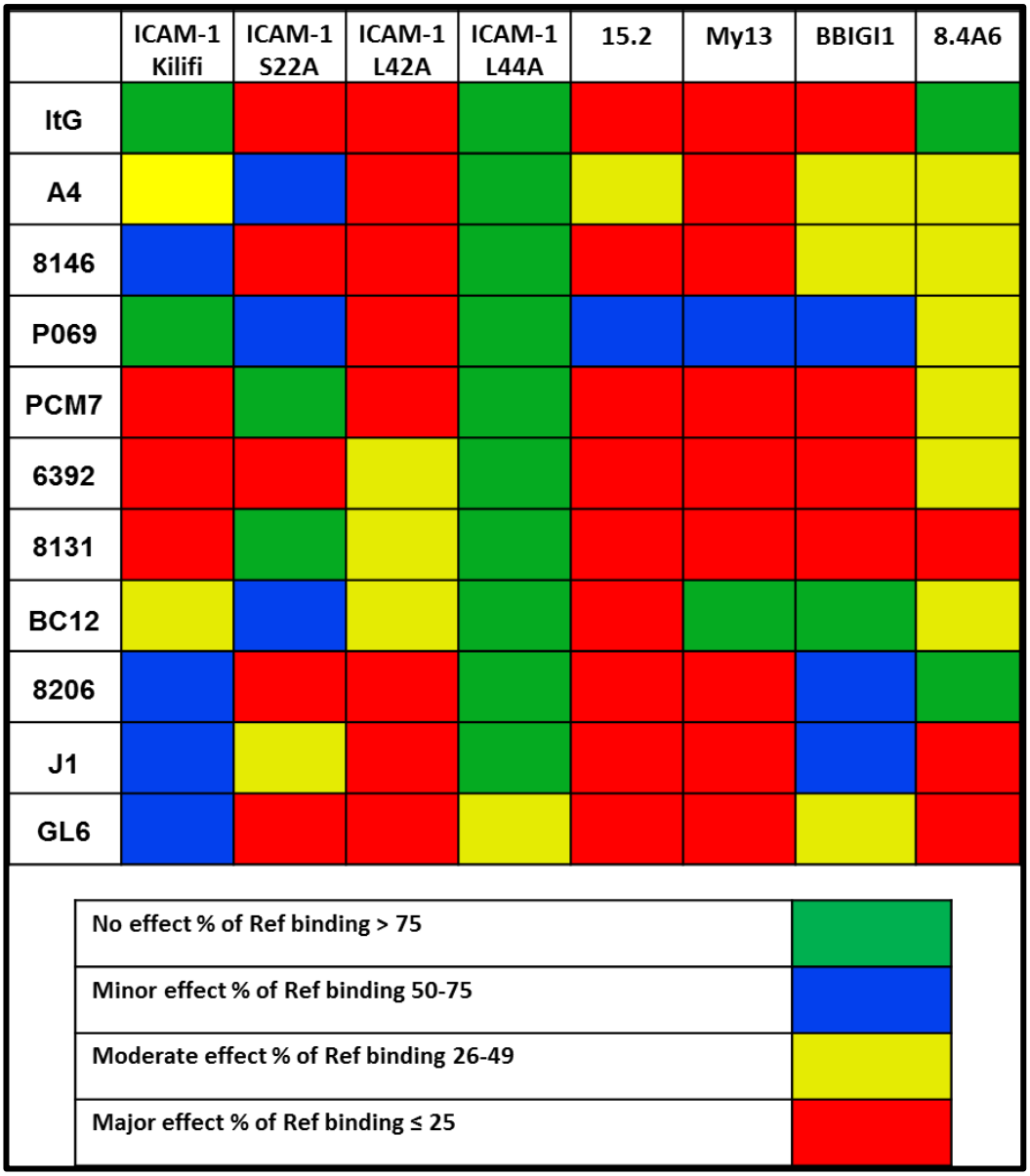

Figure 3.1.14: Summary of the effects of mutant ICAM-1 variants and mAb inhibition on recently ICAM-1 selected isolates.

Alanine replacement mutagenesis and ICAM-1-specific mAbs have provided more details about the binding region on ICAM-1 for *P. falciparum*-IEs (summarised in Figure 3.1.14). The binding between IEs and ICAM-1 was shown to involve the BED face of ICAM-1, containing the DE loop (Tse et al., 2004). The binding phenotypes from earlier studies were categorised based on the isolate's avidity to ICAM-1. Overall, the new binding and inhibition data support the original outcomes that different ICAM-1-binding isolates can use variable contact residues in the DE loop of ICAM-1 to bind. Furthermore, current data

support earlier findings by indicating an important role for L42 for all ICAM-1-binding isolates.

Two ICAM-1-specific mAbs 15.2 and My13, which have been mapped to epitopes including the L42 residue, decreased the binding of all the isolates. The binding of low-avidity-ICAM-1 isolates was more affected by these mAbs than high-avidity-ICAM-1 parasites. 8.4A6 mAb, which targets an epitope on domain two, can also prevent the binding of all isolates. This may be explained by the epitope in domain two being in a position close to domain one or disturbing the structure of this domain, as they have been shown to interact to produce the native ICAM-1 structure (Berendt et al., 1992). Interestingly, most of the isolates were low-avidity ICAM-1 binders similar to A4, which was previously associated with a signature that reveals isolates from SM cases (Ochola et al., 2011).

Flow adhesion on endothelial cell assays more accurately resembles the situation seen in the human circulation than static assays (Chakravorty and Craig, 2005). In the current study, we used TNF-activated HDMEC, which expresses both CD36 and ICAM-1 receptors as well as other endothelial receptors, by using the Cellix system to measure IEs adhesion. The Cellix system has shown comparable results with former flow-based systems on different endothelial cells (data not shown). The binding to TNF- activated HDMEC was nearly the same level for seven out of elevenisolates, comparable to ItG and A4, which bind in the range 200-300 IEs/mm^2. The binding was reduced with both anti-ICAM-1 and anti-CD36 mAbs with the latter showing greater inhibition than anti-ICAM-1. This could be explained by ‘receptor co-operation’ between ICAM-1 and other receptors (Chakravorty and Craig, 2005, Davis et al., 2013). It is possible that ICAM-1 is not the only receptor involved in CM pathogy and, for example, a recent study has shown the ability of IEs to bind to EPCR and associated with SM, including CM (Turner et al., 2013, Esser et al., 2014).

ICAM-1 has been suggested to play a capturing role from the circulation thereby contributing to the pathogenesis of CM (Gray et al., 2003).

However, the role of CD36 in sequestration is not clearly understood. CD36 binding is a typical phenotype for the majority of paediatric isolates and in some studies has been shown to be more linked with binding to isolates from UM cases. It has been suggested that the host uses CD36 to control the parasitemia prior to host immune responses or to reduce pro-inflammatory responses (Ochola et al., 2011).

The molecular basis of the variable binding to ICAM-1 is believed to be due to differences in the contact residues between ICAM-1 and the variant PfEMP1s. PfEMP1 binds to ICAM-1 through a different set of DBLβ domains mostly from groups B or C and it would be challenging to target DBLβ domains in these two groups due to their high sequence diversity. This is observd particularly in approaches to distinguish ICAM-1 binding DBLβ domains from non-binding ones, which has only been fairly successful (Howell et al., 2008). There are ICAM-1 binders among the group A PfEMP-1 that contain a definable DC4 cassette (Bengtsson et al., 2013), but this is still at a very preliminary stage and requires more investigation to see if it could offer a starting point for the development of a vaccine targeting CM by preventing IEs sequestration via ICAM-1 in the brain. The variability in the binding characteristics between IEs and ICAM-1 suggests that it may well be a difficult problem to find a cross-blocking theraputic intervention, although the vital role of the L42 residue and anti-DC4 blocking antibodies gives some support for this approach.

The divergent binding pattern to variants of ICAM-1 of different IEs is similar to that revealed by the causative agent of the common cold, Human Rhinovirus (HRV). The main serotypes of HRV use ICAM-1 to invade the epithelium and two different HRV serotypes have revealed varying adhesion phenotypes to ICAM-1^{Ref} and ICAM-1^{Kilifi}, and their association with variable clinical outcome (Xiao et al., 2004). Very recently, an anti-human ICAM-1 antibody that specifically binds domain one of human ICAM-1, prevented entrance of two major groups of rhinoviruses, decreased virus burden, cellular, inflammation and pro-inflammatory cytokine induction *in vivo*. Notably, this antibody did not

affect ICAM-1 binding to LFA-1, leaving this critical host pathway intact (Traub et al., 2013). Similar approaches might be used to lead to the development of novel treatment candidates to cut malaria morbidity and mortality rates but require a good understanding of the variety of IEs adhesion to ICAM-1.

The essential outcome of this part of the study is the identification of vital targets on the sites of the interactions between parasite ligands and host receptors, which may result in the development of inhibitors which target IEs sequestration. In spite of the presence of rapid and effective parasite-killing drugs, mortality still recorded among children with SM complications, in particular at the immediate period after admission to hospital. Several strategies to improve survival in malaria have been highlighted in a recent review (Miller et al., 2013). Among these is targeting parasite adhesion to the vascular endothelium. Anti-adhesion therapeutics is a promising project in the discovery of novel treatments, including compounds based on the structure of endothelial receptors (Dormeyer et al., 2006). High-throughput screening could identify adhesion blocking molecules that inhibit IE from binding or activating microvascular endothelium (Miller et al., 2013). The good example of this kind of rational-inhibitor design is (+)-EGCG, a polyphenol compound demonstrating significant inhibition ranging from 37 - 80% by the new ICAM-1-binding parasites used in the current study (Patil et al., 2011). The variaiable inhibition by (+)-EGCG might be because of the variable contact residues on PfEMP1 of different patient isolates. The action mode of (+)-EGCG is assumed to be its structural simulation of part of the ICAM-1 binding site for IE based around the L42 loop.

To conclude, the isolates tested in this chapter use variable contact residues on ICAM-1 for their binding. Nevertheless, L42/A inhibits the binding of all isolates, which support the concept of a conserved region used by PfEMP1 to bind on ICAM-1 and that could be used to target interventions.

3.2 A binding analysis of IT4 *P. falciparum* isolates that express ICAM-1-binding DBLβ domains.

3.2.1 Introduction:

The adhesion of IE to the endothelium of vital human organs is crucial to mediate malaria pathology. IE-ICAM-1 adhesion phenotype has been associated with CM (evidence of the involvement of ICAM-1 in CM has been mentioned in the previous chapter). Variable domains of PfEMP1 have been shown to mediate several interactions with human receptors such as some CIDRα domains binding to CD36 (Baruch et al., 1997), and some DBLβ domains binding to ICAM-1 (Smith et al., 2000a, Howell et al., 2008).

DBL domains are members of a parasite adhesion-domain superfamily that are expressed on erythrocyte invasion ligands and named erythrocyte binding ligands (EBLs). DBL domains were originally described as "cysteine-rich domains containing functional binding regions that determine invasion specificity" (Su et al., 1995). The authors coined the term 'DBL' based on the homology described with the cysteine-rich domain of *P. vivax* Duffy binding domains. Therefore, the occurrence of DBL domains was reported in two distinct stages in the *P. falciparum* asexual cycle. First, it is implicated in the complex system in *P. falciparum* of merozoite invasion of erythrocytes, unlike *P. vivax* and *P. knowlesi* that are restricted to the use of single receptors to invade erythrocytes (Higgins and Carrington, 2014). Secondly, DBLs are found in PfEMP1 proteins on the surface of IEs mediating interaction with human receptors such as ICAM-1(Smith et al., 2000a).

The first sequence comparison of PfEMP1 in 1995 showed that DBL domains have diverse degrees of sequence similarities. There were four DBL types defined and called DBL1, DBL2, DBL3, and DBL4. The DBL1 type is what is called DBLα nowadays and is always present at the amino terminal end of PfEMP1. It is followed by a CIDR domain forming what is called the conserved head structure (Su et al., 1995). Then, Rowe *et al.*

(1997) showed that DBLα binds to CR1 and mediates rosetting. In 1998, CIDR1 domains were shown to mediate CD36 binding followed by the identification of DBL-β domain (before known as DBLβ-C2) as ICAM-1 binding domains in early 2000. Later in 2000, the adhesive domains of PfEMP1 were classified based on the available PfEMP1 sequences and some functional binding data into different subclasses α, β, γ, δ, ζ, and ε. (Smith et al., 2000b, Rask et al., 2010).

Crystal structures are available for several DBL domains including some belonging to PfEMP1. The first structure of one of the DBL domains from the PfEMP1 family was completed in 2008 (Higgins, 2008). Although low sequence identity has been observed among DBL domains, they have a conserved skeleton. It was revealed that there is a notable low level of sequence identity, with only 4% of residues identical and around 15% of residues similar in 10 different DBLs studied from both EBA and PfEMP1 variants. Nevertheless, DBL domain structures are built on a conserved skeleton, with a fundamental helical architecture found in all domains. The conserved residues are inside the skeleton of the folds, maintaining the structure of the domain (Higgins and Carrington, 2014).

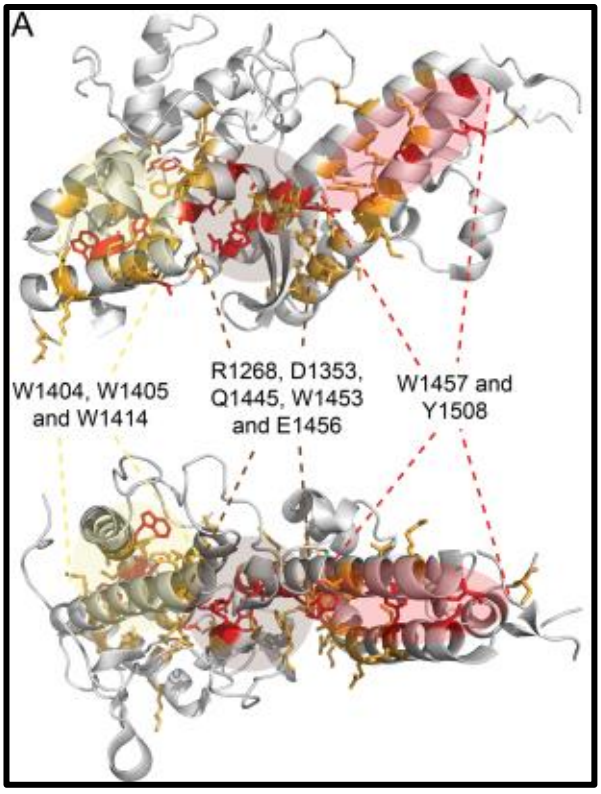

Figure 3.2.1: The structure of a DBL domain. Conservation of the DBL fold. Conserved residues are plotted onto the structure of the DBL3X domain of VAR2CSA, with absolutely conserved residues in red and similar residues in yellow. The conserved residues include three main patches: one stabilizing subdomain 2 (W1404, W1405, and W1414), one stabilizing subdomain 3 (W1457 and Y1508) and one stabilizing the interface between subdomains 2 and 3 (R1268, D1353, Q1445, W1453, and E1456). The figure has been cited from (Higgins and Carrington, 2014).

The DBL domain's composition was described as it contains three subdomains SD1, SD2, and SD3 (Singh et al., 2006). SD1 and SD2 fold together, with SD2 having four helical bundles present in all DBLs and SD1, which does not show conserved secondary structure, wrapped around SD2 (Higgins and Carrington, 2014).

In SD2 of DBL3X of VAR2CSA, there are three conserved tryptophan residues, (W1404, W1405, and W1413) located on the forth helix of SD2. The supposed role of these tryptophans is maintaining the fold through stabilizing contacts with other helices. SD3 is made of a long, two α-helical hairpin joined by two conserved residues with an extra partial helical strand that lies back along the bundle. In addition, there is a third residue (G1360) that mediates a tight turn between the second and third helices. Three disulphide bonds stabilise the distal end of SD3 in almost all cases. A series of salt bridges form a rigid interface that link SD2 and SD3 together by the remaining conserved residues on SD2 and SD3. In addition, there are different loops and helices in domains from both EBA and PfEMP1 proteins. DBL domains from PfEMP1 are often longer and more complex than domains from EBA members (Higgins and Carrington, 2014).

DBL domains were classified into six classes α, β, γ, δ, ζ, and ε based on shared homology blocks. For example, all DBLβ domains share three homology blocks, in addition, DBLβ domains that share one or more HB are sub-classified with a numerical digit (e.g. DBLβ3 and DBLβ5) (Rask et al., 2010). Also, the domain position can be shown by a prefix containing the letter D (for domain) and the number of the domain position from the side of the N-terminal segment e.g. DBLβ3_D4 (Bengtsson et al., 2013). Domain classifications have been associated with particular IE adhesion phenotypes. For instance, DBLβ3 and DBLβ5 subclasses of the DBL domains were shown to bind to ICAM-1. DBLβ3_D4 was in DC4 that has recently been shown mediate the ICAM-1 binding of some of the group A PfEMP1 (Bengtsson et al., 2013). DBL:ICAM-1 interactions still need more investigations for more

understanding of the platform of therapeutic interventions based on this interaction.

Howell *et al*. (2008) expanded the image of the PfEMP1::ICAM-1 complex. They used the sequences of IT4 strains and performed site-directed mutagenesis to DBLβ domains and assessed the binding capacities to identify critical binding residues. Howell and colleagues examined the binding of 21 DBLβ (known before as DBLβ-C2) domain recombinant proteins from all *var* genes in groups A, B and C of the IT4 parasite isolates using magnetic beads. They found that only some of IT4 expressed DBLβ can bind to ICAM-1. The IT4 PfEMP-1 expressing DBL-β ICAM-1 binding isolates will be called IT4-ICAM-1 isolates in this thesis. Biophysical study has characterized the interaction of ICAM-1 with the full extracellular domains from IT4VAR13, a member of IT4-ICAM-1 isolates. Brown et al. (2013) showed that the binding occured on the ICAM-1 N terminus forming a 1:1 complex and is exclusively mediated by DBLβ. It was also shown that PfEMP1 extracellular domains experience minimal structural changes as they bind to ICAM-1 due to their rigid, elongated architecture (Brown et al., 2013).

On the other hand, VAR2CSA shape is compact, that formed by folding itself back towards itself using multiple domains (Srivastava et al., 2010). However, the binding to its ligand was recently found to be mediated mainly by the DBL2 domain which is located at the tip of the folded multi-domains protein (Clausen et al., 2012, Higgins and Carrington, 2014). VAR2CSA is composed of six DBL domains and a single CIDRpam domain, whereas IT4VAR13 follows the structure of a more typical PfEMP1. It was suggested that PfEMP1s have at least two different shapes. Indeed, the development of therapeutic interventions targeting appropriate antigens needs more details about the molecular mechanisms of PfEMP1 recognition of host receptors to mediate sequestration, immune responses and possibly to limit the antigenic variation.

The aim of this section is to examine the binding phenotypes of isogenic IT4-ICAM1 isolates on different sets of mutant ICAM-1s and inhibition of ICAM-1 binding under static conditions. Also, it aimed to investigate the binding to HDMEC under flow conditions.

3.2.2 Methods:

3.2.2.1 IT4-ICAM-1 isolates:

Table 3.1 provides some information about IT4-ICAM-1 isolates used in this study. It includes the alias name given from their original sources with ID and ups category given in vardom database. It can be accessed through the following link ((http://www.cbs.dtu.dk/services/VarDom/)). Also, it shows the available affinities values obtained from Brown *et al.* (2013) study, in nanomolar range. The table also includes the percentage of transfected COS cells bound to five or more ICAM-1/Fc-coated beads cited from (Howell et al., 2008). All isolates were cultured as described in the general methods section. The synchronisations were carried using sorbitol and Plasmion.

ID	Alias	Ups	IT4VARXX$^{DBL\beta}$-ICAM-1^{D1D5}-Fc K_D $m \times 10^{-9}$	rDBL reactivity on bead*
IT4var01	3G8	C	NA	98±4
IT4var13	GC503	B	2.6	100
IT4var14	A4	B	NA	100
IT4var16	ItG	B	51.1	100
ITvar31	P5B6	B	144	100

Table 3.2.1: IT4-ICAM-1 isolates information. The table shows the alias for their originals, ID and ups classification from vardom database. The affinities between rDBLβ and ICAM-1^{D1D5}- Fc cited from (Brown et al., 2013) (NA: not available in the original study). * Last column shows % of transfected cells associated with five or more ICAM-1/Fc-coated beads (Howell et al., 2008).

3.2.2.2 ICAM-1 selection:

Isolates were selected on ICAM-1 and stabilates were made and cryopreserved to minimise the switching that occurs in long-term culture; each stabilate was cultured for up to three weeks. RNA was collected in Trizol on the same day of cryopreservation. The dominant *var* type was confirmed by qRT-PCR after selection. The protocol has been given in general methods.

3.2.2.3 q-RT-PCR:

Described in the general methods.

3.2.2.4 Adhesion assays:

Static and flow protein adhesion assays were used to assess the binding of parasites to CD36 and ICAM-1. Also, flow assays were performed on HDMEC. The binding to CD36 and ICAM-1 on ECs was inhibited by 5 µg/ml anti-CD36 IV-C7 and anti-ICAM-1 15.2 mAbs respectively. All procedures are described in the general methods section, including culturing ECs.

3.2.3 Results:

3.2.3.1 Confirmation of dominant IT4 DBL-β ICAM-1 binding var gene transcript levels after selection on ICAM-1:

In order to maximise the expression of a dominant ICAM-1 binding variant and to improve the adhesion phenotypes related to specific PfEMP1 members, several ICAM-1 selections were carried out and stabilites were frozen to maintain access to the same population during the study.

The analysis of *var* gene transcription in parasite isolates was performed on samples taken at the time of freezing. RNA was harvested from ring-stage parasites, and *var* gene expression profiling was identified by RT-qPCR. Figure 3.2.2 shows that four of the isolates have expressed a single primary IT4-ICAM-1 *var* transcript more than five fold changes. It should be noted that ITvar16 had a secondary *var* transcript of ITvar41.

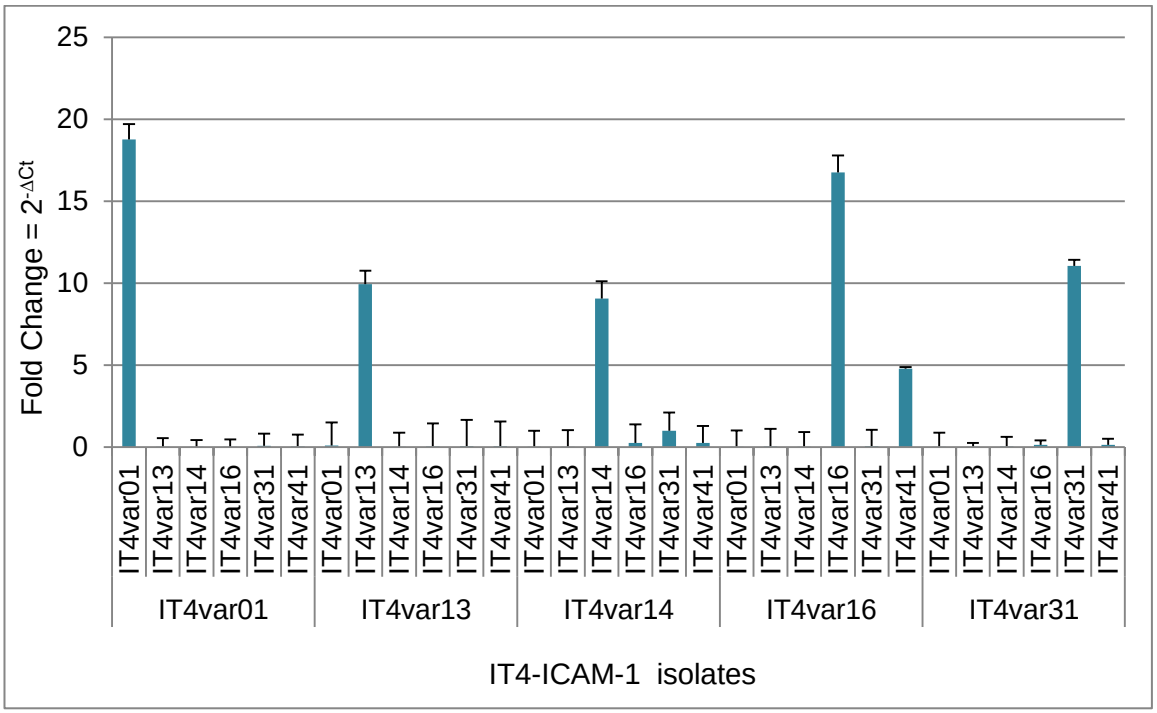

Figure 3.2.2 *var* gene transcription profile after ICAM-1 selection.

3.2.3.2 Static adhesion of IT4-ICAM-1 binding isolates on ICAM-1^{Ref}:

All five isolates were predominantly expressing single ICAM-1 binding PfEMP-1. Based on the level of binding to ICAM-1^{Ref}, IT4var01 and IT4var14 had a similar level of binding of about 2000 IE/mm^2. Each of the other three had different avidities for ICAM-1^{Ref}: IT4var16 had been previously categorised as high ICAM-1 binder, and about 5000 IE/mm^2 were counted. The binding of IT4var13 was just under 900 IE/mm^2. Interestingly, IT4var31 bound far less than other isolates at 134 IE/mm^2, about 35 fold less than the strongest binder in the set of IT4-ICAM isolates (Figure 3.2.3).

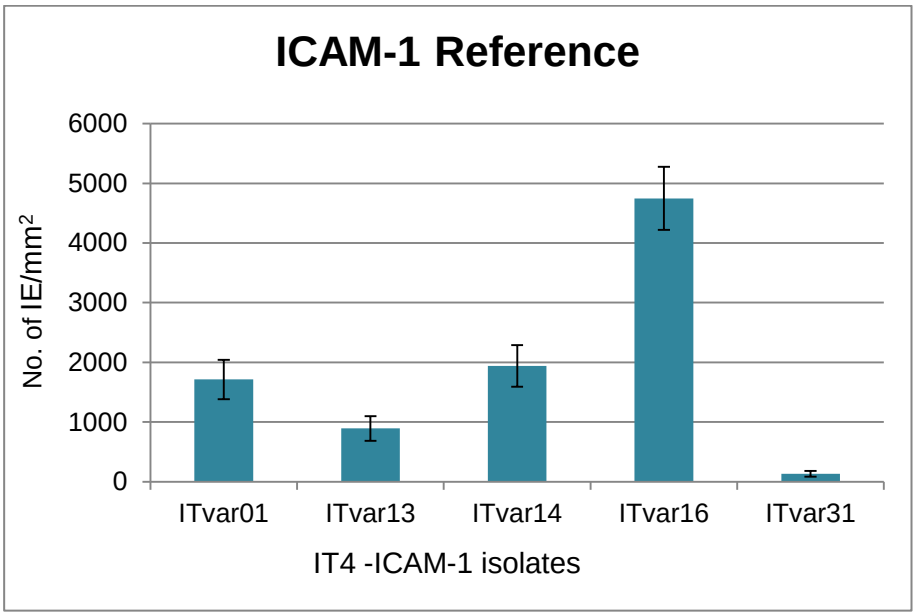

Figure 3.2.3: Static adhesion assay of IEs with IT4-ICAM-1 binding isolates, 2 µl spots at 50 µg/ml ICAM-1Ref were placed onto 6 cm dishes and standard protein static binding assays carried out with IE suspended in binding buffer at a parasitaemia of 3% and a haematocrit of 1%. The results show the mean of binding and the bars represents SE (n >3).

3.2.3.3 Static adhesion of IT4-ICAM-1 isolates binding to mutant ICAM-1 variants:

Despite the variation between the avidities of IT4-ICAM isolates binding to ICAM-1^{Ref}, all isolates except IT*var*01 were significantly affected by the S22/A mutation. In fact, it increased the binding of ITvar01. The natural mutant ICAM-1^{Kilifi} reduced the binding of three isolates to nearly no binding. However, for IT4var16 there was no significant effect and this is in agreement with previous data (Tse et al., 2004). Importantly, as it was shown in the previous chapter, ICAM-$1^{L42/A}$ significantly affected the binding of all isolates compared to their binding to ICAM-1^{Ref}, emphasising the importance of this conserved residue for binding. ICAM-$1^{L44/A}$ reduced the binding for three of IT4-ICAM isolates. It did not affect the binding of IT4var16 at all. On the other hand, it increased the binding of IT4var14 to about 143% compared to the ICAM-1^{Ref} binding. Results are summarised in (Tables 3.2.2- 3.2.5 and Figures 3.2.4-3.2.7)

A) The binding of IT4-isolates to ICAM-1$^{S22/A}$:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	2779	3	307
IT4var13	83	3	20
IT4var14	1221	9	285
IT4var16	336	20	67
IT4var31	38	3	8

Table 3.2.2: Static adhesion assay of IEs with IT4-ICAM-1 binding isolates, ICAM-1$^{S22/A}$ was used at 50 µg/ml. The table shows the mean of the IEs binding, N: number of experiments and the SE.

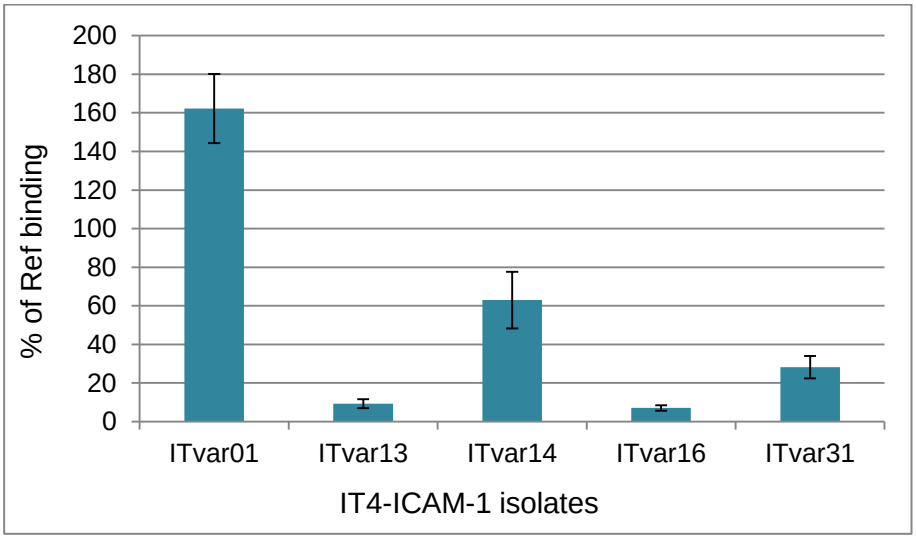

Figure 3.2.4: Static adhesion assay of IEs with IT4-ICAM-1 binding isolates, ICAM-1$^{S22/A}$ was used at 50 µg/ml, the results show the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.2.2).

B) The binding of IT4-isolates to ICAM-1^{Kilifi}:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	29	3	5
IT4var13	31	3	7
IT4var14	897	9	235
IT4var16	3807	20	489
IT4var31	17	3	2

Table 3.2.3: Static adhesion assay of IEs with IT4-ICAM-1 binding isolates, ICAM-1^{Kilifi} was used at 50 µg/ml, the table shows the mean of the IEs binding, N: number of experiments and the SE.

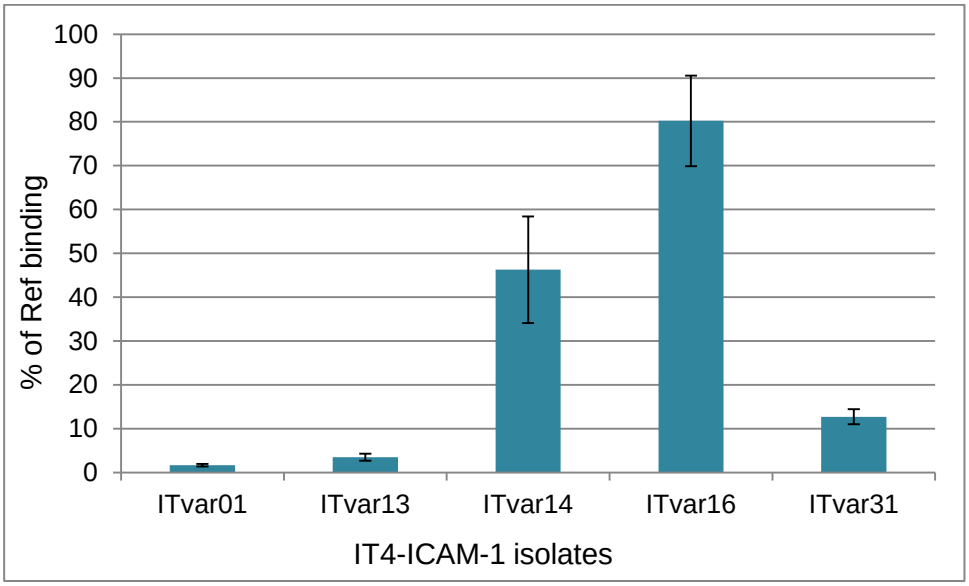

Figure 3.2.5: Static adhesion assay of IEs with IT4-ICAM-1 binding isolates, ICAM-1^{Kilifi} was used at 50 µg/ml, the figure shows the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.2.3).

C) The binding of IT4-isolates to ICAM-1$^{L42/A}$:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	14	3	10
IT4var13	28	3	11
IT4var14	281	3	187
IT4var16	103	4	13
IT4var31	14	3	3

Table 3.2.4: Static adhesion assay of IEs with IT4-ICAM-1 binding isolates, ICAM-1$^{L42/A}$ was used at 50 µg/ml, the table shows the mean of the IEs binding, N: number of experiments and the SE.

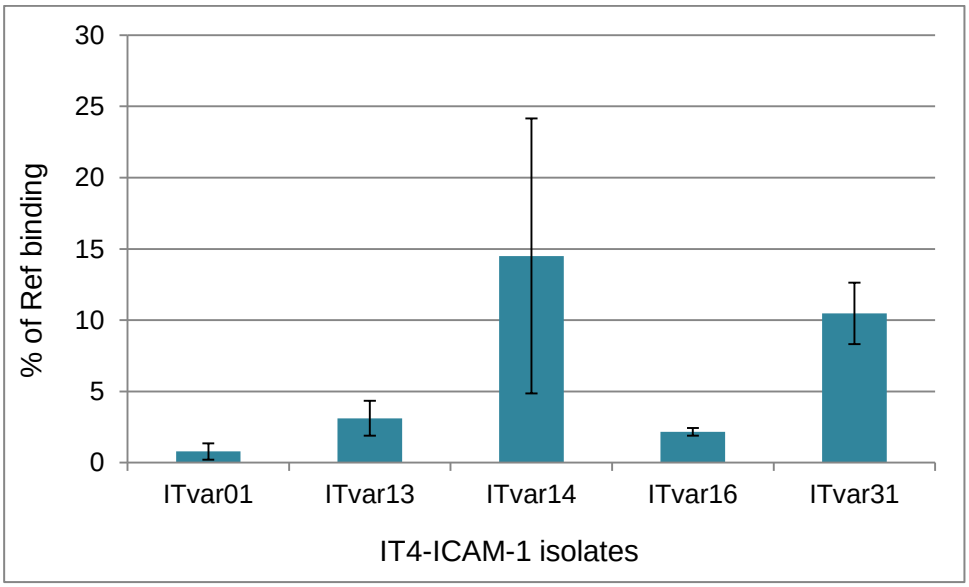

Figure 3.2.6: Static adhesion assay of IEs with IT4-ICAM-1 binding isolates, ICAM-1$^{L42/A}$ was used at 50 µg/ml. The figure shows the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.2.4).

D) The binding of IT4-isolates to ICAM-1$^{L44/A}$:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	481	3	222
IT4var13	276	3	84
IT4var14	2767	3	851
IT4var16	4920	4	648
IT4var31	58	3	23

Table 3.2.5: Static adhesion assay of IEs with IT4-ICAM-1 binding isolates, ICAM-1$^{L44/A}$ was used at 50 µg/ml, the table shows the mean of the IEs binding, N: number of experiments and the SE .

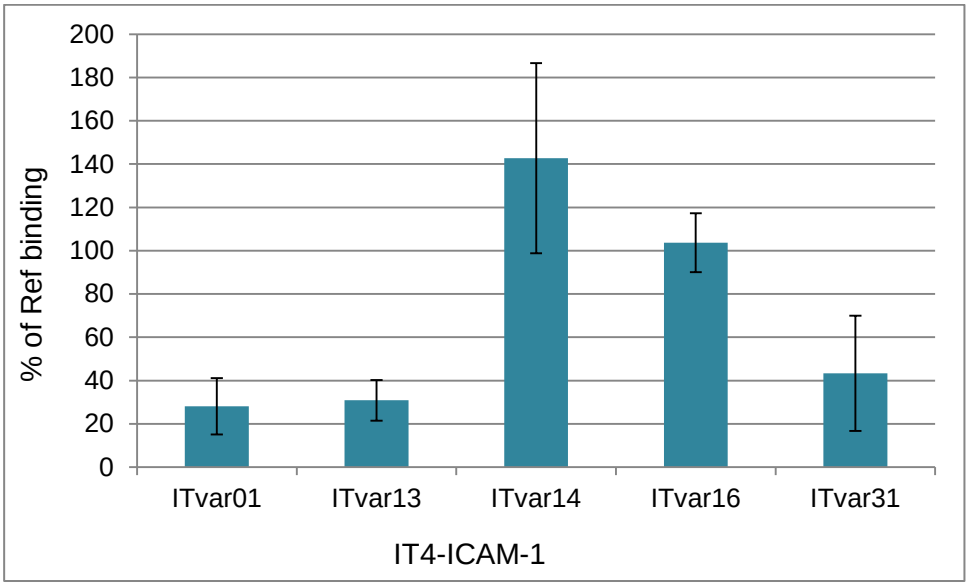

Figure 3.2.7: Static adhesion assay of IEs with IT4-ICAM-1 binding isolates, ICAM-1$^{L44/A}$ was used at 50 µg/ml, the figure shows the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.2.5).

3.2.3.4 Inhibition of IT4-ICAM-1 isolates using different anti-ICAM-1 mAbs:

As mentioned in the previous chapter, the effect of anti-ICAM-1 mAbs on the binding of IE to purified ICAM-1 under static conditions has been studied using specific mAbs reacting with epitopes on Ig-like domains 1 and 2. MAbs 15.2, BBIG-I1 and My13 mapping to domain 1, and 8.4A6 mAb mapping to domain 2 were used in a study that differentiated between the binding sites on ICAM-1 for IE and LFA-1 (Berendt et al., 1992). The previous chapter showed that different mAbs have different inhibitory effects on the isolates. Table (3.2.6) and figure (3.2.8) show the binding of all isolates was nearly completely reduced by mAb 15.2, except for ITvar14 which was inhibited by about 70%, though it should be noted the SE is relatively wide. Two anti-ICAM-1 mAbs, My13 and BBIG-I1, showed essentially a similar inhibitory effect to 15.2. The binding for IT4var01, IT4var13 and IT4var31 was almost totally inhibited. Moreover, IT4var14 and IT4var16 were reduced by about 80% compared to the binding to ICAM-1^{Ref} (Tables 3.2.7 and 3.2.8 and Figures 3.2.9 and 3.2.10). The effect for 8.4A6 anti-ICAM-1 mAb was only significant on one isolate. The inhibition caused by 8.4A6 did not affect the binding of IT4var16 and IT4var31isolates at all. However, IT4var01 was completely inhibited by 8.4A6 and the effect was varied, giving 25% and 50% for IT4var13 and IT4var14 respectively (Table 3.2.9 and Figure 3.2.11).

A) The inhibition of IT4-isolates using 15.2 mAb:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	60	3	12
IT4var13	21	3	8
IT4var14	649	4	249
IT4var16	158	4	48
IT4var31	4	3	1

Table 3.2.6: Static inhibition assay of IEs with IT4-ICAM-1 binding isolates, 15.2 was used at 5 µg/ml, the table shows the mean of the IEs binding, N: number of experiments and the SE.

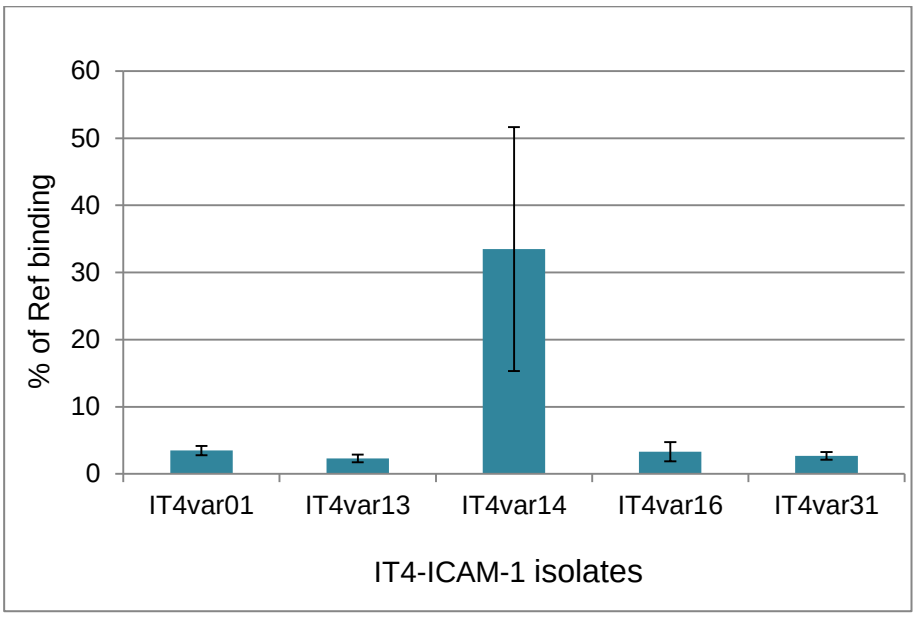

Figure 3.2.8: Static inhibition assay of IEs with IT4-ICAM-1 binding isolates, 15.2 was used at 5 µg/ml, the figure shows the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.2.6).

B) The inhibition of IT4-isolates using My13 mAb:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	48	3	10
IT4var13	22	3	8
IT4var14	300	4	80
IT4var16	685	4	184
IT4var31	3	3	1

Table 3.2.7: Static inhibition assay of IEs with IT4-ICAM-1 binding isolates, My13 mAb was used at 5 µg/ml, the table shows the mean of the IEs binding, N: number of experiments and the SE.

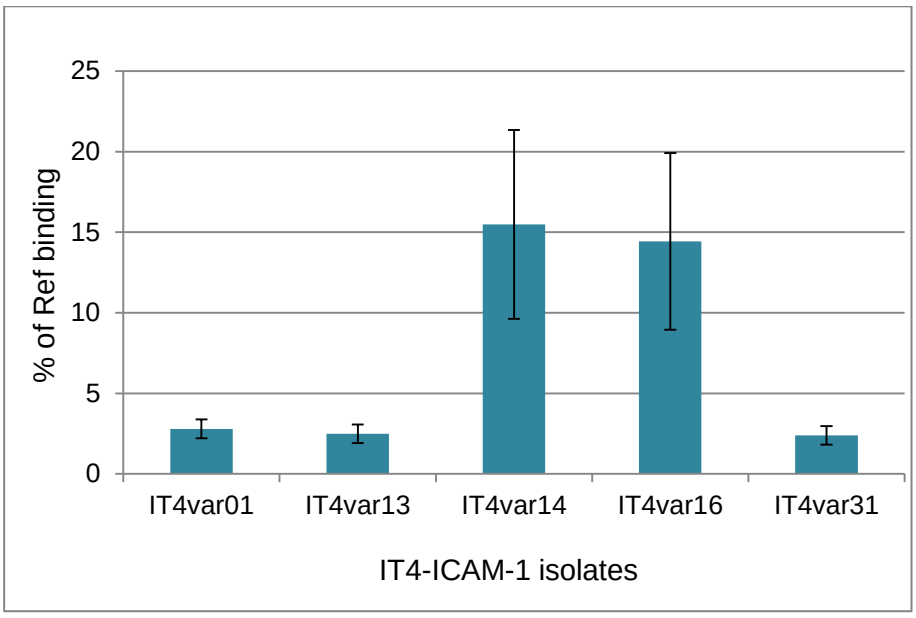

Figure 3.2.9: Static inhibition assay of IEs with IT4-ICAM-1 binding isolates, My13 mAb was used at 5 µg/ml, the figure shows the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.2.7).

C) The inhibition of IT4-isolates using BBIG-I1 mAb:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	63	3	15
IT4var13	26	3	5
IT4var14	503	4	129
IT4var16	984	4	182
IT4var31	5	3	1

Table 3.2.8: Static inhibition assay of IEs with IT4-ICAM-1 binding isolates, BBIG-I1 mAb was used at 5 µg/ml, the table shows the mean of the IEs binding, N: number of experiments and the SE.

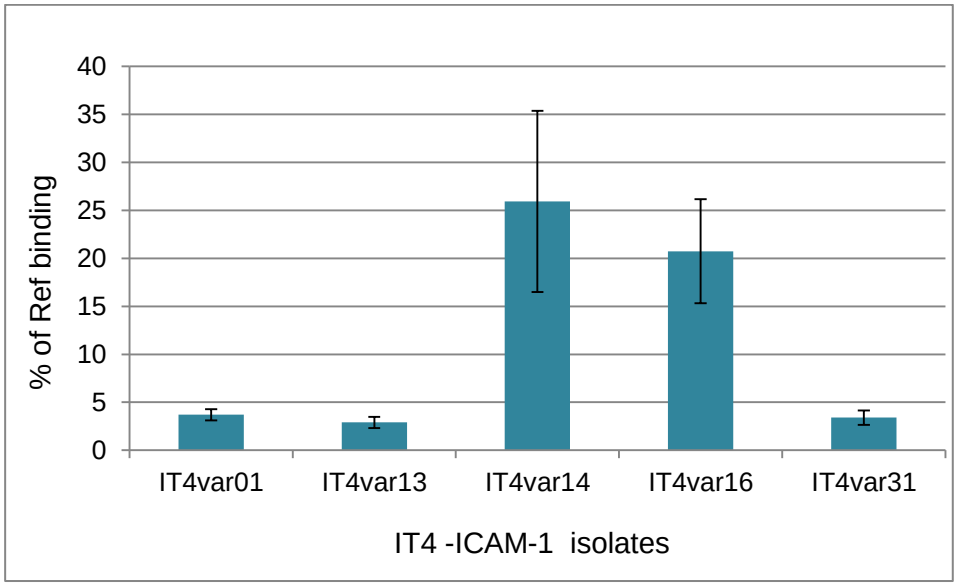

Figure 3.2.10: Static inhibition assay of IEs with IT4-ICAM-1 binding isolates, BBIG-I1 mAb was used at 5 µg/ml, the figure shows the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.2.8).

D) The inhibition of IT4-isolates using 8.4A6 mAb:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	53	3	11
IT4var13	652	3	183
IT4var14	885	4	366
IT4var16	5494	4	1597
IT4var31	140	3	51

Table 3.2.9: Static inhibition assay of IEs with IT4-ICAM-1 binding isolates, 8.4A6 mAb was used at 5 µg/ml, the table shows the mean of the IEs binding, N: number of experiments and the SE.

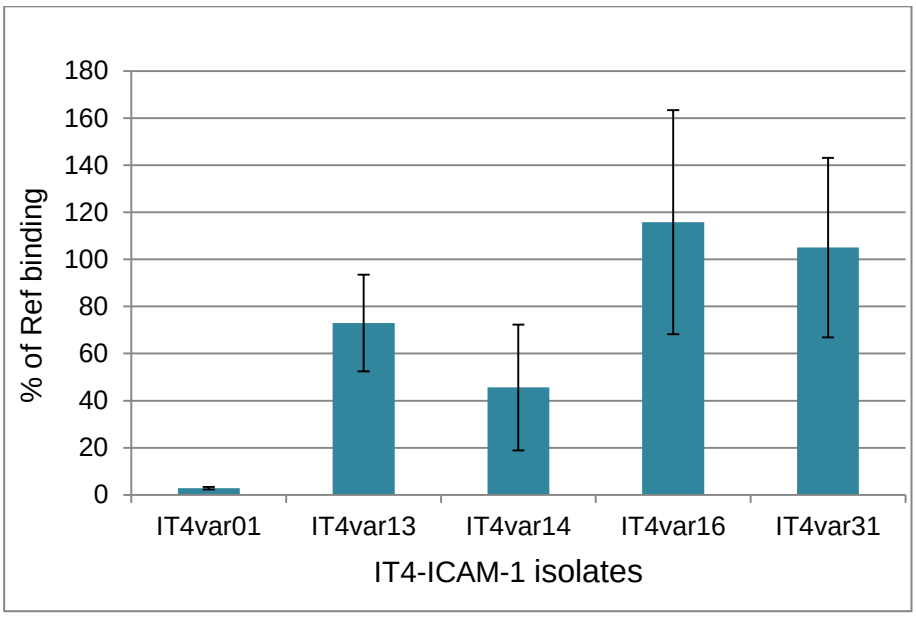

Figure 3.2.11: Static inhibition assay of IEs with IT4-ICAM-1 binding isolates, 8.4A6 mAb was used at 5 µg/ml, the figure shows the %ICAM-1^{Ref} binding and the bars represent SE (n can be found in table 3.2.9).

3.2.3.5 The binding of IT4-ICAM-1 isolates on HDMEC under flow conditions:

The binding to ICAM-1^{Ref} was variable from protein based static adhesion assays. Despite this variation, all isolates bound to HDMEC in a relatively similar pattern in the range of 200-300 IE/mm^2, with a slight increase to 464 IE/mm^2 for ITvar01 (Figure 3.2.12). Further investigation using an anti-ICAM-1, 15.2 mAb, revealed more than 85% of binding was inhibited for IT4var13. However, the inhibition was less for IT4var01 and IT4var31 inhibiting the binding by 40% and 30% respectively (Table 3.2.10 and Figure 3.2.13). Furthermore, because HDMEC expresses CD36, when the binding was blocked by anti-CD36 mAb, it was reduced by about 90% in all of the isolates. In fact, the binding of IT4var31 was inhibited by more than 95% (Table 3.2.11 and Figure 3.2.14).

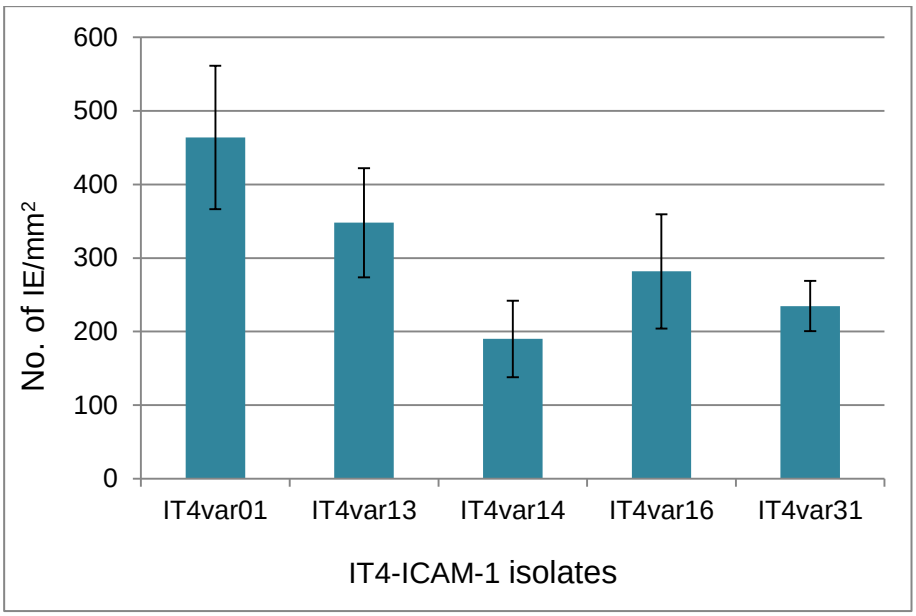

Figure 3.2.12: HDMEC flow endothelial cell adhesion assay of IT4-ICAM-1 isolates. HDMEC seeded on channels pre-coated with fibronectin; IE were passed on confluent cells for five minutes followed by washing by binding buffer for two minutes before counting 6 fields in two different channels. The parasitaemia was 3% and a haematocrit of 2%. The results show the mean of and the bars represents SE.

ICAM-1 inhibition on HDMEC for IT4-isolates using 15.2 mAb:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	276	3	37
IT4var13	46	3	11
IT4var14	91	3	10
IT4var16	115	15	18
IT4var31	165	2	18

Table 3.2.10: Flow ICAM-1 inhibition of IEs with IT4-ICAM-1 binding isolates on HDMEC, 15.2 mAb was used at 5 µg/ml. The table shows the mean of the IEs binding in the presence of 15.2 mAb, N: number of experiments and the SE.

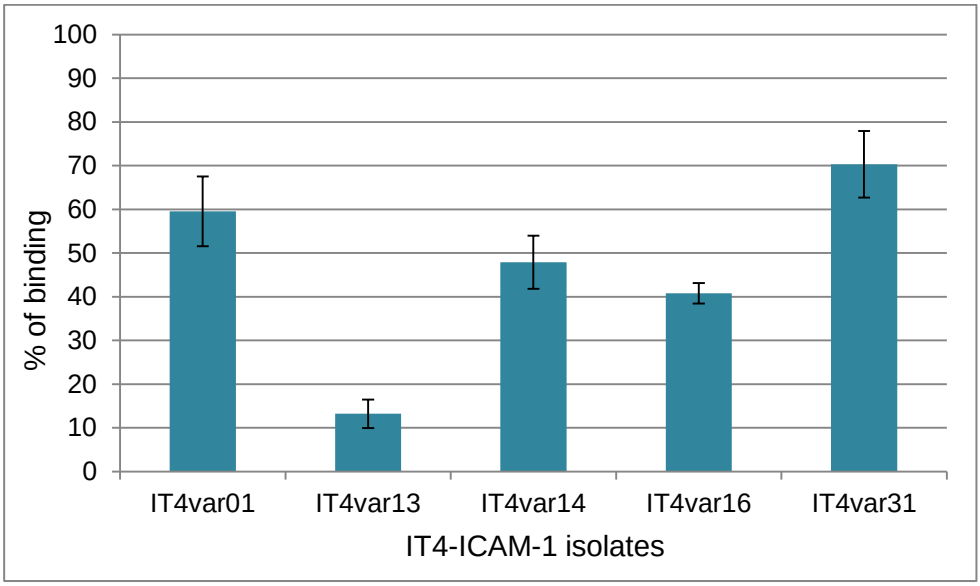

Figure 3.2.13: Flow ICAM-1 inhibition of IEs with IT4-ICAM-1 binding isolates on HDMEC using 15.2 mAb at 5 µg/ml. The figure shows the % binding against no inhibitory effect and the bars represents SE (n can be found in table 3.2.10).

CD36 inhibition on HDMEC for IT4-isolates using IVC7 mAb:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	57	3	8
IT4var13	51	3	18
IT4var14	22	8	3
IT4var16	26	3	3
IT4var31	6	2	3

Table 3.2.11: Flow CD36 inhibition of IEs with IT4-ICAM-1 binding isolates on HDMEC, IVC7 was used at 5 µg/ml. The table shows the mean of the IEs binding in the presence of IV-C7 mAb, N: number of experiments and the SE.

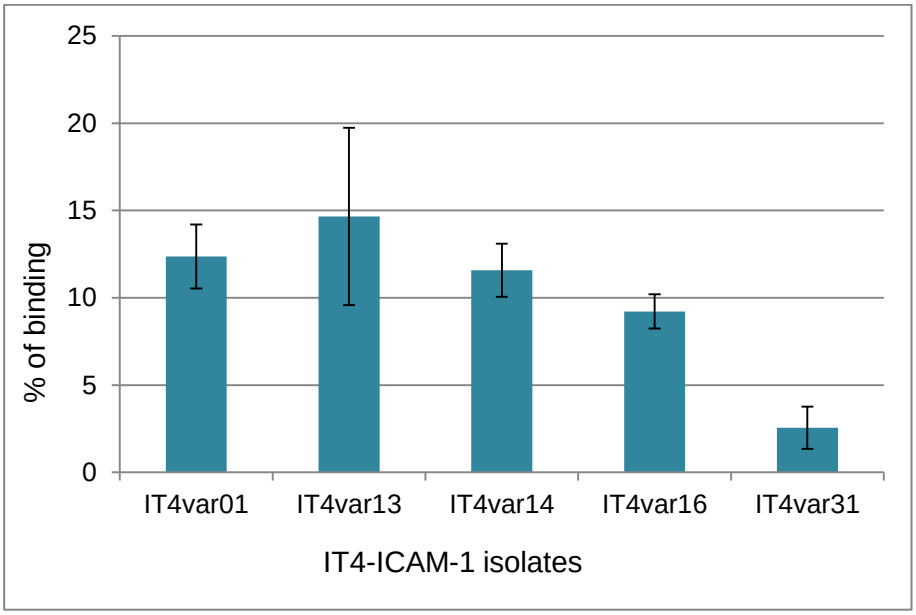

Figure 3.2.14: Flow CD36 inhibition of IEs with IT4-ICAM-1 binding isolates on HDMEC using IV-C7 mAb at 5 µg/ml. The figure shows the % binding against no inhibitory effect and the bars represents SE (n can be found in table 3.2.11).

3.2.4 Discussion:

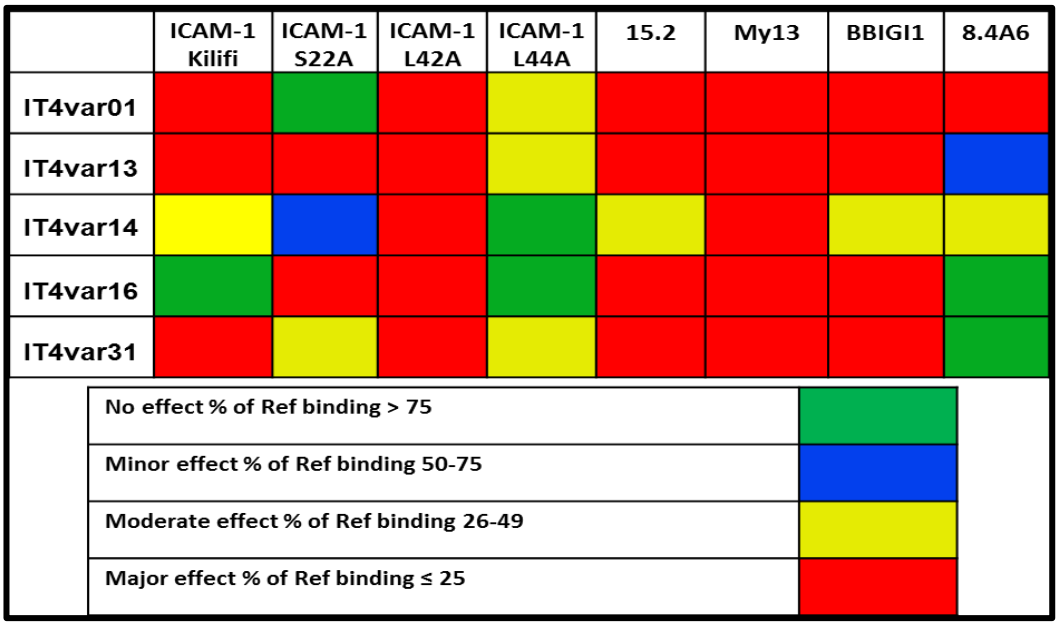

Figure 3.2.15: Summary of the effects of mutant ICAM-1 variants and mAb inhibition on IT4-ICAM-1 isolates.

Studying PfEMP1 interactions with host receptors is significant to understand the mechanism of malaria pathogenesis. It is hoped it will lead to vaccine development once appropriate targets have been identified, for example, the PfEMP1::ICAM-1 interaction is thought to be involved in CM although not exclusively. Previous studies have demonstrated that PfEMP1-ICAM-1 interaction is entirely mediated by a single DBLβ domain and have also shown that there is an overlap between the binding site of IE on ICAM-1 and other receptors such as LFA-1, Mac-1 and Rhinoviruses but has distinct elements. Moreover, the binding site of different parasites is disrupted by different mutations leading to subtle differences between the various parasite variants.

In the current chapter, the binding of isogenic IT4-ICAM-1 isolates was characterised on purified ICAM-1 variants under static conditions. The difference between this part and the previous one is mainly using laboratory clones that express known ICAM-1- binding PfEMP1 variants confirmed by qRT-PCR from one genotype, IT4. Also, ICAM-1 blocking by different mAbs was investigated. It was not surprising to see aspects

of distinct adhesion for all PfEMP1 variants based on the use of ICAM-1 variants binding and inhibition using mAbs against ICAM-1.

Mutant ICAM-1s and anti-ICAM-1 mAbs approaches have illustrated that isogenic IE expressing variant PfEMP1 members bind to different residues in the DE loop of ICAM-1 (Summarised in Figure 3.2.15). However, incorporating current data with data from the previous chapter supports the use of the conserved region as a basis for designing cross-variant inhibitors of adhesion, with a significant role for L42 for all ICAM-1-binding isolates examined so far. Using SPR Brown *et al.* (2013) observed that the binding of PfEMP1 occurs to an overlapping binding site on ICAM-1. This was shown by the competition of two different DBLβ domains to bind to ICAM-1. The dissociation of the first bound domain decreased upon the exposure of the second domain. The degree of ICAM-1 binding variation observed in the previous chapter is also observed for the IT4-ICAM-1 isolates even though they are genetically identical.

Howell et al. (2008) determined that rDBLβ of some IT4 isolates bound to ICAM-1 and suggested that variant single rDBLβ domains were capable of binding ICAM-1 almost equally based on their method. In contrast, this is not the case when the whole PfEMP1 is expressed on the surface of IEs, as seen in IE binding studies. It was shown that PfEMP1 architecture experiences slight changes upon binding to ICAM-1 (Brown et al., 2013). However these changes were not *in vivo*, where it might introduce significant structural effects.

Interestingly IT4VAR31 was the lowest ICAM-1 binder among the isolates tested here. This is consistent with previous adhesion characterisation and predicted to be due to secondary structure folding of the binding domain (Smith et al., 2000a). It was also reported that ITVAR31 DBLβ bound ICAM-1 with the lowest affinity among IT4-ICAM-1 isolates investigated before (Brown et al., 2013). Binding to HDMEC is exactly following the pattern observed for lab-adapted ICAM-1 isolates as described in the previous chapter (Madkhali et al., 2014). However,

IT4var31, the lowest binder on purified ICAM-1 bound to HDMEC similarly to other isolates. ICAM-1 mAb treatment showed about 30% inhibition suggesting a role of CD36 in mediating the binding.

The affinities of DBLβ of IT4-ICAM-1 reported by the Brown et al. study (Brown et al., 2013) correlated with IE adhesion level on ECs but did not correlate with the IE adhesion level on purified proteins in the current study. The affinity of DBLβ of IT4var13 and IT4var16 was 2.6 and 51.1 nM respectively, but the binding of IT4var13 was lower than the of IT4var16 as shown by static protein assay. On the other hand, the level of the binding of IT4var13 was higher than all IT4-ICAM-1 isolates on HDMEC except for IT4var01 that was not included in Brown et al. study. The high affinity of IT4var13 was supported by the fact that it showed the most reduced binding when ICAM-1 was blocked on HDMEC and the least affected by anti-CD36 mAb. CD36 binding varied between IT4 isolates expressing different PfEMP1 variants under flow conditions ((Figure 4.2.2) shown in the chapter4.2). This is consistent with a recent study examined rolling velocities to CD36 under dynamic flow conditions (Herricks et al., 2013). The authors thought that PfEMP1 sequence variability or surface expression levels play an essential role in mediating the whole binding avidity of IEs. The modelling of the interactions between PfEMP1 and human receptors were based on recombinant proteins. It should be considered that binding properties may be changed by other domains on whole PfEMP1 and more possible changes can take place on the surface of IEs when surrounded by many structural molecules (Janes et al., 2011).

It is also interesting to know that all IT4-ICAM-1 isolates are DBLβ5 except IT4var31, which is DBLβ3 (and the smallest in terms of domain composition, more details are found in chapter 4.2). Janes and colleagues investigated if DBLβ subclasses can predict ICAM-1 binding based on the recombinant DBLβ-ICAM-1 binding data. It was shown that all DBLβ5 domains from IT4 genotype bound to ICAM-1. Moreover, an extra DBLβ3 domain bound ICAM-1, IT4var31. It was suggested DBLβ5

could be a marker for ICAM-1 adhesion phenotype. This was not only seen in IT4 isolates but also from an Indian isolate that bound strongly to ICAM-1 called JDP8-ICAM-1, although only one isolate of JDP8 has been examined so far. Based on both adhesion data in this study and kinetic parameters from Brown *et al.* study, it can be suggested that IE with isolates expressing DBLβ5 PfEMP1 bind at higher levels and more avidity than IE with isolates expressing DBLβ3 PfEMP1 (at least among IT4 isolates) on purified ICAM-1. However, this is not to argue that DBLβ5 PfEMP1s are associated with disease severity, which has not been tested. Another criticism against overplaying the role of DBL β5 is due to the existence of only one isolate that expresses DBLβ3 among IT4-ICAM-1 isolates.

In fact, from other genotype, 3D7, there was a DBLβ3 PfEMP1 that bound to ICAM-1. However, adhesion data of the 3D7-ICAM-1 binder that express DBLβ3 was shown based on different adhesion method and so probably no valid comparison can be argued from this data. The current study should have included DBLβ3 3D7-ICAM-1 binder isolate to widen the adhesion phenotype of DBLβ3 isolates. Particularly, when it is known that this is a Group A isolate and based on the shared combinations of short tandem domains, known as DCs, it is DC4 which has been associated with SM (Bengtsson et al., 2013). Group A *var* genes are more conserved than the others and have been linked with severe malaria (Jensen et al., 2004). There is, therefore, a deficiency about the prediction of the ICAM-1 binding based on a single domain subclass. Moreover, the transcription of DBLβ3 was more associated with SM patients and associated with group A PfEMP1 (Bengtsson et al., 2013). Indeed, this is different from predicting the association of DBLβ subclasses with disease outcome, which is still not established. More information about the binding of DC4 isolates to ECs and the expression of these isolates from clinical samples may provide a useful link for therapeutic interventions.

3.3. The endothelium and cytoadhesion assays perspective: a comparison between the binding of IT4-ICAM-1 isolates on activated HUVEC and HBMEC.

3.3.1. Introduction:

It has been more than a century since Marchiafava and Bignami found the pivotal observation that IEs are sequestered in the brain microvasculature, affecting the blood flow by causing a mechanical obstruction. Cytoadhesion is a form of sequestration that has a major role in malaria pathogenesis either directly by blockages of blood vessels or indirectly by inflammation mediation, coagulation defects and endothelium dysfunction (Storm and Craig, 2014). The endothelium plays distinctive roles in several physiological processes including the blood flow regulation, coagulation, permeability, inflammation, and innate and adaptive immunity (Aird, 2012). ECs have, also, significant heterogeneity in their structure which is detected by the expression of certain receptors on their surfaces which are so called vascular “ZIP codes or molecular signatures”. The variation of the surface receptor phenotypes plays a critical role in the pathogenesis of several diseases, including malaria (Aird et al., 2014). These variations, beside the variations of molecules exported to surface of IEs, result in variable binding properties of the IEs to host endothelia and consequently variable malaria outcomes (Moxon et al., 2011, Aird et al., 2014).

One of the major advancements in malaria research was the ability to culture *P. falciparum in vitro* in 1976. This came in parallel with the successful isolation and culturing of ECs *in vitro*. Together, this allowed the study of *P. falciparum* adhesion phenotypes to explore therapeutic interventions to control malaria morbidities and mortalities (Udeinya et al., 1981). From this, the role of the interactions between parasite proteins on the surface of IEs with host endothelium has been studied. Studying adhesion of IEs to ECs *in vitro* has been performed using different methods. It was first described using confluent HUVEC on

coverslips under static conditions (Udeinya et al., 1981). Then, to simplify the culturing conditions and expand the number of adhesion studies, an alternative model based on transfected melanoma cells was described (Schmidt et al., 1982). Indeed, these methods have contributed to revealing significant information about the adhesion of IE, despite some concerns raised about receptor expression levels in transfected melanoma cells (C32). Then, in order to investigate more about the interactions between IEs and individual receptors, purified receptors were placed in petri dishes, and incubated with parasites to facilitate the study of IEs binding (Ockenhouse et al., 1991). This, for example, has allowed the in-depth study of interactions such as the use of mutant proteins for determining the binding sites that mediate specific interactions.

Until now, static protein and cell adhesion assays are still commonly used and produce useful results. Nonetheless, they do not resemble an accurate model of the cytoadherence mechanisms that occur in hosts (Nash *et al*., 1992; Rogerson *et al*., 1997). The fact that static assays exclude the roles of variable forces affecting the binding of IEs inside the vasculature raised the need for a model that included the shear stress controlling the blood flow. Thus, the flow adhesion assay was developed to enable adhesion studies in the relevant environment that mimic, albeit approximately, physiological conditions (Nash et al., 1992, Cooke et al., 1994). This enables investigators to describe the dynamics and kinetics of the interaction of the IEs with their ligands, i.e. the concepts that describe the behaviours of IEs on different receptors such as tethering, rolling and firm adhesion linked to the function of human receptors on ECs (Cooke et al., 1994). For example, ICAM-1 is thought to play tethering role and rolling on ECs, whereas IEs do not require rolling on CD36. Although most phenotypes observed using static assays show similar behaviour under flow, an exception was found that IT4var16 (ItG), a high ICAM-1 binder on static assay, was a relatively very weak binder on flow systems using HUVEC (Gray et al., 2003).

To improve the flow model, the use of microfluidics has been adopted to study parasite adhesion, which allows for further refinement about the behaviours of IEs on different receptors considering the size of the channels used in microfluidics. For example, the use of the commercial Cellix system in our lab has given an advantage of using very small quantities of materials to run the experiments (Madkhali et al., 2014). This could be particularly useful in field studies where enough materials usually hamper the studies. The Cellix system is currently being used in Malawi, although some challenging issues are being encountered in using complex equipment in this setting(personal communication).

A very recent study has shown ECs in brain microvasculature might have a different response to curvature than ECs from non-brain cells, regardless of their size, such as HUVEC and HDMEC. The curvature in this context means the reciprocal of the monolayers of endothelial cells radius (Ye et al., 2014). Analysis of the influence of curvature and shear stress on cell morphology has shown no significant change in HBMECs. In contrast, HUVECs showed a significantly increased elongation under shear stress in comparison to static conditions. Nonetheless, these changes were smaller than induced by curvature, emphasising the vital role for curvature in controlling cell morphology (Ye et al., 2014).

The aim of this part of the thesis was comparing the pattern of binding of IT4-ICAM-1 isolates on HUVEC and HBMEC. This was carried out using flow adhesion assays.

3.3.2 Methods:

Parasite and endothelial cell cultures, and the flow adhesion assay were described in the general methods. The parasite isolates details have been given in Table 3.2.2.1 (Previous chapter). ICAM-1 expression on ECs was confirmed using FACS as described in the general methods.

3.3.3 Results:

3.3.3.1 Receptor expression:

Preliminary sets of FACS experiments confirmed the expression of the positive endothelial cell marker CD31 with and without TNF stimulation. EPCR expression was higher if not stimulated and slightly reduced upon TNF stimulation. This is consistent with previous reports that confirmed this expression of ECs receptors.

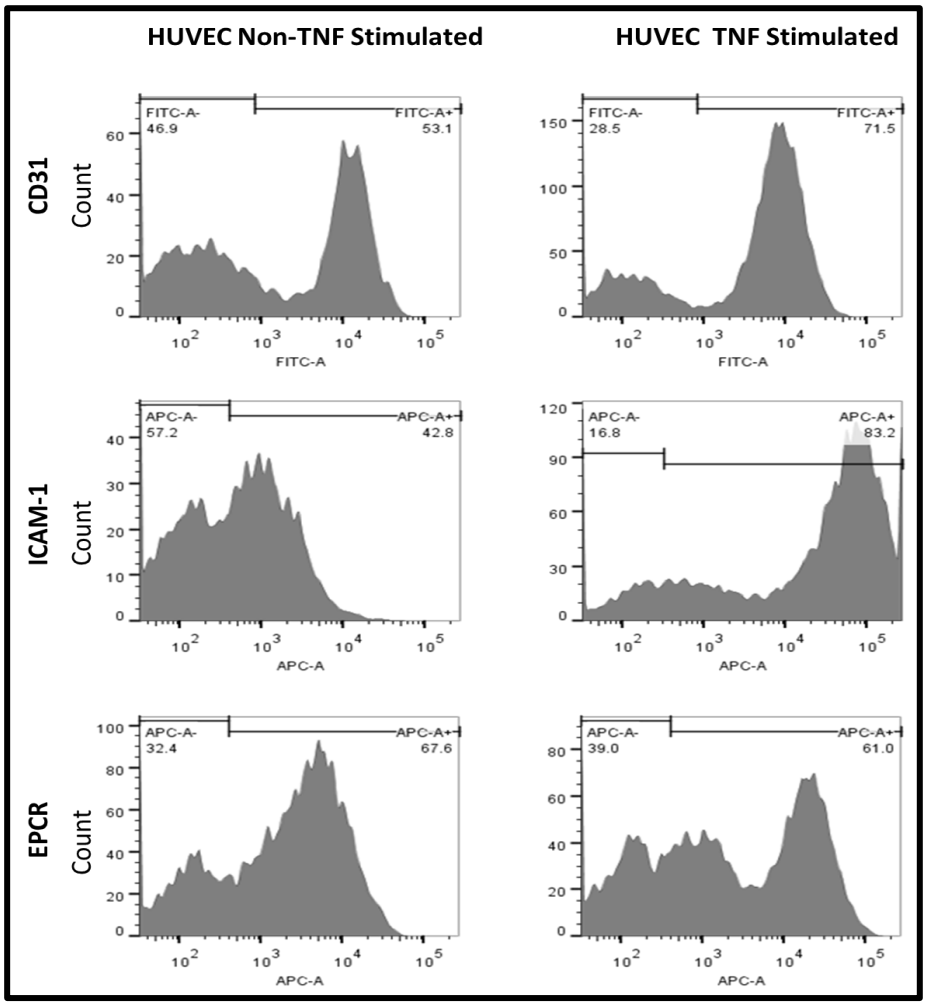

Figure 3.3.1: Preliminary FACS analysis of ICAM-1 and EPCR expression on HUVEC without (left panel) and with (right panel) TNF stimulation. TNF stimulation was for 24 hrs using 10 ng/ml. Endothelial positive marker (CD31), ICAM-1 and EPCR are shown at the upper, the middle and the bottom panels respectively.

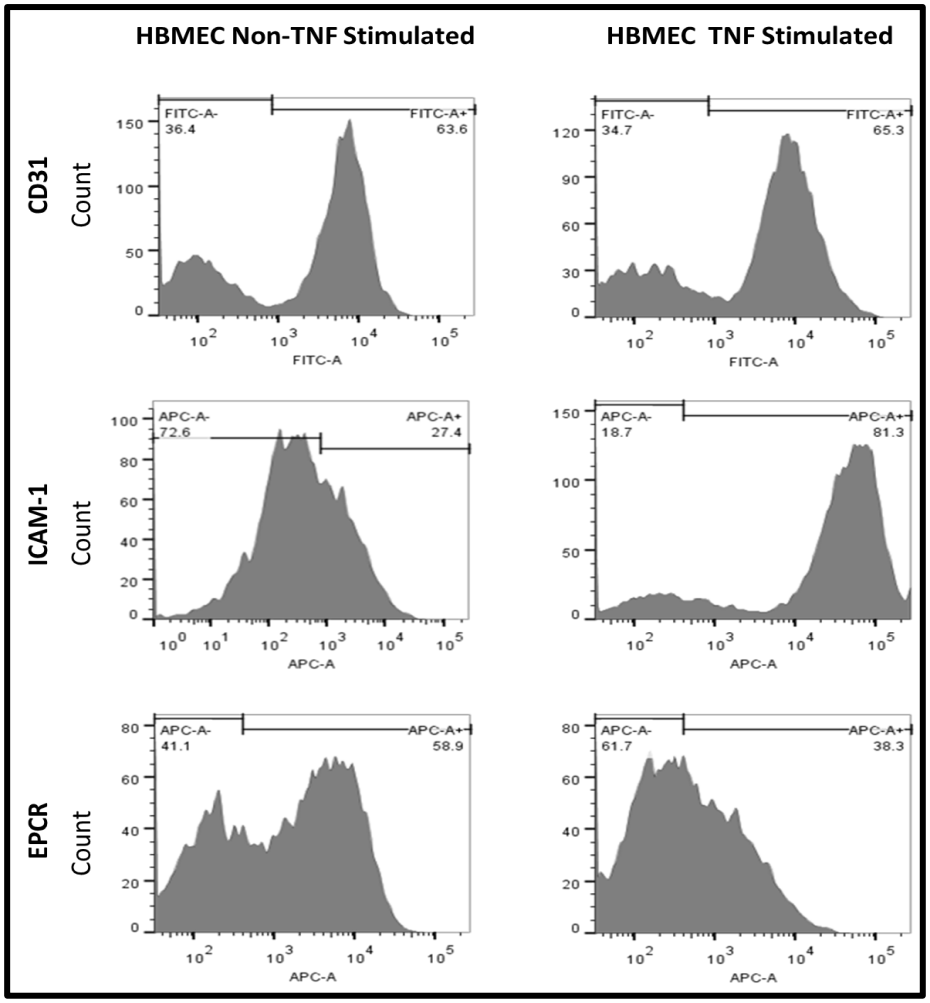

Figure 3.3.2: Preliminary FACS analysis of ICAM-1 and EPCR expression on HBMEC without (left panel) and with (right panel) TNF stimulation. TNF stimulation was for 24 hrs using 10 ng/ml. Endothelial positive marker (CD31), ICAM-1 and EPCR are shown at the upper, the middle and the bottom panels respectively.

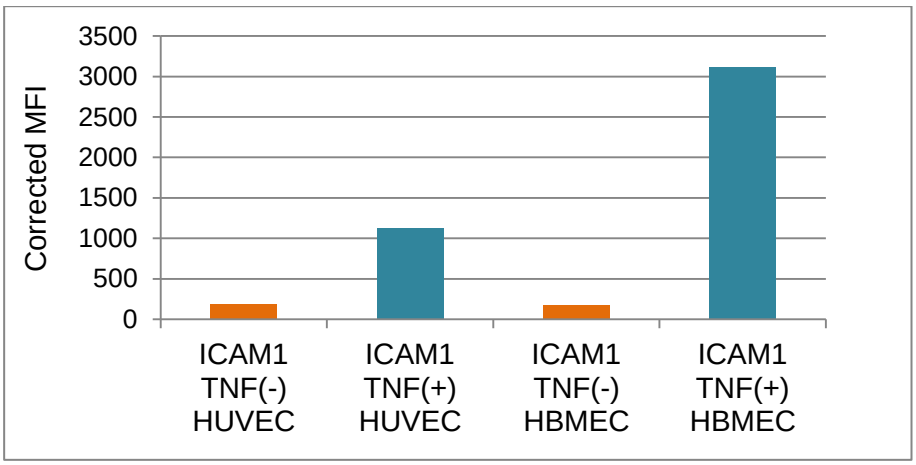

Figure 3.3.3: Preliminary FACS analysis of HUVEC and HBMECs confirming ICAM-1 expression without TNF stimulation and with TNF stimulation. The graph shows the corrected mean fluorescence intensity.

3.3.3.2 The binding of IT4-ICAM-1 isolates on HUVEC and HBMEC under flow conditions:

All the isolates tested were known ICAM-1 binders from data derived using static and flow adhesion assays as shown in previous chapters. The pattern of binding on HBMEC was the same as that on HUVEC. The quantities of IEs bound on HBMEC were comparable to the binding to HUVEC (Figure 3.3.4, Tables 3.3.1 and 3.3.2). However, anti- ICAM-1 (15.2) affected the binding on HBMEC more than HUVEC for all isolates except one (Figure 3.3.5, Tables 3.3.3 and 3.3.4). Previous characterisation has shown that IT4var16 bound relatively low to HUVEC under flow condition despite it being recognised as a higher avidity binder to purified ICAM-1 and cells under static condition. The binding was nearly similar to IT4var31, which has lower avidity to ICAM-1 as shown in the previous section. Importantly, this phenotype has also been noticed to HBMEC as well.

Parasites	mean IE binding/mm^2	N	SE
IT4var01	377	3	79
IT4var13	166	3	24
IT4var14	240	3	28
IT4var16	56	2	11
IT4var31	30	2	1

Table 3.3.1: the binding of IT4-ICAM-1 isolates to HUVEC. The table presents the means of IE binding/mm^2 and the number of experiments and the SE.

Parasites	mean IE binding/mm^2	N	SE
IT4var01	296	3	64
IT4var13	184	3	36
IT4var14	248	3	49
IT4var16	74	2	22
IT4var31	29	2	20

Table 3.3.2: the binding of IT4-ICAM-1 isolates to HBMEC. The table presents the means of IE binding/mm^2 and the number of experiments and the SE.

Parasites	mean IE binding/mm^2	N	SE
IT4var01	15	3	6
IT4var13	32	3	14
IT4var14	76	3	7
IT4var16	7	3	5
IT4var31	10	2	4

Table 3.3.3: ICAM-1 inhibition of IEs with IT4-ICAM-1 binding isolates on HUVEC when 15.2 mAb was used at 5 µg/ml. The table shows the mean of the IEs binding in the presence of 15.2 mAb, N: number of experiments and the SE.

Parasites	mean IE binding/mm^2	N	SE
IT4var01	18	3	4
IT4var13	2	3	1
IT4var14	27	3	2
IT4var16	3	3	2
IT4var31	6	2	2

Table 3.3.4: ICAM-1 inhibition of IEs with IT4-ICAM-1 binding isolates on HBMEC when 15.2 mAb was used at 5 µg/ml. The table shows the mean of the IEs binding in the presence of 15.2 mAb, N: number of experiments and the SE.

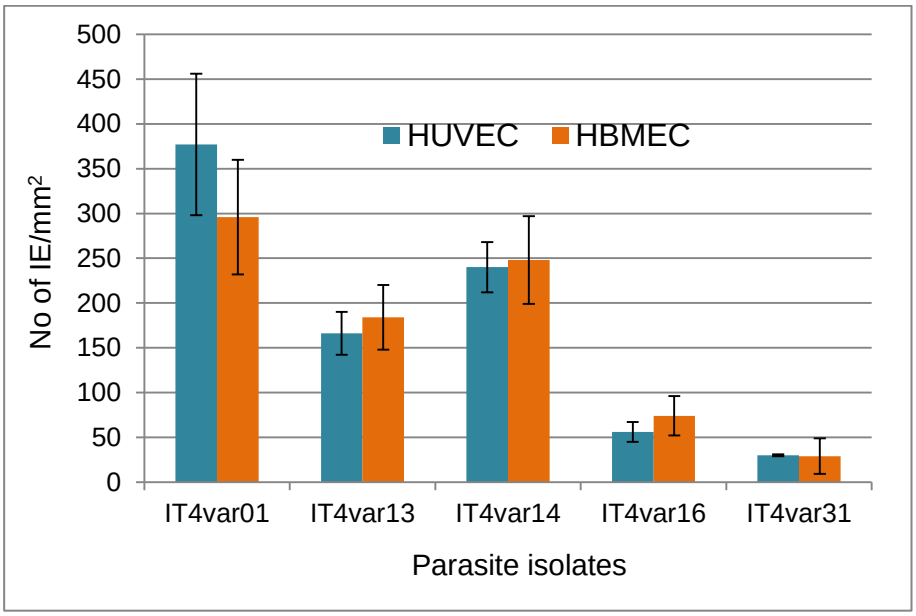

Figure 3.3.4: The binding of IT4-ICAM-1 isolates to HUVEC and HBMEC. The results show the mean and the bars represents SE (n can be found in tables 3.3.1 and 3.3.2).

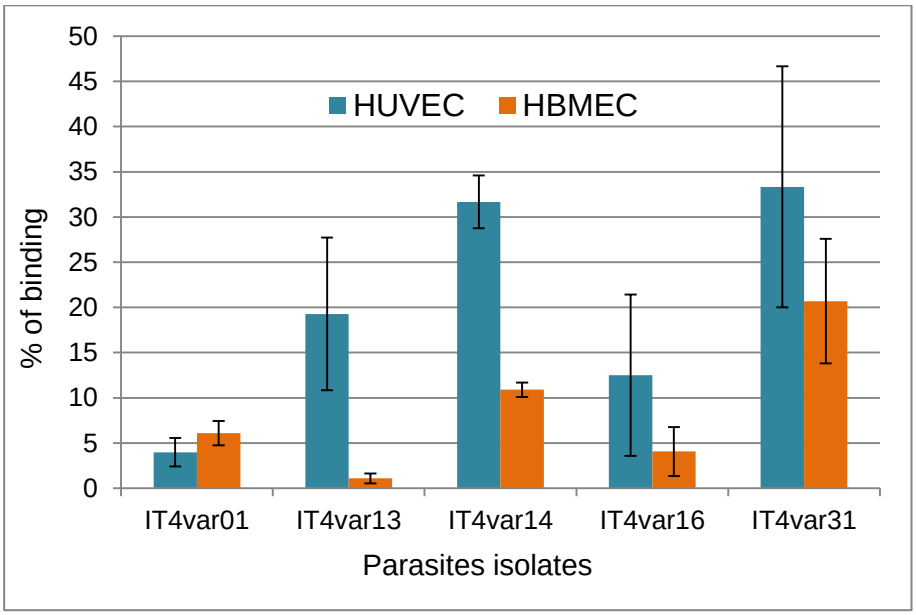

Figure 3.3.5: ICAM-1 inhibition of IEs with IT4-ICAM-1 binding isolates on HUVEC and HBMEC using anti-ICAM-1 (15.2) mAb at 5 µg/ml. The figure shows the % binding against no inhibitory effect and the bars represents SE (n can be found in tables 3.3.3 and 3.3.4).

3.3.4 Discussion:

Diverse endothelial receptors have been associated with binding to different variants of PfEMP1 (Rowe et al., 2009, Turner et al., 2013, Esser et al., 2014). Among these receptors is ICAM-1, which is expressed on diverse ECs including HUVEC and HBMEC. Previous adhesion studies investigating the role of ICAM-1 in cytoadhesion were carried out using HUVEC due to the difficulty of HBMEC isolation and routine culturing *in vitro* in the early studies (Udeinya et al., 1981). HUVEC is similar to HBMEC in their abilities to express ICAM-1 and not expressing CD36. In contrast, HDMEC expresses both ICAM-1 and CD36. However, HUVEC is different from HBMEC, because HUVEC is isolated from large cells, whereas HBMEC is isolated from microvascular ECs. This chapter aimed to characterise the binding of IT4-ICAM-1 isolates on HBMEC and HUVEC to examine their binding patterns under flow conditions.

The adhesion that occurred on HBMEC was almost similar to the adhesion on HUVEC (Fig 3.3.3). In the previous chapters, it has been shown that the binding on HDMEC was in the range of 200-300 IE/mm^2 for most of the isolates including IT4var16 and IT4var 31. However, the binding of these two isolates was low on both HUVEC and HBMEC under flow conditions. The low binding of IT4var31 was consistent with the binding data from protein adhesion results in 3.2 chapter and biophysical analysis (Brown et al., 2013). The binding of IT4-ICAM-1 isolates on HDMEC was discussed before and thought to be CD36 mediated as shown by mAb inhibition data. Although the IT4-ICAM-1 isolates were proven as ICAM-1 binders having subtle differences in affinities ranging from 2.8 nM to 144 nM. The low binding of the IT4var16 to HUVEC is observed for HBMEC as well. This emphasises the complexity of binding that occurs in the cellular context, because in protein assay the IT4var16 was shown to have highest avidity to ICAM-1, whereas the binding to ECs that express ICAM-1 was relatively low binding. Indeed, PfEMP1 architecture including its structure, length, domain compositions plays such role for optimising the binding (chapter 4.2 will analyse binding data based on the domain compositions).

The dynamics inside the human body must be considered to understand the pathological events of malaria. In this context, the adhesion cascade of leukocytes is the most studied example and has given insights into the rheological events that take place at the molecular and cellular levels. This cascade was long recognised to have three major steps; rolling, activation and arrest. However, recent updates have considered other additional steps; slow rolling, adhesion strengthening, spreading, intravascular crawling before cellular transmigration (Ley et al., 2007, Robert et al., 2013). Indeed, ICAM-1 plays a significant role in this cascade by mediating binding to the LFA-1, which is recognised as a mobile molecule.

Mobile LFA-1 is explained by the ability to switch the molecular conformational state transiently to mediate its biological role, though the

mechanism is not fully understood. It has been highlighted that LFA-1 can mediate lateral mobility across the membrane in order to regulate integrin activation and adhesion to ICAM-1. The mobility is altered upon chemokine stimulation. Similarly, it might be considered that the mobile PfEMP1 can be affected by several variables including stimulation effects, the architecture of the PfEMP1 variant, and other possible effects. For example, the knobs form an average of 135 nm protrusions on the IEs, and the DBLβ of IT4var13 was found at about 15 nm above the IE membrane enabling ready access to the ligand. The position of this domain at the tip of the extracellular domain could facilitate efficient recognition and increase the possibility of binding to other ligands explaining the co-operation seen in endothelial binding (Brown et al., 2013).

A parasite sub-line of 3D7 expressing two distinct *var* genes on a single IE adhered to both ICAM1 and PECAM1 and bound efficiently to ECs, however more investigations are required to support these findings to re-evaluate the extent of any breakdown in the mutually exclusive *var* gene expression model (Joergensen et al., 2010) Not only that but, also, it is started to recognise that one adhesion form can influence another adhesion form contributing to the parasites sequestration. This was demonstrated by a single IE that mediated cytoadhesion and rosetting simultaneously via two different receptors (Adams et al., 2014). These several interactions inside the host must be put into consideration to widen the view of the multi-cellular events that take place to understand the malaria pathophysiology.

It is known that ICAM-1 is induced upon inflammatory stimulation, and it binds to diverse ligands including LFA-1, Mac-1 and even to monocytes which express ICAM-1. The interactions between proteins inside the hosts are exposed to many influences including competitive interactions with several cellular factors and post-translational modifications (Herce et al., 2013). In a recent study on experimental CM in mice, glycocalyx was lost in brain vessels more than in UM (Hempel et al., 2014). This effect

enhances the interactions of leukocytes and platelets with the endothelial cells which have been shown to play a potential role in malaria pathophysiology (Wassmer et al., 2011, Hempel et al., 2014). Loss of endothelial glycocalyx may be caused by several mechanisms including inflammation and hypoxia which are crucial in CM pathology (Hempel et al., 2014).

In addition, it was suggested that HBMEC were different from other small and large vasculatures in their interactions with some agents including α-thrombin which is involved in the coagulation cascade. In Moxon et al. (2013) study, thrombomodulin was found to play a role in CM. The recent finding of EPCR involvement in SM has enhanced the understanding that multiple processes take place inside the host to cause disease, such as coagulation as well as more standard inflammatory responses.

In fact, many events besides the cytoadhesion must be considered during the infection including inflammatory responses, platelet coagluopathology and endothelial dysfunction. Thus, the host might contribute to the disease outcome by induced endothelial damage that can take place by different process independent of cytoadherence including metabolic acidosis and plasma uric acid (N'Dilimabaka et al., 2014). Also, persistent endothelial activation and inflammation were observed up to one month after the infection. This was recognised by the elevated plasma levels of soluble ICAM-1, angiopoetin 2, and C-reactive protein (Moxon et al., 2014). It is possible that damaged ECs increase the disease severity by increasing the risk of developing co-infection contributing to the high mortality in Sub-Saharan Africa. Mallewa *et al.* (2013) found that the interaction between *P. falciparum* and viral infections could increase the risk of developing severe outcomes (Mallewa et al., 2013).

Unfortunately, adhesion studies *in vivo* are hampered due to the lack of appropriate animal models that represent human disease pathophysiological manifestations (Craig et al., 2012a). The considerable information about the host-parasites interactions has been provided

through *in vitro* cytoadhesion studies. There is an appreciable number of adhesion studies carried out in the field that used static proteins assays (Ockenhouse et al., 1991, Chaiyaroj et al., 1996, Newbold et al., 1997, Rogerson et al., 1999, Heddini et al., 2001, Chilongola et al., 2009, Ochola et al., 2011, Almelli et al., 2014). There are variations in the outcomes of these studies (Craig et al., 2012b). However, there is a need for multi-expert decision to standardise a model for adhesion assay (cells of choice and system) to ensure a comprehensive usage of the ongoing research to study adhesion phenotypes. It is thought that the need to implicate the flow adhesion system using endothelial cells may well bridge the gaps between the roles of different adhesion receptors. Particularly, a valid model is, now, available to investigate the rosetting phenotypes using flow assays. Recently, it has been shown that a dual binding phenotype occurred on ECs with rosetting for the same isolate (Adams et al., 2014).

However, using ECs in field studies with fresh patient isolates is quite difficult (Janet Storm personal communication). However, it is important to use ECs at least to avoid the major disadvantage of protein adhesion study that focus on individual receptors. It is now apparent that different receptors mediate different functions such as static and rolling adhesions by CD36 and ICAM-1 respectively, which may work synergistically (Chakravorty and Craig, 2005, Rowe et al., 2009). Recently seven extracellular domains from IT4var19, a HBMEC selected isolate, bound to multiple ECs and most domains bound almost equally to all endothelial cell types. However, individual domains have shown differences in the level of binding to specific cell types (Avril et al., 2013).

In conclusion, despite the differences observed between HUVEC and HBMEC in a recent report (Ye et al., 2014), HUVEC was shown to be a reliable model for ICAM-1 adhesion phenotypes that occur on HBMEC.

4.1: UpsC PfEMP-1 analysis:

4.1.1 Introduction:

Malaria successful transmission continues in the presence of human hosts as carriers, vectors and appropriate environment. WHO reported 198 million total malaria cases in 2012 (uncertainty range: 124–283 million) and the vast majority of cases were UM. Ineffective case management might lead to the development into SM, contributing to the morbidity and mortality of the disease. Some efforts targeting malaria transmission have been successful (WHO, 2013). However, asymptomatic carriers render malaria transmission control problematic by providing vital reservoirs of parasites for mosquitoes. The ongoing host reservoirs remain a major obstacle for control programs in endemic countries due to the sequestered parasites in humans avoiding immune responses and facilitating transmission. This sequestration is mediated by variable PfEMP1 members encoded by *var* genes.

It has been mentioned before that in spite of their great diversity, most *var* genes can be classified based on the upstream sequence (ups), chromosomal location and direction of transcription into three major groups (ups A, B and C) and two intermediate groups (B/A and B/C). UpsA and upsB genes are subtelomeric genes that are oriented tail to tail, whereas ups C genes are found in the centre of the chromosomes and are oriented head to tail in a tandem repeat manner (Kraemer and Smith, 2003, Lavstsen et al., 2003). Recent data have suggested strong associations between upsA PfEMP1 and SM (Jensen et al., 2004, Avril et al., 2012, Bengtsson et al., 2013, Lavstsen et al., 2012, Turner et al., 2013). In contrast, upsC and some of upsB were associated with UM (Jensen et al., 2004, Kyriacou et al., 2006, Rottmann et al., 2006).

Evidence about these associations comes mainly from *var* genes expression from infected individuals studied by qRT-PCR (Mugasa et al., 2012, Kyriacou et al., 2006, Rottmann et al., 2006). Also, data acquired from the reactivity of immune sera with laboratory isolates *in vitro*, such

as selection of 3D7 parasites on pooled plasma from semi-immune children from Ghana and Tanzania (Jensen et al., 2004). The result showed that up-regulated *var* genes were among the group A and group B/A. In contrast, group C members were mostly down-regulated suggesting their association with mild malaria. However, the adhesion of IEs with identified PfEMP1 variants that are potentially linked with UM has been rarely addressed (Janes et al., 2011). The first part of the current chapter will be more on PfEMP1 variants that are favour the establishment of chronic malaria, a concept that is often ignored in the usual focus on SM.

The establishment of chronic malaria is linked with a shift in PfEMP1 expression and it is thought that this trait of PfEMP1 is towards variants that are less adapted for optimal adhesion (that cause serious effects upon the host) (Buckee and Recker, 2012). However, some studies have shown that UM is more associated with CD36-adhesion phenotype (Ochola et al., 2011, Newbold et al., 1997). The linkage between human receptor usage and PfEMP1 expression in mild disease has been controversial. For example, a very recent report found that binding to CD36 is associated with CM more than ICAM-1 (Almelli et al., 2014). However, there are some concerns about the adhesion method used in this study due to the concentrations of used proteins and time of incubation.

The molecular mechanisms of the adhesion of isolates found associated with UM cases in the cellular context should be investigated. Studying the adhesion phenotypes of upsC PfEMP1 proteins will provide more information to understand their role in causing chronic infections. It was hoped to broaden the vision about these essential members particularly as being important within the malaria eradication/ elimination programs. The project has many challenges to find a reliable conclusion based on available data. Nevertheless, current data confirms the diversity of *var* genes that can be implicated with adhesion phenotypes. In other words, there is no distinct adhesion phenotype that can be found from the limited

number of isolates examined in this study. For example, all upsC isolates of IT4 examined here bound to CD36, and one isolate bound to ICAM-1. In contrast, HB3 isolates did not bind either CD36 or ICAM-1.

The aim of the current chapter is characterisation of the binding phenotypes of IEs with different PfEMP1 variants from genetically distinct parasites to improve our understanding of their association with clinical data from field studies.

Part I:

i- Assess upsC isolates' abilities to bind CD36 and ICAM-1 under static and flow conditions.

ii- Characterise upsC isolates binding phenotypes on HDMEC, HUVEC and HBMEC under flow conditions.

Part II:

i- Collect the adhesion data from flow assays of IEs isolates with known PfEMP1 domain compositions and analyse their adhesion phenotypes based on the form of PfEMP1 expressed.

4.1.2 Methods:

4.1.2.1 Parasite culture:

All isolates were grown as described in general methods. A table of upsC isolates describing their alias, their *var* gene ID and the literature they were used in, is provided below (Table 4.1.1). The synchronisation was performed using sorbitol and Plasmion.

Alias	ID	The source and *var* expression confirmation
3G8	IT4var01	(Janes et al., 2011)
4E12	IT4var37	
P5C2	IT4var43/66	
BA06	HB3var35	(Noble et al., 2013)
BH08	HB3var29	
CH05	HB3var31	
DF06	HB3var30	

Table 4.1.1: UpsC isolates from IT4 and HB3 parasites. The table presents the alias of the parasites from their original sources and their ID which is used in the text of the current thesis.

4.1.2.2 Selection for knobby IE (Plasmion flotation):

Described in the general methods.

4.1.2.3 Adhesion assays:

Static and flow protein adhesion assays were used to assess the binding of IE to CD36 and ICAM-1. Also, flow assays on endothelial cells were performed using HDMEC and HUVEC. The binding to CD36 and ICAM-1 on ECs was inhibited by 5 µg/ml of anti-CD36 IVC7 and 5 µg/ml anti-ICAM-1 15.2 mAbs respectively. All these procedures are explained in the general methods section, including culturing ECs.

4.1.2.4 Adult Hyperimmune sera (HIS) reactivity:

Described in the general methods.

4.1.3 Results:

4.1.3.1 Characterisation of upsC isolates binding to CD36:

All IT4 isolates bound to CD36 with different avidities. The binding of IT4var37 was about 1500 IE/mm^2, being the highest isolate bound to CD36. On the other hand, the binding of all of the HB3 isolates was nearly at the level of the negative background (PBS which usually gives few IEs after several washes (1-5 IE) (Table 4.1.2 and Figure 4.1.1). The same pattern was also observed under flow conditions. IT4var37 bound to CD36 with higher avidity than other IT4 isolates (Table 4.1.3 and Figure 4.1.2).

A) Static adhesion of upsC isolates to CD36:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	1257	3	42
IT4var37	1497	3	303
IT4var43/66	986	3	139
HB3var29	17	2	1
HB3va30	8	2	1
HB3var31	13	2	1
HB3var35	8	2	1

Table 4.1.2: Static adhesion of IE with upsC isolates to CD36. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

B) Flow adhesion of upsC isolates to CD36:

Parasite	mean IE binding/mm^2	N	SE
IT4var01	370	3	59
IT4var37	417	3	51
IT4var43/66	330	3	54
HB3var29	2	2	1
HB3var30	1	2	1
HB3var31	1	2	1
HB3var35	1	2	1

Table 4.1.3: Flow adhesion of IE with upsC isolates to CD36. The table presents the means of IE binding/mm^2, the number of experiments and the SE.

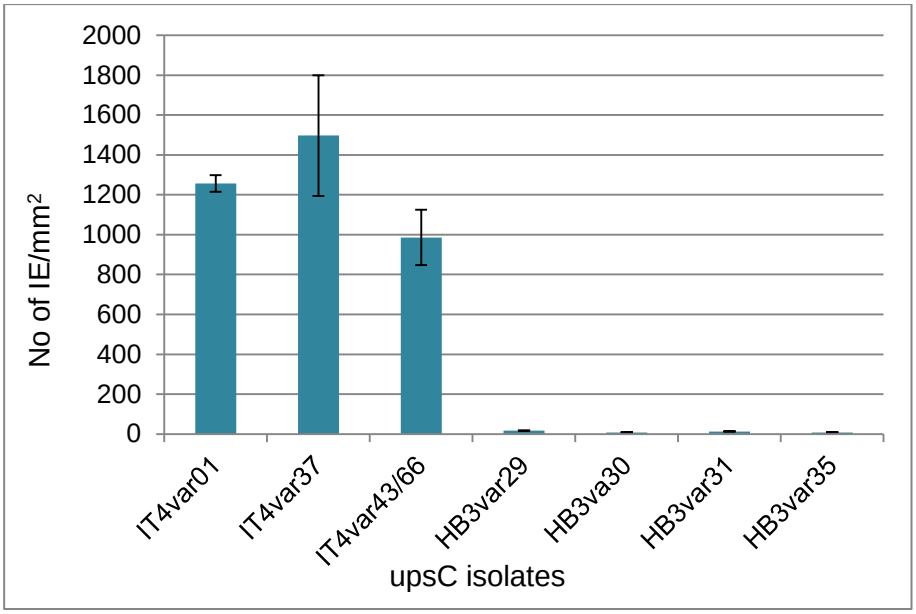

Figure 4.1.1: Static adhesion assay, the binding of upsC isolates to CD36. CD36 was used at 50 µg/ml. The figure shows the mean of binding (IE/mm^2) and the bars represents SE (Numbers are given in table 4.1.2).

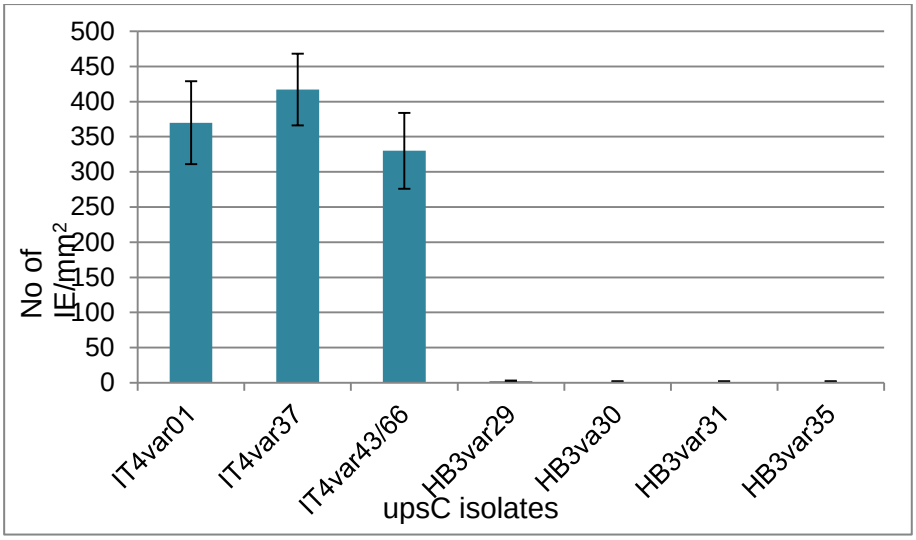

Figure 4.1.2: Flow adhesion assay, the binding of upsC isolates to CD36. 50 µg/ml of CD36 was used. The figure shows the mean of binding (IE/mm^2) and the bars represents SE (Numbers are given in table 4.1.3)

4.1.3.2 Characterisation of upsC isolates binding to ICAM-1:

Among all isolates, only IT4*var*01 bound with high avidity to ICAM-1, the binding was 1713 IE/mm^2. The binding seen with IT4var43/66 to ICAM-1 is thought due to the presence of IT4var31 expressing parasites from the origin of the isolate (Janes et al., 2011). Whereas IT4var37 was the highest CD36 binder, it and all the HB3 isolates did not bind to ICAM-1 at all under static and flow conditions (table 4.1.3 and figures 4.1.3 and 4.1.4).

A) Static adhesion of upsC isolates to ICAM-1:

Parasites	mean IE binding/mm^2	N	SE
IT4var01	1713	3	331
IT4var43/66	186	3	108

Table 4.1.4: Static adhesion of IE with upsC isolates to ICAM-1. The table presents the means of IE binding/mm^2, the number of experiments and the SE. Data of other isolates are not shown because they showed no detectable binding to ICAM-1.

B) Flow adhesion of upsC isolates on ICAM-1:

Parasite	mean IE binding/mm^2	N	SE
IT4var01	523	2	84
IT4var43/66	16	2	3

Table 4.1.5: Flow adhesion of IE with upsC isolates to ICAM-1. The table presents the means of IE binding/mm^2, the number of experiments and the SE. Data of other isolates are not shown because they showed no detectable binding to ICAM-1.

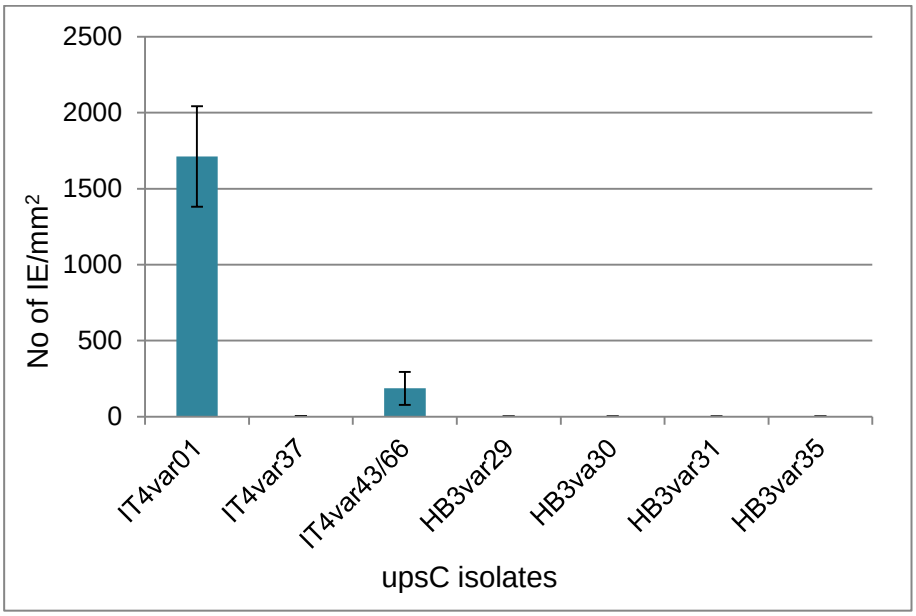

Figure 4.1.3: Static adhesion assay of upsC isolates to ICAM-1. ICAM-1 was used at 50 µg/ml. The figure shows the mean of binding (IE/mm^2) and the bars represents SE (n can be found in Table 4.1.4).

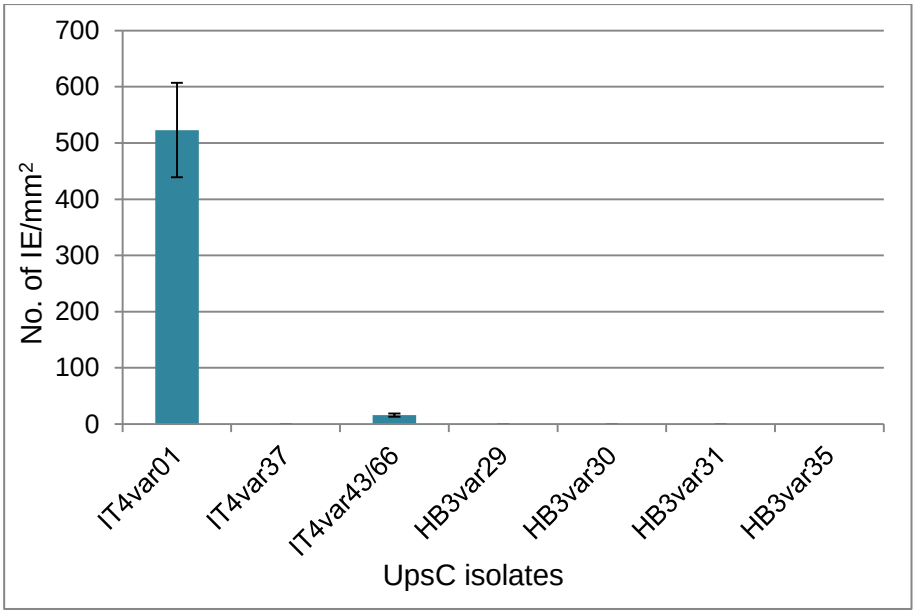

Figure 4.1.4: Flow adhesion assay of upsC isolates to ICAM-1. ICAM-1 was used at 50 µg/ml. The figure shows the mean of binding (IE/mm^2) and the bars represents SE (n can be found in Table 4.1.5).

4.1.3.3 Characterisation of upsC isolates on HDMEC under flow conditions:

The binding of upsC isolates to HDMEC was similar to the pattern of the binding to purified CD36. IT4var37, which bound to CD36 but did not bind to ICAM-1, was the highest HDMEC binder, even higher than isolates that bound to both CD36 and ICAM-1 such as IT4var01 and other IT-ICAM-1 isolates. In contrast, HB3 isolates were comparable to the binding of negative background (Table 4.1.4 and Figures 4.1.5). There was no difference for HB3 isolates when CD36 and ICAM-1 were blocked using anti-CD36 and anti-ICAM-1 mAbs. On the other hand, IT4var37 was almost totally inhibited by anti-CD36 mAb and no effect was observed upon ICAM-1 blocking, supporting the adhesion data. The effect of CD36 blocking was about 90% for all IT4 isolates as found before (previous chapters).

Parasites	mean IE binding/mm^2	N	SE
IT4var01	464	3	98
IT4var37	628	2	104
IT4var43/66	152	2	34
HB3var29	8	2	1
HB3var30	11	2	1
HB3var31	8	2	1
HB3var35	6	2	1

Table 4.1.6: the binding of IE with upsC isolates to HDMEC. The table presents the averages of IE/mm^2 and the number of experiments and the SE.

A) Flow adhesion of upsC isolates to HDMEC:

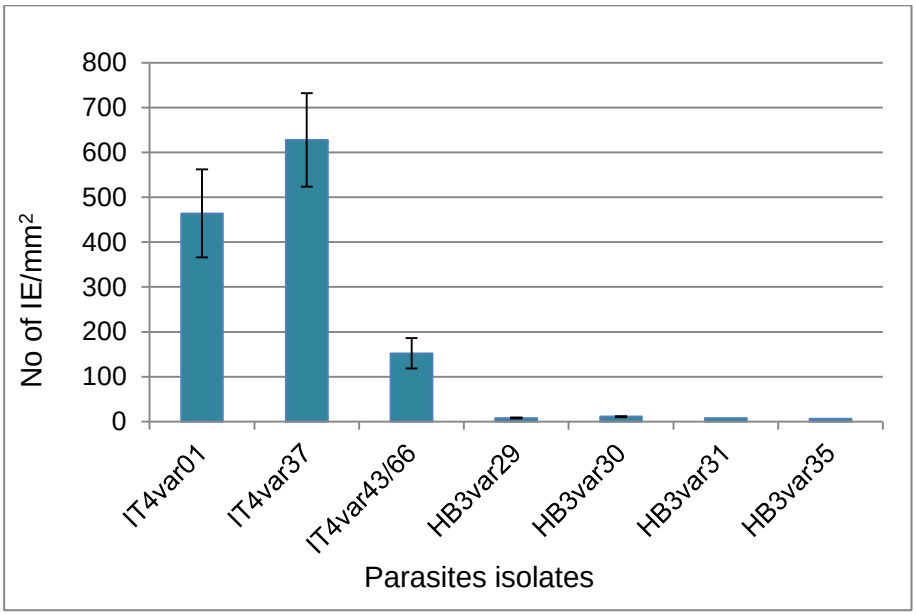

Figure 4.1.5: Flow adhesion assay, the binding of upsC isolates to HDMEC. The figure shows the mean of binding (IE/mm^2) and the bars represents SE (n can be found in Table 4.1.6).

B) Inhibition of upsC isolates on HDMEC using anti-CD36 and anti-ICAM-1 mAbs under flow condition:

A) CD36 inhibition	Parasites	mean IE/mm^2	N	SE	B) ICAM-1 inhibition	Parasite	mean IE/mm^2	N	SE
	IT4var01	57	3	8		IT4var01	236	3	37
	IT4var37	41	2	15		IT4var37	647	2	136
	IT4var43/66	19	2	7		IT4var43/66	75	2	10

Table 4.1.7: The binding of IE with upsC isolates to HDMEC in the presence of 5 µg/ml IV-7 (anti-CD36 mAb) (A) and in the presence of 5 µg/ml 15.2 (anti-ICAM-1 mAb) (B). The Table presents the means of bound IE/mm^2 and the number of experiments and the SE.

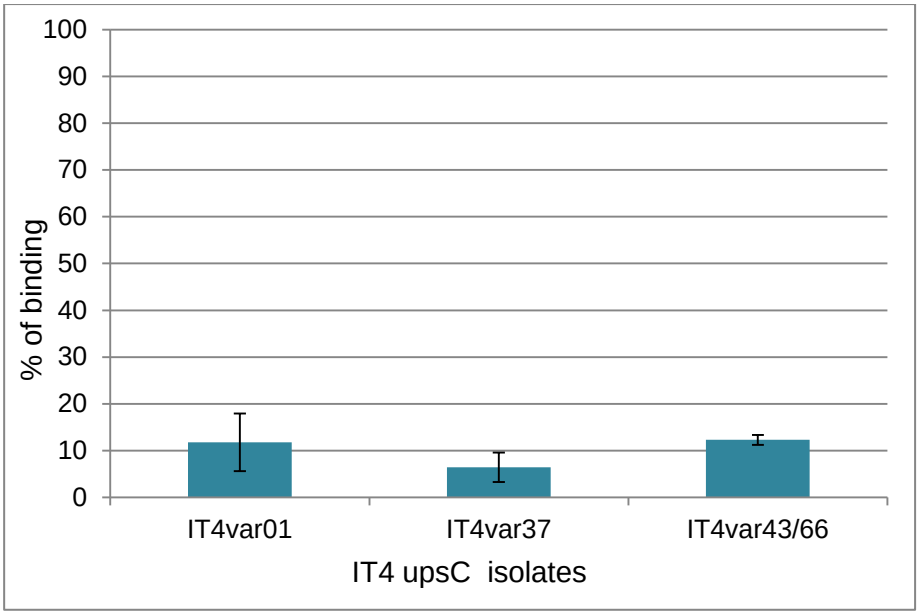

Figure 4.1.6: HDMEC CD36 inhibition of IT4 upsC isolates in the presence of 5 µg/ml IV-C7 (anti-CD36 mAb). The graph presents the % of binding compared to the binding on HDMEC with no mAb effect. (n can be found in Table 4.1.7)

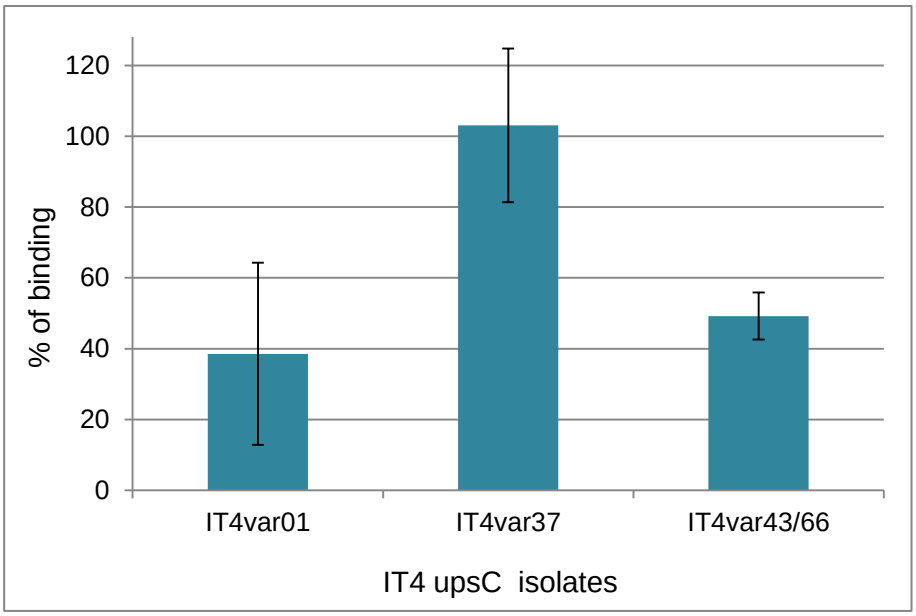

Figure 4.1.7: HDMEC ICAM-1 inhibition of IT4 upsC isolates in the presence of 5 µg/ml 15.2 (anti-ICAM-1 mAb). The graph presents the % of binding compared to the binding on HDMEC with no mAb effect. (n can be found in Table 4.1.7)

4.1.3.4 Characterisation of upsC IT4 and HB3 PfEMP-1 isolates on HUVEC under flow conditions:

The binding to HUVEC is similar to the ICAM-1 binding pattern. Only IT4var01 bound to HUVEC with high avidity confirming the results of adhesion protein static and flow assays. HB3 isolates did not bind to HUVEC and HBMEC, similar to what was observed on HDMEC. IT4var01 binding was reduced by anti-ICAM-1 mAb, as shown in chapter three (Table 4.1.8 and Figure 4.1.8).

Parasites	mean IE binding/mm^2	N	SE
IT4var01	377	3	79
IT4var37	8	3	3
IT4var43/66	19	3	5
HB3var29	1	2	1
HB3var30	3	2	1
HB3var31	1	2	1
HB3var35	2	2	1

Table 4.1.8: the binding of IE with upsC isolates to HUVEC. The table presents the means of IE binding/mm^2 and the number of experiments and the SE.

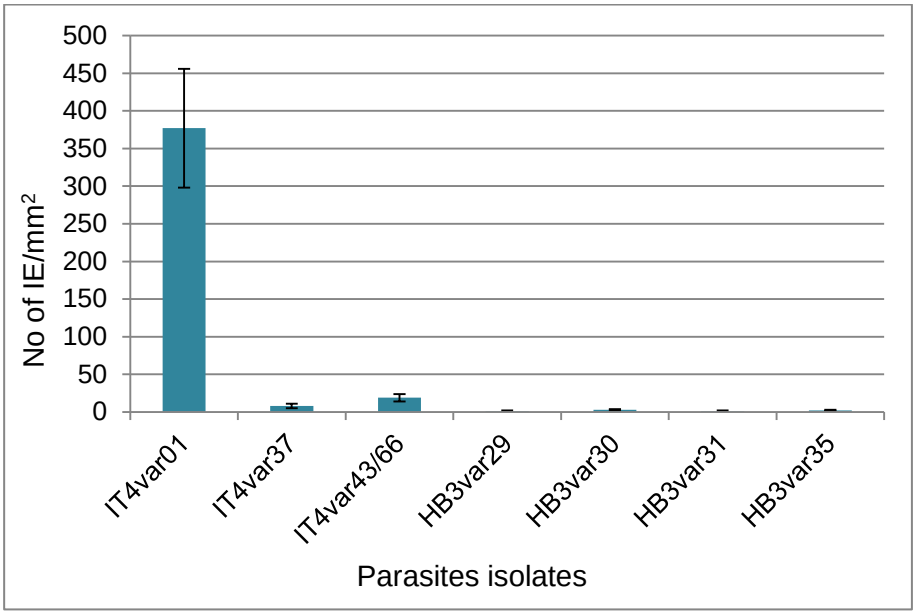

Figure 4.1.8: Flow adhesion assay, the binding of upsC isolates to HUVEC. The figure shows the mean of binding (IE/mm^2) and the bars represents SE (Numbers are given in table 4.1.6).

4.1.3.5: Cross reactivity of UpsC HB3 isolates with HIS:

The IT4var14 control isolate showed clear labelling with BC6 mAb that recognises IT4var14 specifically (Smith et al., 1995). The optimal functional concentration of HIS was 30 mg/ml for IT4var14 (figure 4.1.9). Thus, this concentration was used to examine the reactivity of HB3 isolates that were suspected to export functional PfEMP1 on their IE surfaces. Figure 4.1.10 demonstrates that about 40% of all HB3 upsC isolate populations were positive with HIS compared to human sera used for parasite culture obtained from individuals who have not previously been exposed to malaria. Also, the corrected MFI presented is at a very similar level after correction with the normal human sera (figure 4.1.11).

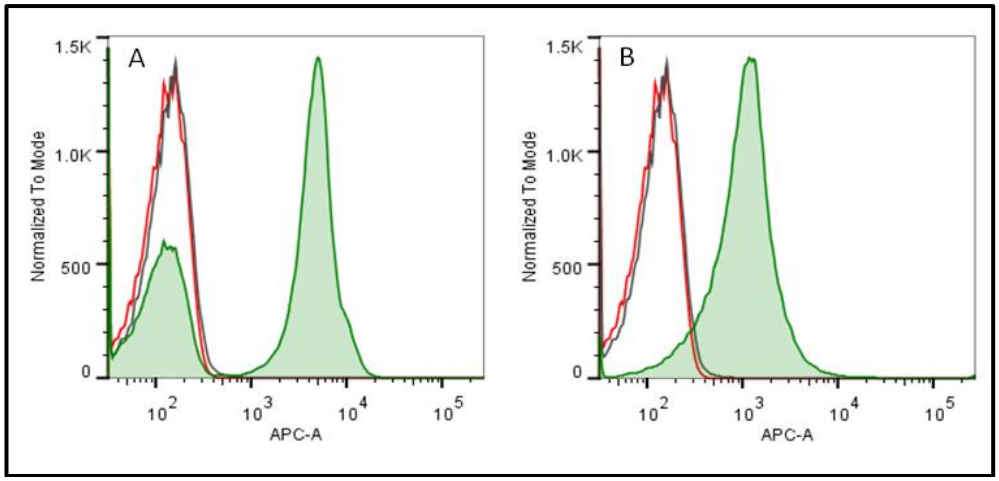

Figure 4.1.9: FACS analysis of IEs IT4var14. IEs were labelled with A) BC6 and B) 30 mg/ml HIS both highlighted in green. Also, human sera used for culture was used as a control (red lines) and anti-IgG isotype control (grey lines).

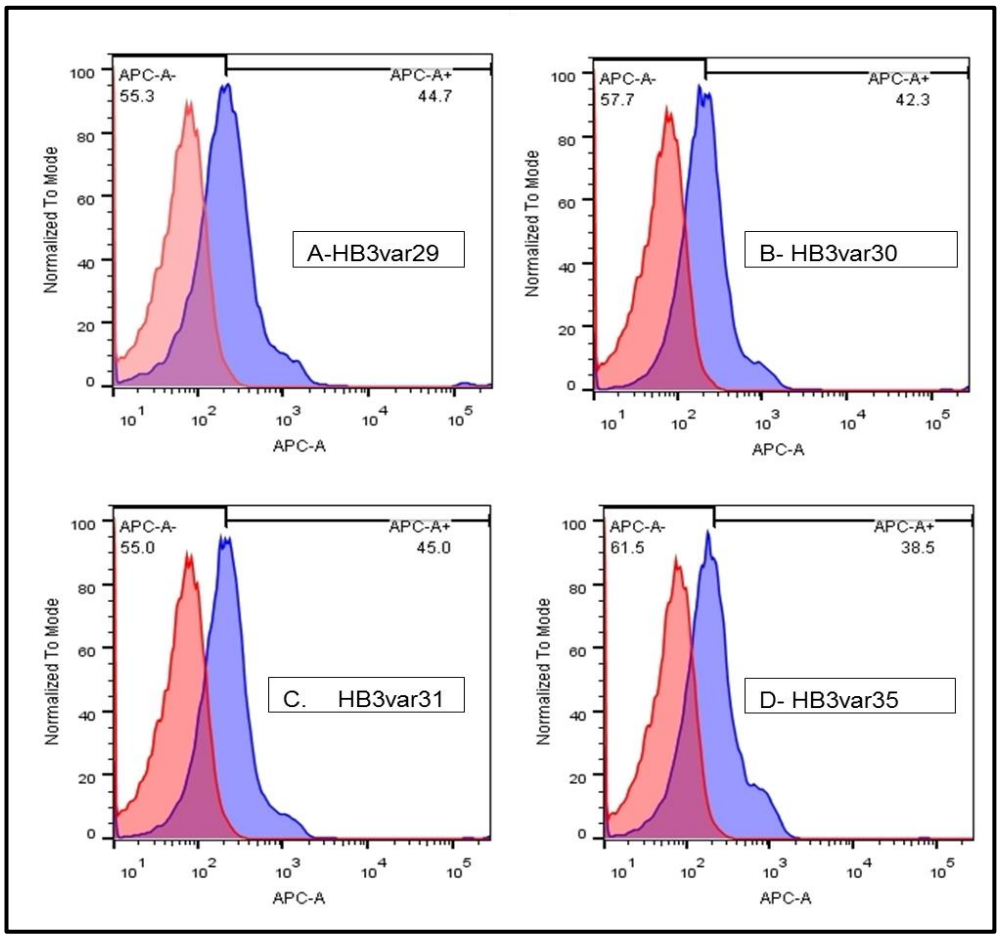

Figure 4.1.10: FACS analysis of IEs with UpsC HB3 isolates; A) HB3var29 B) HB3var30, C) HB3var31 and D) HB3var35. IEs were labelled with human sera used for culture as a negative control (orange highlighted) and 30 mg/ml HIS (violet highlighted). The percentage of reactive IEs with HIS is shown by APC^{+} population of each isolate.

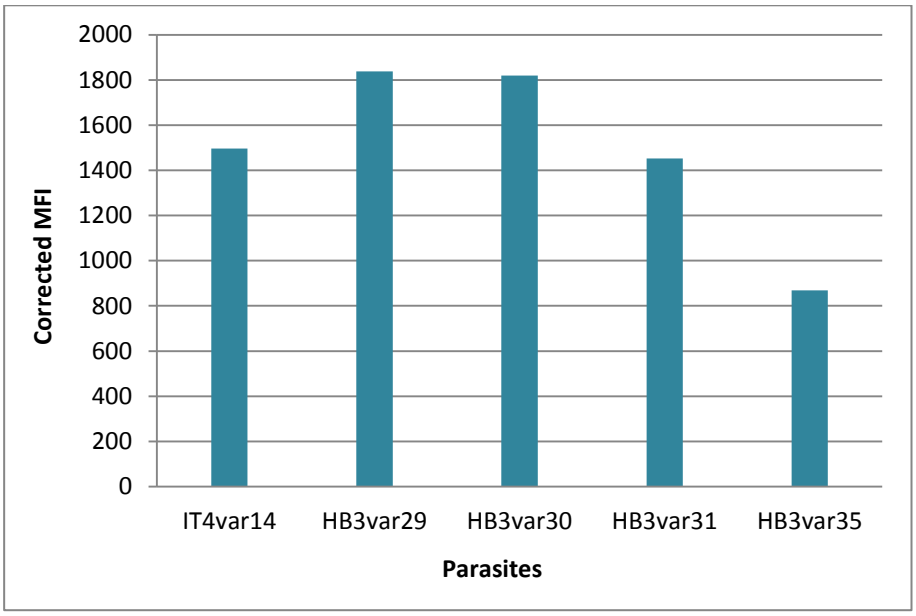

Figure 4.1.11: FACS analysis of IEs with UpsC HB3 isolates. The graph shows the corrected mean fluorescence (MFI) intensity for the four upsC HB3 isolates.

4.1.4 Discussion:

One of the fundamental questions about malaria pathogenesis is how chronic infections are maintained despite limited *var* gene repertoire in a single parasite genome (50-60 *var* genes). The associations between particular gene expression, malaria outcomes, ups-classifications, and host factors are not fully understood. However, the correlation of specific *P. falciparum* adhesion phenotypes with malaria outcomes has been partially improved in the last decade. Studies have found the expression of group C PfEMP1 variants as a characteristic of asymptomatic or chronic disease. This association is usually based on either the reactivity of semi-immune individuals or the relative *var* expression of isolated parasites from infected individuals (Jensen et al., 2004, Kyriacou et al., 2006, Rottmann et al., 2006) but no adhesion data on primary ECs have been acquired from the field.

One study has assessed the binding of some IT4 variants including three group C variants (Janes et al., 2011). However, Janes and colleagues used quite different type of cells based adhesion assays, using a number of transfected CHO cell lines each of which expressed different human

receptors including CD36, ICAM-1, E-selectin and VCAM-1. It should however be noted that they did use a similar protein-based adhesion assay to what was used in this study. We aimed in this study to assess the binding phenotypes of some of the upsC variants that were examined in the Janes *et al.* study using primary endothelial cells as well as investigating the binding to the commonly used human receptors CD36 and ICAM-1. To be more representative, isolates from two distinct genotypes IT4 and HB3 were used in this study.

The binding of all upsC isolates to ICAM-1 was not expected from both genotypes due to their lack of DBLβ-ICAM-1 binding domains, except IT4var01. The binding of IT4var01 to ICAM-1 was discussed in the previous chapter as it is among IT4-ICAM-1 isolates. The thought about ICAM-1 role in only mild malaria based on the finding that upsC isolates can bind to ICAM-1 can be argued. IT4var01 is the only one of the DBLβ domain-containing PfEMP1s (total 23) from the IT4 parasite isolates that are upsC and bind ICAM-1. It was previously reported that upsC variants can be found in all disease manifestations despite their relatively low expression (Rottmann et al., 2006). It was also shown that a solo domain sub-classification cannot be easily described as a predictor for ICAM-1 binding. Likewise, the ups classification also cannot exclude the binding of ICAM-1 to one ups group. Having a single PfEMP1 in the whole genome which is upsC and binds to ICAM-1 may not be enough reason to simply conclude that ICAM-1 is more likely to be associated with mild malaria rather than SM. In fact, the evidence that supports the role of ICAM-1 in mediating SM is stronger than its association with UM, even though in some reports the SM link did not reach significance. The available data suggest that upsC PfEMP1s seem to be under less selection to bind ICAM-1, at least in IT4 and HB3 parasites.

IT4 isolates from both upsB and upsC were CD36 binders. This is consistent with previous findings that ups B and C PfEMP1 variants are more associated with CD36 (Robinson et al., 2003, Kraemer and Smith, 2006, Cabrera et al., 2014). The role of CD36 in malaria pathogenesis is

not clear. The association between CD36 and mild malaria was mainly drawn by the finding that the majority of PfEMP1 variants encode CIDR1 domains which provide the binding motif for CD36 in the head structure of PfEMP1 (Robinson et al., 2003, Baruch et al., 1997, Smith et al., 1998). Also, several reports showed that CD36 adhesion phenotypes in the field are associated with uncomplicated cases and not SM (Newbold et al., 1997, Ochola et al., 2011).

However, binding to CD36 cannot be generalised to all upsC PfEMP1. This is demonstrated by HB3 isolates and their incapacity to bind to CD36 and HDMEC and other ECs. Consistently with this study Xiao *et al.* (1996) reported very low binding of HB3 parasites to a variety of ECs including HMEC-1, HBEC-5i, HUVEC and C32 despite selecting six times to obtain higher binding, and even though after repeated selections CD36 and ICAM-1 inhibition did not affect the binding, considering that CD36 and ICAM-1 were expressed by C32 and HUVEC respectively. Moreover, Rowe's lab team published (2012) a selection protocol to enhance the binding of parasites to HBEC-5i (Claessens and Rowe, 2012). They used HB3 parasite and the initial binding was very low on different ECs including HBEC-5i and HDMEC under a static conditions. This last point is important as static conditions generally show higher binding than in flow condition, which was used in this study. Note, the next part of this thesis compares binding data based on PfEMP1 length and will show results of upsA and upsB binding to ECs.

The lack of ability of HB3 isolates to bind ECs raised the question of whether these isolates were knobby or not. Therefore, the selection of knobby populations was confirmed using Plasmion, which was routinely practiced during this study. The plasmion flotation used to enrich the knobby parasites was easy and straightforward. It was clear under the microscope that there were high levels of parasitaemia after plasmion enrichments. The results would have been more confirmed testing the transcription of *kahrp*$^{+}$ by RT-PCR. However, there were some parasites transcribed *kahrp* but did not float in gelatin (Janes et al., 2011).

It is not easy to claim that PfEMP1 has been successfully exported and presented in structural confirmation in the absence of a universal marker. The alternative avenue was to test the HB3 isolates' cross reactivity with adult hyper immune sera (HIS) on FACS, even though it is known that upsC are highly diverse. However, all the isolates were cross reactive with HIS which could suggest that PfEMP1 is expressed and reactive considering that it is the a significant immunodominant molecule on the surface of IEs. However, this is not enough as there are some molecules are immunogenic other than PfEMP1. Previous studies have used flow cytometry to show that PfEMP1 is the prime target of immune antibody reactivity (Chan et al., 2012). Evidence of the involvement of PfEMP1 was mainly derived from the recognition of the IEs surface by HIS was abolished after trypsin treatment. However, there are other antigens on the surface of IEs including the RIFINS and BAND 3 are known to be sensitive to trypsin (Williams and Newbold, 2003).

The lack of binding to ECs or at least minimal binding capacities does not exclude the adaption to bind elsewhere to ensure chronic infections. It is possible that upsC parasites are adapted to not induce high binding that might harm the host. However, the mechanism of this is still unknown. It might be seen through the tolerance of group C expressing parasites for several environmental stress conditions (Rosenberg et al., 2009).

The achievement of chronic infection is acquired by immune evasion through antigenic variation that allows parasites to switch between PfEMP1 variants displayed on IEs by differential expression of around 60 members per parasite genome. In the chronic infections, parasites must avoid intensive inflammatory response induction that can harm the hosts. However, they must be sequestered as mature asexual parasites are not found in the circulation and to avoid splenic destruction. The binding of HB3 isolates suggests that some parasite variants may not require attachment at higher quantities to the ECs, potentially increasing the risk of affecting the hosts. It is known that the parasitemia is higher in the acute malaria than the chronic. This is obvious particularly with the high

parasitemia that often occurs in SM, which is theoretically resulting from high sequestered parasites. The low sequestration level might best represented by following the typical parasitemia wave that was observed in a natural infected child. Dondorp *et al.* (2005) was able to identify a method to calculate the biomass of sequestered parasites. It was given that the plasma concentration of *P. falciparum* histidine-rich protein 2 (PfHRP2) is correlated with sequestered-parasite biomass. The sequestered-parasite biomass in Thai patients suffering from SM was 10-fold higher than those with UM. This supports the low adhesion levels of isolates representing chronic infections (Dondorp et al., 2005).

HB3 and IT4 were isolated from Central America and Southeast Asia respectively. Their *var* gene repertoires have been compared with the reference *P. falciparum* genome sequence, 3D7, which represents African isolates. The parasite genomes of IT4, HB3 and 3D7 have nearly the same proportions of *var* genes in each *var* group regardless of their distinct *var* repertoire origins (Kyes et al., 2007). IT4 isolates are commonly used for adhesion assays *in vitro*. They, overall, showed relatively comparable binding to CD36 and consequently to HDMEC. This is clearly demonstrated in this study and many other studies using different methods of adhesion assays. One reason for their extensive use is that they maintain the asexual life cycle *in vitro* and do not transform to gametocytes easily, unlike other isolates such as 3D7 and HB3.

Probably, as mentioned above, one of the differences between HB3 and IT4 parasites is the ability of HB3 parasites to transform to gametocytes easily. Should HB3 upsC variants have a role in reducing the binding in the hosts or binding to other tissues, such as adipose tissue, to induce the generation of sexual stages to ensure the transmission of the parasites to vectors? If yes, how it can be achieved? It is suggested that parasites may have evolved to control their biomass inside hosts to switch to gametocytes (Cunnington et al., 2013). This is supported by recent findings about exosome-like vesicles (EVs) which mediate communication between IEs to deliver genetic materials that are

proposed to affect behavioural changes such as the requirement of gametocytes production. However, the role of EVs in controlling parasites biomass is still unexplored.

4.2 An analysis of the binding phenotype of PfEMP1 variants based on their length.

4.2.1 Introduction:

PfEMP1 is a major pathogenic virulence factor on the surface of IEs, where it mediates the binding to human tissues including the endothelium. Researchers remain uncertain about predicting the ability of PfEMP1 binding to ECs based on sequence features alone. Many studies have attempted to categorise adhesion phenotypes based on variable PfEMP1 features. The most common is the ups classification, as found in the previous part of the current chapter. Also, sub-classifications of single binding domains have been used such for DBLβ and ICAM-1. However, it has been shown that sub-classifications of single domains are not exclusively proper predictors for IE binding on ECs. Similarly, not all upsC share a common adhesion phenotype to use for binding predictions.

However, another recent theoretical classification of PfEMP1 repertoires has divided them into long and conserved (mainly Ups group A) and short and diverse (mainly Ups group B and C) (Buckee and Recker, 2012). This classification was suggested after a significant, non-random link between the number of domains composing *var* genes and the extent of their sequence conservation. Most of the PfEMP1s have a tandem DBL-CIDR domain at the N-terminus, known as the semi-conserved head structure. Short PfEMP1s have an extra DBL and CIDR to form 4 domain extracellular units. In contrast, long PfEMP1s have some more domains. Understanding the *var* gene diversity and classifications can be significant for designing vaccine and chemotherapies to target malaria outcomes. The aim of this part of the thesis is to collect the adhesion data from flow assays of parasite isolates with known PfEMP1 domain compositions and analyse their adhesion phenotypes based on this composition.

4.2.2 Methods:

Flow adhesion assays have been carried out as described before. The isolates were grouped into long and short PfEMP1 variants based on their compositions found in the vardom database or their original references (Figure 4.2.1). Three extra isolates from HB3 were included; HB3var05, long and upsA, and HB3var13 and HB3var27, both short and upsB.

ID	location	ups	No. of DBL and CIDR domains	PfEMP1 structure
IT4var01	Cen	C	6	NTS DBLα CIDRα DBLβ DBLγ DBLδ CIDRγ ATS
IT4var13	Sub	B	7	NTS DBLα CIDRα DBLβ DBLδ CIDRγ DBLγ DBLz ATS
IT4var14	Sub	B	7	NTS DBLα CIDRα DBLβ DBLδ CIDRγ DBLγ DBLz ATS
IT4var16	Sub	B	6	NTS DBLα CIDRα DBLβ DBLγ DBLδ CIDRβ ATS
IT4var31	Sub	B	4	NTS DBLα CIDRα DBLβ DBLγ ATS
IT4var37	UN	C	4	NTS DBLα CIDRα DBLδ CIDRβ ATS
HB3var05	Sub	A	6	NTS DBLα CIDRδ DBLγ DBLδ CIDRβ DBLβ ATS
HB3var13	Sub	B	4	NTS DBLα CIDRα DBLδ CIDRβ ATS
HB3var27	Cen	B	4	NTS DBLα CIDRα DBLδ CIDRβ ATS
HB3var29	Cen	C	4	NTS DBLα CIDRα DBLδ CIDRβ ATS
HB3var30	Cen	C	4	NTS DBLα CIDRα DBLδ CIDRβ ATS
HB3var31	Cen	C	4	NTS DBLα CIDRα DBLδ CIDRβ ATS
HB3var35	Cen	C	4	NTS DBLα CIDRα DBLδ CIDRγ ATS

Figure 4.2.1: Schematic representation of isolates from IT4 and HB3. It includes their locations, cen: central and sub: subtelomeric. It also shows the ups classification and more importantly the number of DBL and CIDR domains that compose the PfEMP1. The aqua (blue) colour is used for large (long) domains for all the figures in this chapter, and orange is used for short (small).

4.2.3 Results

The adhesion data of all isolates to CD36 (Figure 4.2.2), ICAM-1 (Figure 4.2.3) HDMEC (Figure 4.2.4), HUVEC (Figure 4.2.5) shows the pattern of binding of IEs with PfEMP1 variable structures. The aqua colour is used for large (long) domains for all the figures in this chapter, and orange is used for short.

4.2.3.1 The binding of short PfEMP1:

All HB3 isolates used in this study did not bind to either CD36 nor to ICAM-1 on both static and flow adhesion assays. A minor exception was HB3var13 (short and upsB) which bound transiently to CD36 under flow conditions, however almost all bound IE were removed during the washing step. The binding of this isolate on HDMEC was extremely low, and this was similar to HUVEC and HBMEC that do not express CD36. Also, blocking CD36 on HDMEC did not alter the binding. The binding of IE expressing other short HB3 proteins to ECs was negligible. There were two short PfEMP1s from the IT4 lineage; IT4var31 and IT4var37. Generally, all IT4 isolates used in this study bound to CD36. Interestingly, the strongest CD36 binder, IT4var37, as previously shown in the previous section, did not bind to ICAM-1 in both static and flow adhesion assays. It did bind to HDMEC but not CD36 negative ECs; this is basically because this PfEMP1 does not have a DBLβ domain. In contrast, the other short PfEMP1 from IT4 lineage was IT4var31, which has DBLβ3 that makes it able to bind to purified ICAM-1 as well as ICAM-1 expressing ECs.

4.2.3.2 The binding of long (large) PfEMP1:

All long PfEMP1 from IT4 bound to different ECs with highly variable avidities. Among all the HB3 isolates used there was only HB3var05 that is long and upsA. HB3var05 did bind to all ECs used in this study under flow conditions, higher than other HB3 isolates. The binding was relatively similar on three ECs variants at about 100 IE/mm^2. Moreover, the binding was not affected by anti-CD36 and anti-ICAM-1, supporting

the protein adhesion assays. In contrast, the binding of all IT4 IEs with long PfEMP1 on HDMEC was higher than IEs with HB3var05; IT4var14 was lowest among them and it was nearly twice as high as HB3var05.

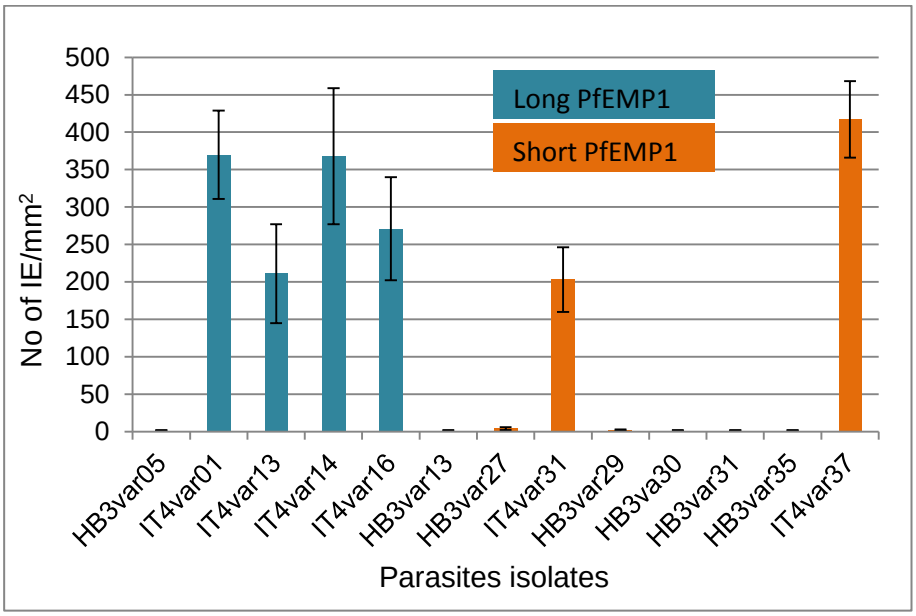

Figure 4.2.2: Flow adhesion assay to CD36. The binding of IEs with long and short PfEMP1 isolates from IT4 and HB3 lineages.

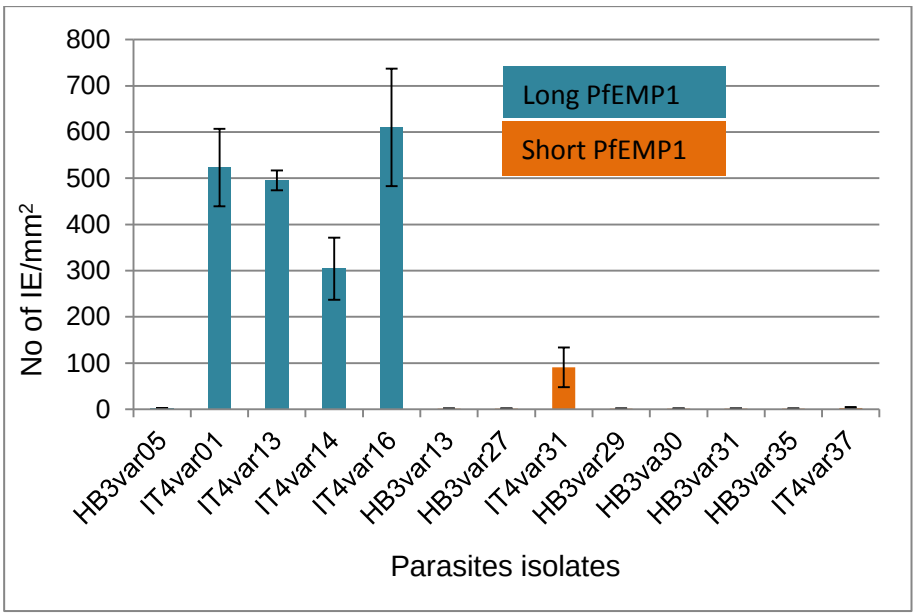

Figure 4.2.3: Flow adhesion assay to ICAM-1. The binding of IEs with long and short PfEMP1 isolates from IT4 and HB3 lineages.

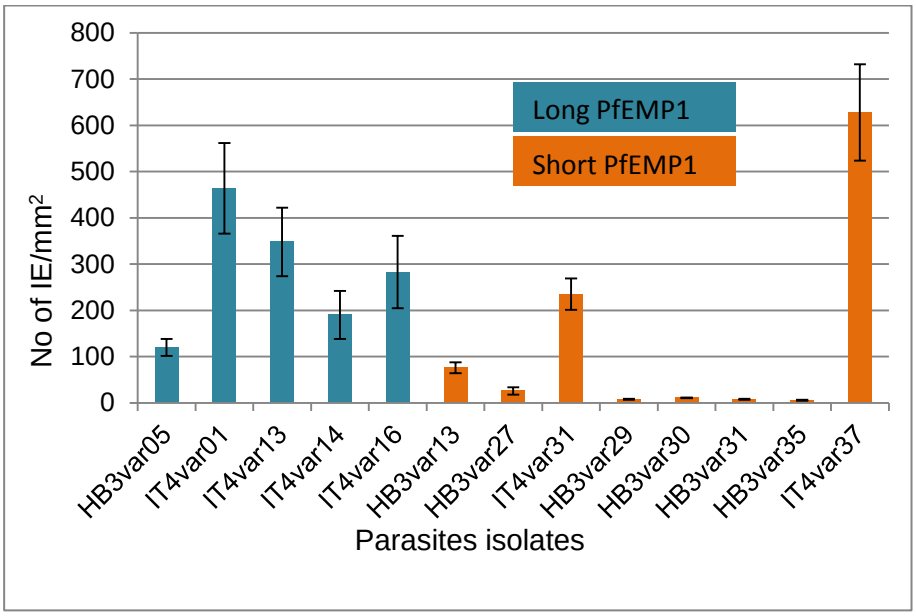

Figure 4.2.4: Flow adhesion assay to HDMEC. The binding of IEs with long and short PfEMP1 isolates from IT4 and HB3 lineages.

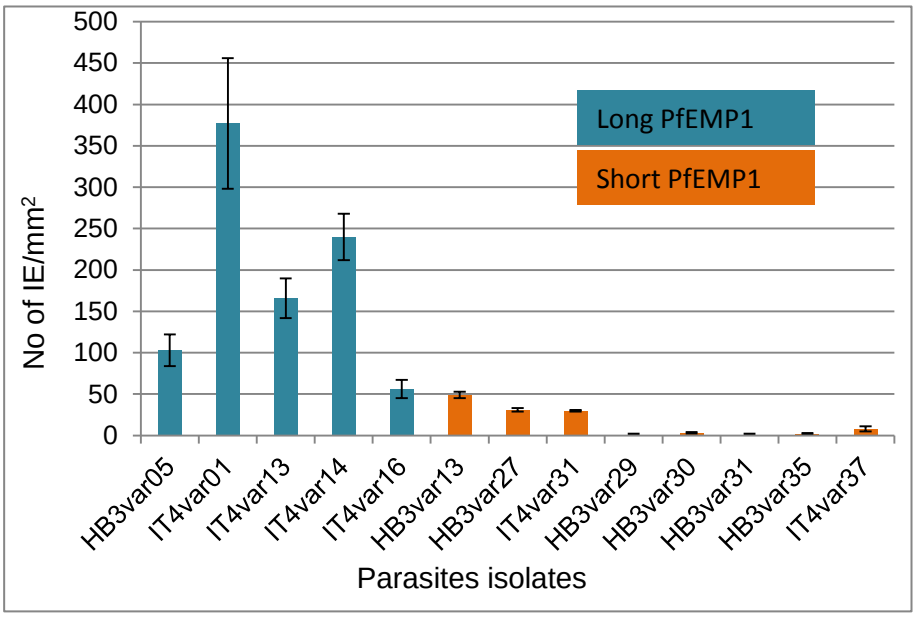

Figure 4.2.5: Flow adhesion assay to HUVEC. The binding of IEs with long and short PfEMP1 isolates from IT4 and HB3 lineages.

4.2.4 Discussion:

The compositions of genomic repertoires of var genes are structurally organised. A major outcome of the analysis conducted by Buckee and Recker (2012) was that *var* genes can be divided into either 'short and diverse' or 'long and conserved'. The adhesion data of isolates from these two different lineages were analysed to test the hypothesis that PfEMP1 length might be considered a major factor for IE adhesion. Isolates were grouped based on the number of DBL and CIDR domains composing PfEMP1. They were classified into either short PfEMP1 containing only four domains or large PfEMP1 containing more than four domains.

Overall, long PfEMP1 of both HB3 and IT4 can bind to all ECs examined in this study with variable avidities and different receptors usage. Also, IT4 isolates either short or long can bind ICAM-1 provided that they express ICAM-1-binding DBLβ domains. CD36 binding phenotype is associated with CIDRα2–6 sequence types and linked to ups B and C proteins. By contrast, CIDRα1, β, γ and δ are associated with upsA variants and do not show association with CD36 adhesion phenotype. The expression of CIDRα2–6 was in all IT4 isolates investigated in the current study. All IT4-ICAM-1 isolates either short or long bound to CD36, confirming earlier findings that CIDR-CD36 binding domains are found in both small and large PfEMP1 proteins and have nearly 40% sequence identity (Robinson et al., 2003, Janes et al., 2011). Also, the binding avidities were variable and, interestingly, the shortest had the higher avidity on both HDMEC and purified CD36. This goes in agreement with recent experiments tested the rolling velocities of a group of IT4 isolates to CD36 and to examine the effect of flow rate on adherent IEs cell shape. It was suggested that the binding of IT4 isolates to CD36 is selected for optimal binding regardless the size of PfEMP1 variants (Herricks et al., 2013).

However, the situation is completely different regarding the HB3 isolates. All HB3 isolates seemed to lack, based on sequence, the components for

ICAM-1 binding and therefore their binding to ICAM-1 was not expected. Remarkably, the binding to CD36 was comparable with the negative background, except for one isolate that was able to bind transiently to CD36 but binding was quickly removed and could not be counted. This is perhaps due to the formation of bonds rate, K_{on}, and dissociation rate, K_{off}. It is thought that adhesion occurs based on the distance separation between the host receptors and IEs ligands. Usually, high binding results from high K_{on} and low $K_{off.}$ Whereas, the transient binding could be due to that both parameters being high, allowing adhesion to occur but be quickly disassociated.

In the previous chapter, it was concerned about the ability of HB3 to express PfEMP1 properly. However, the major finding in this chapter is ups A HB3 isolate, HB3var05, bound to all ECs. These data at least provide some support that HB3 can express PfEMP1 to allow adhesion. Therefore, the lack of binding in the upsC isolates is unlikely to be due to non-expression of PfEMP1.

HB3var05 bound to all ECs and was not blocked by anti-CD36 or by anti-ICAM-1. The adhesion to ECs is mediated by numerous human receptors. It was very difficult to examine the particular receptor that was mediating the adhesion of this isolate using mAbs blocking assays, due to limited experimental time. Fortuitously, very recent data examined the association of DC5 type PfEMP1 with SM; it was shown that the adhesion by DC5 was mediated by PECAM-1 (CD31). Importantly, HB3var05 was categorised as a DC5 type PfEMP1 (Berger et al., 2013). This is in agreement with the current study that binding to ECs was not inhibited by anti-CD36 or by anti-ICAM-1.

DC5 PfEMP1 expression was cross reactive with serum from SM patients. DC5 is composed of DBLγ12-DBLδ5-CIDRβ4-DBLβ9. However, it was shown that two recombinant proteins, DBLδ5-CIDRβ4, play a central role of DC5 for the cross reactivity. Nevertheless, the binding to PECAM-1 was not reduced by antibodies raised against these two domains, but rather it was inhibited by IgG against the whole DC5

domains (Berger et al., 2013). From these observations it was suggested that DC5 PfEMP1s seem to be compact. In other words, it means the domains of the molecules fold back on itself rather than being an extended molecule offering “beads on a string” structure where a single domain may behave independently of other domains. This is similar to VAR2CSA that mediate PAM. Although, the association of DC5 with SM is still at a very early stage, the suggestion of using compact structures may not support the hypothesis that the PfEMP1 length could facilitate an optimal adhesion; it could be the structure, the shape and the domains composition of the PfEMP1.

The binding to ICAM-1 and HUVEC, that express ICAM-1 but not CD36, was relatively low for IT4var31, the only short PfEMP1 among IT4-ICAM-1 isolates. Interestingly, it is also the only one that expresses DBLβ3 as described previously. It is difficult to conclude that the lower avidity is because of the architecture of the PfEMP1 on the IE or sub-classification of the DBL domain. It is more likely neither of the reasons because IT4var16, which is long and has DBLβ5, is a higher ICAM-1 binder on purified proteins but shows nearly to the same level of binding as IT4var31 on HUVEC and HBMEC. Thus, these controversial observations could be better interpreted if there was adhesion data for isolates that are long and express DBLβ3 from the same lineage. However, none of IT4-ICAM-1 isolates have DBLβ3 except IT4var31.

Similar to CD36, different avidities were observed for binding to HDMEC regardless of the PfEMP1 length. Binding could be mainly due to CD36 because CD36 inhibition reduced this by almost 90%. This could also be explained by co-operation occurring between CD36 and ICAM-1 on HDMEC, often referred to synergism but in this adhesion context probably better seen as cooperation until formal synergism can be demonstrated.

The other notion is that IEs of IT4 isolates tend to bind with higher avidities to ECs, unlike IEs with HB3 isolates. It is unknown if their origin has an impact on this less adapted binding or not. The comparison

between isolates within and between different genotypes is rarely addressed. Ideally, this is should be carried by obtaining PfEMP1 variants from different genotypes that are identical at least in their domains composition and compare their adhesion phenotypes to ECs. To the best of the knowledge, in vitro, there is no available comparison data that characterised the adhesion phenotypes of IEs with the PfEMP1 expression profiles from distinct genotypes. It is difficult to identify the IEs expressing PfEMP1 that have the same domains composition. Especially, there are heterogeneous parasite populations in the clinical samples, but assuming that a single genetically distinct clone was isolated; IEs population usually express a single PfEMP1 variant. This is hampered by the difficulty to find a single genotype express different PfEMP1 variants in clinical samples and allows them to grow in vitro without altering their *var* gene expression to investigate their binding phenotypes. This lack of knowledge has precluded a conclusive link of the adhesion abilities based on the genotype of the parasites.

Cytoadhesion in PAM is mediated by the interaction between VAR2CSA and CSA expressed on placental proteoglycans. Interestingly, CSA is expressed elsewhere in the microvasculature, but it does not mediate VAR2CSA binding of IEs. Very recent study has addressed the tropism of the placenta-VAR2CSA specific adhesion using synthetic membranes with variable CSA gaps intervals. It was found that VAR2CSA-CSA binding was highly dependent on the CSA distance at different hydrodynamic settings (Rieger et al., 2015).

The ratio of short PfEMP1s in HB3 isolates is larger than IT4 isolates, albeit they both have a higher ratio of large PfEMP1 proteins compared to 3D7 (Kyes et al., 2007). The impact of expressing higher numbers of small PfEMP1 proteins in 3D7 and HB3 is not clear. One major difference between the three commonly used parasite lines, HB3, IT4 and 3D7 is the presence of conserved type 3 *var* gene in IT4 and 3D7 whereas it is absent in HB3. Type 3 *var* gene is also present in many other parasites that have been studied. This work did not attempt to correlate disease

outcomes with adhesion phenotypes and the length of PfEMP1. However, considering that most upsA are long and more linked to SM, whereas ups B and C contain the diverse and short PfEMP1s, VAR3 is an exceptional protein because it is highly conserved, very short and upsA. The expression of VAR3 has been associated with SM (Wang et al., 2012) but no adhesion data are available for this type of var gene. Also, no functional role has been identified for VAR3. Rask et al. (2010) showed VAR3 is not found in *P. falciparum* genome IGH, nor in *P. reichenowi* genome that infects chimpanzees. On the other hand, DC5 is dominantly present in *P. falciparum* genomes and even *P. reichenowi*, which could suggest a specialised evolutionary role of these *var* genes. It might be that *P. falciparum* first were not specialised to sequester like other older parasites. Then, the presence of DC5 which is found to bind to CD31 probably which I call “used to be the first target” on human tissues to highly abundance before stimulation did not mean to harm other hosts. Then, the existence of VAR3 in human parasites and not others may indicate that from this point the virulence increased, as seen in newer isolates. Especially, considering that VAR3 is associated with SM. There might a recombining role of VAR3 especially because it exists in up to three different copies in a single parasite genotype (Gardner et al., 2002).

The limited available isolates representing the variety of classifications covered in this chapter is a weakness for drawing conclusions from these experiments. An extra nine isolates obtained from Copenhagen’s groups were obtained but none of them grew in our lab. However, the need of performing limited cloning dilution become apparent at the end of the study, and it is known that it costs really long time to have the required PfEMP1 type.

In conclusion, it seems that is that long PfEMP1 adapted to bind more than the short, but this may merely be due to the lack of variety of DBLs for adhesion. Therefore, it is suggested that it is the domain constitution rather than size that seems to be important.

5. General discussion:

Malaria is still a major concern for global health, infecting about 128 million in 2013 (WHO, 2014). Malaria infections vary from asymptomatic, mild and in some cases the disease becomes severe and fatal. The vast majority of malaria cases are chronic infections which cause a major concern for malaria elimination programmes by facilitating continuous transmission by the mosquitoes. One of the major concerns in the field is the shifting of malaria burden from the typical susceptible populations of young children and pregnant women to older children and adults (Cotter et al., 2013).

Malaria pathogenesis is developed during the asexual intraerythrocytic stage where *P. falciparum* modifies the host erythrocytes by exporting repertoires of parasite proteins on surface of IEs. PfEMP1 is one of these proteins that mediate different functions including sequestration. Cytoadhesion is one of the sequestration forms which can be lethal through the obstruction of blood flow, mediating inflammatory responses, and/ or causing endothelial activation and dysfunction. However, sequestration is also considered to be a strategy to ensure transmission and not to harm the hosts.

Different adhesion phenotypes to ECs can be observed *in vitro* and can be quantified and categorised based on the avidities between ligands and receptors. However, some isolates did not bind to ECs, suggesting that the parasites might bind to other host cells during the absence of the vectors. It is known that another complexity of the life cycle is the ‘suspended’ transmission in the time of the year with little or no rain, particularly in low transmission settings. The complexity of the parasite’s life cycle is demonstrated by the explosive numbers of mosquitos just in three days following the rains when an adult mosquito needs at least eight days to grow following their hatching (Sohn, 2014). Thus, it is not only complicated to understand the sudden appearance of the vector during dry seasons, but it may also be complicated to discover the impact

of the ‘hidden’ parasites during the absence of the vector in chronic infections.

Chronic infections have been associated with the expression of upsC and some upsB PfEMP1 variants. Theoretically, most upsC or small PfEMP1s could bind to CD36. The association between CD36 and chronic malaria was mainly drawn from the finding that the majority of PfEMP1 variants encodes CIDR1 sub-domains that encode the binding motif of CD36 in the head structure of PfEMP1 (Robinson et al., 2003, Baruch et al., 1997, Smith et al., 1998). Also, several reports showed that CD36 adhesion phenotypes in the field are associated with uncomplicated cases and not in SM (Newbold et al., 1997, Ochola et al., 2011). However, others have demonstrated a correlation between CD36 adhesion with SM (Almelli et al., 2014). In this study, adhesion phenotypes of IEs with variable PfEMP1s have been characterised. However, seven PfEMP1 variants of each genotype IT4 and HB3 have shown extremely variable binding adhesion phenotypes based on the parasite’s genotype. It was not surprised to find all IT4 isolates can bind to CD36 and HDMEC. But, surprisingly, none of HB3 isolates in this study bound to CD36 or ICAM-1 regardless their variant type.

Previous studies also observed that HB3 isolates bind to ECs with lower avidities prior to any selection effects, suggesting other sites for binding. The lack of binding to ECs, or at least minimal binding capacities, does not exclude the adaption to bind elsewhere that might induce the immunomodulation to ensure chronic infections. It is possible that there are some parasites that have adapted not to induce high binding that could harm the host. Interestingly, in the late 1990s, Urban and colleagues studied the binding of IEs to leukocytes. It was shown that binding to dendritic cells modulated the immune responses and delayed parasite killing. This was shown through the finding that some parasites able to produce intimate contact with leukocytes prevented their maturation. In contrast, there were some parasites not able to mediate leukocyte adhesion phenotypes that completed their maturation normally.

The authors reported some evidence that not all parasites bind to the endothelium in their hosts despite their ability to express variant antigens associated with this phenotype (Urban et al., 1999).

It was concluded that not all upsC encoded PfEMP1 proteins can bind to CD36. It seems there are some other factors that play an unknown role to enhance the transmission of the parasite. Importantly, group C expressing parasites have been found to be more tolerant to several environmental stress conditions (Rosenberg et al., 2009). Recent work has suggested the epigenetic dysregulation of virulence gene expression in SM (Merrick et al., 2012). There might be some epigenetic regulation for chronic infection that controls the adhesion.

On the other hand, several reports have associated SM with upsA PfEMP1 variants. The associations between particular gene expression, malaria outcomes, ups-classifications, and host factors are not fully understood. However, the correlation of specific *P. falciparum* adhesion phenotypes with malaria outcomes has been partially improved. Up to date, three common endothelial adhesion phenotypes have been observed in different outcomes. It seems there is an overlap between the human receptors usage and disease severity and PfEMP1 variants. Recent findings suggest that EPCR interactions with conserved PfEMP1 are associated with SM more than CD36 and ICAM-1 (Figure 5.1). The figure illustrates that upsA variants and EPCR binders are more conserved to be associated with SM. However, the figure has implicated ICAM-1 adhesion phenotypes more towards SM but not excluded from mild malaria, making the upsB PfEMP1 variants shared between SM and mild malaria AM. In contrast, binding to CD36 is more linked to mild malaria but, also, is not excluded from SM. This might be a reasonable theoretical demonstration of the diversity of common endothelial adhesion receptors and different PfEMP1 variants and disease severity.

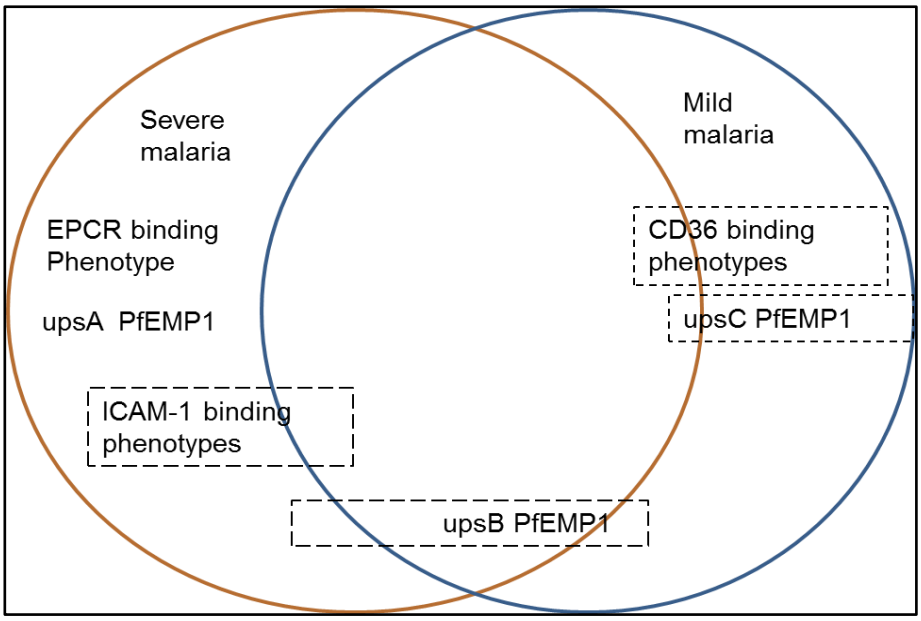

Figure 5.1: diversity of adhesion phenotypes and possibilities of involvements of all PfEMP1 groups and their associations with disease severity.

ICAM-1 has received more attention during this study to improve our current understanding of PfEMP1::ICAM-1 interactions. ICAM-1 plays an essential role in capturing leukocytes in inflammatory responses. It also mediates binding to HRV and *P. falciparum* IEs. The binding site of ICAM-1 to PfEMP1 has been studied using alanine replacement mutagenesis and ICAM-1-specific mAbs. The binding region on ICAM-1 for *P. falciparum* IEs was revealed to involve the BED face of ICAM-1 which is three β-strands of ICAM-1 named B, D, and E, including the DE loop (Tse et al., 2004). In the current study, recently lab-adapted patient isolates selected on ICAM-1 were used to expand the number of isolates compared to the original work. Essentially, similar findings have emphasized the original thoughts about the significant role of L42 as a conserved residue for all ICAM-1-binding genetically distinct isolates despite high variable use of different contact residues in the DE loop of ICAM-1 (Madkhali et al., 2014). Thus, therapeutic approaches might be encouraged targeting the conserved residues of ICAM-1 as an adjunctive treatment of malaria SM symptoms.

Despite the concern of malaria drug resistance, rapid and effective parasite-killing drugs are available, particularly in Africa. However, malaria still causes death among children with SM syndromes. Several strategies have been highlighted to enhance survival in malaria, one of which is targeting the binding of parasite to the endothelial cells (Miller et al., 2013). The key outcome of host-parasites interaction studies is the identification of vital targets mediating the interaction between parasite ligands and host receptors and using this for the development of inhibitors that block IE sequestration. Anti-adhesion therapeutics is an encouraging project in the discovery of novel treatments. For example, levamisole, an available drug, disturbs CD36 dephosphorylation, which causes an inhibition of CD36-dependent binding. Based on the studies of the DE loop of ICAM-1, 36 anti-cytoadhesion mimeotopes were identified *in silico* to provide novel effective therapeutics. One of these compounds is (+)-EGCG that showed a significant inhibition effect ranging from 40%–80%, including by the new ICAM-1-binding isolates used in the present study (Patil et al., 2011). It is thought that variable contact residues on PfEMP-1 of different isolates cause the variation of inhibition by the inhibitor. The mode of action of (+)-EGCG is assumed to be its structural simulation of part of the ICAM 1 binding site for IE based on the L42 loop. More recently, the core of (+)-EGCG was successfully replaced with tetrahydroisoquinoline ring. This has enabled the production of a small library of compounds targeting the DE-loop of ICAM-1. Thus, a novel cytoadhesion inhibitor was identified as a hit compound to encourage the anti-adhesion development. It is thought that it is chemically accessible and designed to interfere with PfEMP1::ICAM-1 interaction (Gemma et al., 2014).

The isolates used here have shown high diversity of ICAM-1 binding. On the PfEMP1 side, only some DBLβ domains have proved to bind to ICAM-1. Recent studies have explored ICAM-1-DBLβ binding using different approaches, including structure modelling and biophysical analysis, especially for IT4 isolates. These efforts have provided useful hints to understand PfEMP1::ICAM-1 interactions. However, none of the

previous studies characterised the binding of the used isolates on ICAM-1 using standard binding assays including the binding to primary ECs. Thus, it was attempted to examine the binding of IT4-ICAM-1 isolates to understand the binding phenotypes and compare it with available biophysical data. Two major differences between this part and the previous one are IT4-ICAM-1 are genetically identical and expressing known PfEMP1 confirmed by q-RT-PCR. IT4-ICAM-1 isolates have also shown diverse contacts of ICAM-1 residues but have also confirmed the use of the conserved L42 residue in the DE loop. This diversity of ICAM-1 usage has led to some varying thoughts about ICAM-1 involvement in SM, which is more often associated with upsA PfEMP1s, whereas many of the IT4 variants are upsB. Even where there are known groups of DBLβ variants showing ICAM-1 binding it would be difficult to target the DBLβ domains in these groups due to their extensive sequence diversity. This is seen particularly in approaches to discriminate ICAM-1 binding DBLβ domains from non-binding ones, which has only been partially successful. There are ICAM-1 binders among the group A PfEMP-1 that contain a definable DC4 cassette, but this work is still at a very preliminary stage and needs more investigation to see if it could provide a starting point for the development of a vaccine targeting CM by inhibiting IE sequestration via ICAM-1 in the brain. The variability in the binding characteristics between IE and ICAM-1 suggests that it could be a difficult problem to find a cross-blocking therapy, although the central role of the L42 residue and anti-DC4 blocking antibodies provide some support for this approach.

Limitations and future studies:

It should be admitted that doing cytoadhesion assays *in vitro* for large number of isolates is not simple nor convenient due to the variation of the results occurring due to technical issues largely inherent with the assay. For example, static assays need very careful handling during the incubation and washing, and if the performance during these shaking steps is not appropriate, the whole assay can be dismissed. Also, in the

cell-based adhesion assay, if the control isolates did not demonstrate the sort of expected binding level to ECs, then it would not be worth carrying out the assay on that day. This caused the necessity of repeating the assays several times to reduce the SE.

Another issue is that the limited available isolates represent a restricted variety of classifications which could be a weakness for the whole picture drawn from the experiments. It was tried to grow an extra nine isolates obtained from Copenhagen groups, but none of them grew in our lab. The need for performing limited cloning dilution to extend the available clones become apparent at the end of the study, and it is known that it costs really a long time to have the required PfEMP1 types, and so could not be included here.

In future, one of the aims is extending the number of isolates from HB3 genotype. The target isolates are HB3var03 (among DC13 isolates), HB3var10, HB3var17, HB3var21 and HB3var48. All these are long form PfEMP1 variants representing different ups groups, which should give useful hints about the findings of this study about the adhesion capabilities of upsC HB3 isolates to different host receptors. Also, ICAM-1 selection on beads looking for HB3-ICAM-1 isolates that either express DBLβ3 or DBLβ5 such as HB3var02 (among DC4 isolates) and HB3var34, which is a central upsC but has six domains including DBLβ5, could be carried out to maximise the chance of obtaining other interesting isolates.

Also, evidence has demonstrated that blocking the activity of Plasmepsin V which is essential for PfEMP1 exportation reduced the display of PfEMP1 and consequently cytoadhesion. It could be speculated that some parasites that maintain chronic infection regulate their PfEMP1 expression through regulation Plasmepsin V (Sleebs et al., 2014). Future work will investigate the expression of Plasmepsin V in upsC HB3 isolates and compare it with different parasites. Others have proposed that PHIST domains interact with the ATS domain of PfEMP1 at a conserved epitope which might be disrupted and affecting the parasite

cytoadhesion (Mayer et al., 2012). These interactions in HB3 upsC isolates should be investigated to find a cause their impaired cytoadhesion.

It would of interest to examine the binding of parasites to ECs that have been co-incubated with early ring stage IEs for 12 hours. Ideally, the adhesion assay should be carried using the same population that incubated with ECs. This would be followed by RNA extraction to study the effects that could occurr in response to the parasites stimulation even before adhesion of mature trophozoite stages. Then, flow adhesion assay will be conducted on the IEs stimulated ECs and non-infected erythrocyte stimulated ECs.

Interestingly, A4 was associated with a signature that reflects isolates from SM cases (Ochola et al., 2011). In the current study, most of the lab-adpted patient isolates were low-avidity ICAM-1 binders similar to A4. It is thought that there might be other severity induction-cause rather than just the binding. For example, dual binding could occur between the leukocytes and the parasites stabilising a major complex *in vivo*. It is thought that a competition experiment between the binding of LFA-1 and parasites to ICAM-1 on ECs preliminary might discriminate between the high and low-avidity parasites.

Another valuable addition to strengthening the part that compares the binding between HUVEC and HBMEC is including EPCR binders from IT4, 3D7 and HB3 isolates.

Another future work should be carried out in the shared borders of the KSA and Yemen. The adhesion phenotypes from this region have not been studied previously. The study would look at the gene expression profiles, and the adhesion phenotypes on ECs, ideally under physiological flow conditions. It should also include markers of immune response and endothelial activation and dysfunction. Also, measuring PfHRP2 will be done to relate the adhesion phenotypes with the sequestered biomass in the infected people. Also, citizens in Jazan suffer

from several genetic disorders such as sickle cell anaemia and thalassemia; it would of interest to understand, whether individuals have developed a protection against the disease such as by acquiring EPCR polymorphisms.

In brief, sequestration might not be the only mediator of the SM. There are many studies that have compared concentrations of inflammatory mediators and shown that they are higher in malaria infected individuals than non-infected people. Nowadays, vascular endothelium dysfunction is thought to play an essential role to SM pathogenesis. The activation of the ECs can be stimulated by the adhesion of IEs or the inflammatory response mediators. This may well disturb microvascular blood flow. Thus, sequestration, inflammation, and endothelial dysfunction are perhaps closely related; vascular endothelial activation may be caused by the sequestered IEs and inflammation whereas activation of vascular endothelium can increase IEs binding to human receptors (Storm and Craig, 2014). A comment was published in the Nature in December 2014 that discussed the thought that microbiologists are paying attention to the microbes' virulence factors variables and usually ignoring the host variables. It was stated that a microbe is not able to cause illness without a host response. Several potential consequences resulting from interactions between the microbe and the host cause sickness. It was thought that scientists should focus on the interactions between a microbe and a host rather than the pathogen alone (Casadevall and Pirofski, 2014).

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Appendices:

Appendix 1:

Static adhesion of new ICAM-1 binding isolates to CD36:

Parasites	Mean IE binding/mm^2	SD
ItG	1321.4	427.0
A4	1153.0	379.7
8146	1462.2	1020.3
P069	2308.6	419.2
PCM7	363.3	171.9
6392	606.0	330.4
8131	923.7	485.2
BC12	326.2	133.0
8206	NA	NA
BC31	5818.9	1837.7
J1	916.8	96.1
GL6	1321.4	427.0

Static adhesion of IEs with selected ICAM-1 binding isolates to CD36. The table presents the means of IE binding/mm^2 and the standard deviation.

Appendix 2:

PUBLICATION AND PRESENTATIONS

Some work in this thesis has been presented for publication

An Analysis of the Binding Characteristics of a Panel of Recently Selected ICAM-1 Binding Plasmodium falciparum Patient Isolates. MADKHALI, A. M., ALKURBI, M. O., SZESTAK, T., BENGTSSON, A.,PATIL, P. R., WU, Y., ALHARTHI, S., JENSEN, A. T., PLEASS, R. & CRAIG, A. G. 2014. *PLoS One,* 9**,** e111518.

Also, a poster was presented at meeting

9th Annual BioMalPar Conference on the Biology and Pathology of the Malaria Parasite. 13-15 May 2013. EMBL, Heidelberg, Germany.

Post Graduate Research Conference day, LSTM, 2013. The poster has been awarded among the best poster presentations.

8th European Congress on Tropical Medicine and International Health. 10- 13 September 2013. Copenhagen, Denmark.

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