Study of platelet-mediated clumping phenotypes of

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Nuffield Division of Clinical Laboratory Sciences

St Cross College

Supervisors

Prof David J. Roberts

Dr Peter C. Bull

By

Frank Gekara Onyambu

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DEDICATION

To my parents for their unending support, love and patience

David Onyambu Gekara

Drucillah Mong'ina Onyambu

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SUMMARY

Platelet-mediated clumping of *Plasmodium falciparum*-infected erythrocytes (IEs) is a common property of field isolates associated with severe disease (Pain, Ferguson *et al*. 2001). Platelet receptors CD36 (Pain, Ferguson *et al*. 2001), P-Selectin (Wassmer, Taylor *et al*. 2008) and gC1qR (Biswas, Hafiz *et al*. 2007) mediate clumping. To characterize the molecular specificities of the clumping phenotype, I cloned clumping parasite line IT/C10 by limiting dilution. I characterized *var* gene expression in the IT/C10 clones using generic primers for the DBL tag region (Bull, Berriman *et al*. 2005). Clumping assays were conducted in the presence of specific reagents to delineate host factors hypothesized to contribute to development of the clumping phenotype. Finally, I conducted a clinical study with isolates from children with malaria in Kilifi, Kenya.

This study shows that in parasite line IT/C10, platelet-mediated clumping is associated with Itvar30 suggesting a prominent role for the PfEMP-1 encoded by this *var* gene in development of platelet-mediated clumping. For IT/C10 parasites, platelet activation appears to be involved in platelet-mediated clumping. Platelet P-Selectin appears to mediate clumping using lectin-dependent interactions. To further elucidate the mechanisms that mediate clumping by host platelets, I have used a panel of platelet antagonists to delineate specific platelet activation pathways. Our results show that platelet activation plays an important role in platelet-mediated clumping. Finally, in this study, platelet-mediated clumping was associated with parasitaemia, but not with disease severity.

DECLARATION

I performed the work in this thesis except for the following

1. Miss Sindu Nair and Prof David Roberts conducted the cloning of parasite line IT/C10 in Oxford.
2. Dr Sue Kyes conducted the radioactive work for the Northern blot experiments in the laboratory of Prof Chris Newbold.
3. Dr George Warimwe and Dr Peter Bull sequenced the DBL tags of the Kilifi isolates in a wider on-going study within which this study is nested.
4. The clinical examinations of children were conducted by clinicians at Kilifi District Hospital, Kenya.

Contents

Chapter 1

Introduction

1.1 Background

Malaria is a major public health problem in the world. In 2010, it is estimated that there were over 200 million clinical cases of malaria and approximately 700,000 deaths (World Health Organization 2011). Most of the malaria burden was borne by developing countries, whereby Sub-Saharan Africa accounted for over 70% of the cases while South-East Asian countries accounted for approximately 25% of all malaria cases (Snow, Craig *et al*. 1999). It may not be coincidental that the inhabitants of these regions are the poorest people in the world. The economic burden of malaria on the developing world that falls mainly on African nations, was estimated to be 12 billion dollars per year at the turn of the millennium (Sachs and Malaney 2002). Malaria is thought to be partly responsible for the slow economic growth of some areas (Gallup and Sachs 2001). In areas of high malaria transmission, children under 5 years are the most affected by malaria deaths, an age distribution related to the slow acquisition of antibodies to the malaria parasite. Malaria is thought to also contribute more widely to death rates in these areas, since malaria-specific control measures often lead to a decrease in non-malaria related deaths (Molineaux 1997).

In the past decade, there has been an unprecedented decrease in malaria-related mortality and morbidity (World Health Organization 2011, Murray, Rosenfeld *et al*. 2012), although the exact cause of the decrease in malaria-related morbidity and mortality is not entirely understood. The decrease in malaria related deaths can be explained by a combination of factors, including successful case-management of disease, increased access to effective

anti-malaria drugs particularly, Artemisinin-Combination therapies, widespread use of effective preventive measures such as distribution of insecticide-treated bed nets and greater financial commitment from the developed world towards malaria control and perhaps eventual eradication (O'Meara, Bejon *et al*. 2008, O'Meara, Mangeni *et al*. 2010). Although there is reported decrease in malaria transmission, the population growth rates in malaria-endemic areas, may lead to an increase in malaria cases in future. There is also reported emerging drug and insecticide resistance. Therefore, there is continuing need to develop more effective methods to prevent and treat malaria.

A vaccine for malaria that reduces morbidity and mortality from severe forms of malaria is thought to be the ideal and long-term solution for the malaria problem. There has been a series of substantial leaps forward in the study of the parasite including successful cultivation of asexual *Plasmodium falciparum* malaria parasites *in vitro* (Trager and Jensen 1976) and the completion of the genome sequencing of the *Plasmodium falciparum* line 3D7 genome (Gardner, Hall *et al*. 2002).

Furthermore, there is evidence of renewed global commitment to funding research leading to development of a vaccine for malaria and more generally for the basic research of this complex disease. Notable recent initiatives include the European Union Network of Excellence (*BioMalPar*), Bill and Melinda Gates Foundation, Malaria Vaccine Initiative (MVI) and Global Alliance for Vaccines and Immunizations (GAVI). For example over 2.6 billion dollars was spent on malaria prevention and eradication programmes in 83 endemic countries.

1.1.1 History of malaria

Malaria-like symptoms have been recorded throughout history. The first mention of malaria is found in Chinese text over 4000 years ago in the *Canon of Medicine* edited by Emperor Huang Ti (Bruce-Chwatt 1988). The remains of King Tutankhamun of Egypt who died in 1323 BC have suggested that he may have died from malaria (Arman, Adams *et al*. 2013). Early Indian medical literature refers to a deadly fever especially common in the rainy season (Bruce-Chwatt 1988). Malaria was also prevalent in ancient Greece in 400BC. Hippocrates, in reference to what we now believe to have been malaria, noted that people who drank stagnant waters suffered from quartan fevers and had enlarged spleens (Bruce-Chwatt 1988).

The causative agent of malaria was discovered on 20th October 1880, when Alphonse Laveran, stationed in Algeria, using a light microscope, noticed crescent-shaped bodies among the erythrocytes of a fellow soldier suffering from intermittent fevers (Bruce-Chwatt 1988). Laveran's discovery remained unnoticed until 1896 when Canadian physician George McCallum observed sexual stage Haemoproteus parasites wriggling among blood cells of a crow**.** These were sexual stage parasites similar to those observed by Laveran. The transmission of these parasites remained unexplained until 1897 when Ronald Ross showed that mosquitoes fed on *Plasmodium reticulum*-infected blood from birds had parasites in their digestive tract. For their discoveries Ronald Ross and Alphonse Laveran received the Nobel Prize in 1902 and 1907 respectively. The mosquito was recognized as the vector for malaria but Italian scientist Grassi was the first to show that it belonged to the Anopheles genus where he showed that an anopheles mosquito fed on malaria-infected blood could transmit malaria (Grassi and Bignami 1899).

1.1.2 Plasmodium parasite lifecycle

In Africa, three mosquito species are known to be the main vectors that transmit malaria namely; *Anopheles gambiae*, *A. arabiensis,* and *A. funestus* (Coetzee 2004). Malaria is caused by protozoan parasites of the Plasmodium genus, for a long time there were four plasmodial species known to cause human malaria, namely, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. However, since 2004 the Simian malaria parasite *P. knowlesi* is now considered a fifth Plasmodium species that infects humans (Singh, Kim Sung *et al*. 2004, Cox-Singh, Davis *et al*. 2008). *P. falciparum* is the most important of the four since it is responsible for almost all malaria-related mortality and morbidity and is, not surprisingly, is the most studied of all the Plasmodium species. Traditionally *P. vivax* was thought to be 'benign' but currently this has been refuted (Baird 2007, Baird, Schwartz *et al*. 2007, Barcus, Basri *et al*. 2007, Cerutti, Boulos *et al*. 2007). *P. malariae* is found worldwide but causes limited symptoms. *P. ovale* is found only in a few places and causes mild malaria (Mueller, Sie *et al*. 2007, Mueller, Zimmerman *et al*. 2007).

The Plasmodium parasite has a complex life consisting of a sexual and an asexual stage of the life cycle. The asexual part of the life cycle that takes place in the human host is represented in Figure 1.1 below.

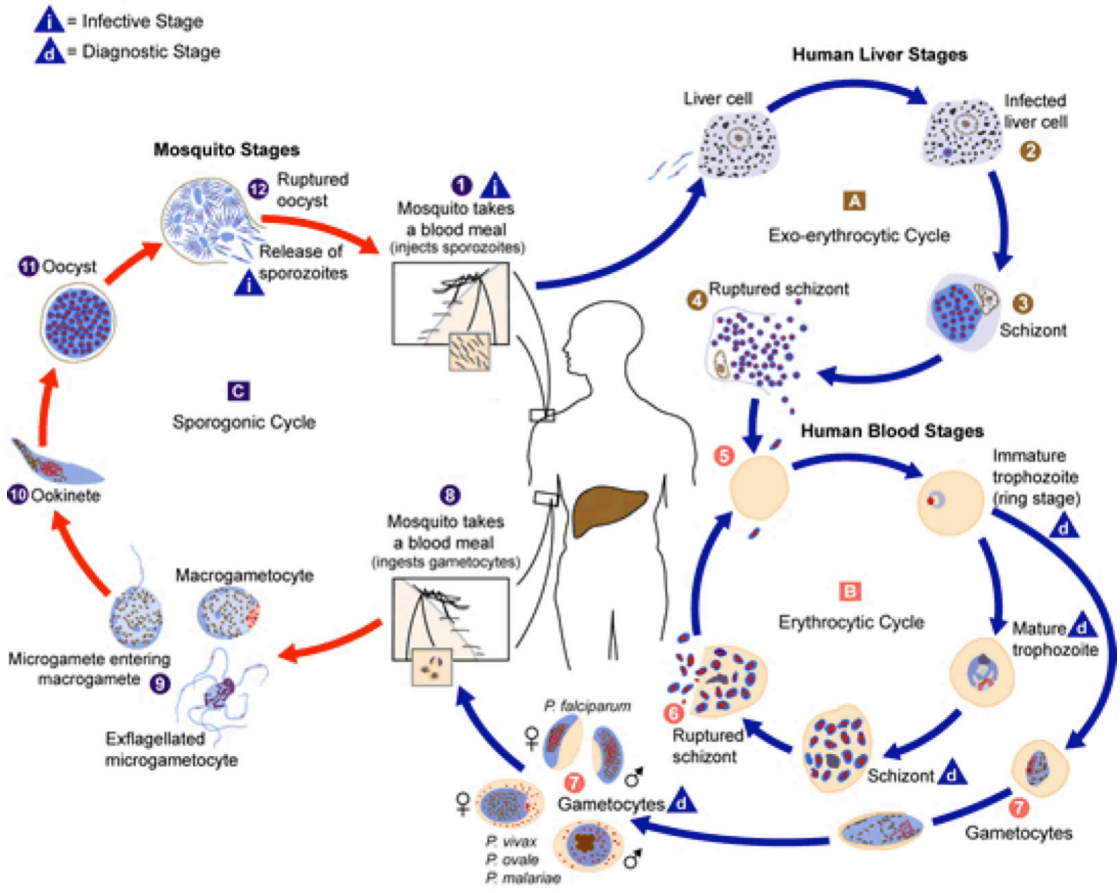


Figure 1.1 Representation of *P. falciparum* development life cycle

A female mosquito inoculates sporozoites into the blood stream of a human host (1), the sporozoites move to the liver where they invade hepatocytes (2), the sporozoites multiply asexually and grow into schizonts (3) the schizonts rupture releasing merozoites into the bloodstream (4), the merozoites infect erythrocytes (5), the merozoites grow from the trophozoite to the schizont stages and rupture releasing more merozoites (6), some merozoites differentiate into the sexual stage becoming gametocytes (7), a female mosquito ingests male and female gametocytes during a blood meal (8), in the stomach the male gametocyte penetrates the female gametocyte to form a zygote (9), the zygote becomes motile and elongates forming an ökinete (10), the ökinete invades the mid-gut wall and develops into an öocyst (11), the oocyst grows, ruptures and releases sporozoites (12), which make their way to the salivary glands. (Source: http://www.cdc.gov/malaria/)

a) Sexual stage

The sexual stage of parasite development takes place in female anopheles mosquitoes. Transmission begins when a female anopheline mosquito ingests gametocytes from an infected host during a blood meal. The sexual stage life cycle is illustrates in Figure 1.1. The mosquito ingests male and female gametocytes from the blood stream of an infected host during a blood-meal. The male gametocyte penetrates the female gametocyte to form a zygote. The zygote develops into a motile öokinete that migrates to the basal site of a mid-gut cell where it develops into an öocyst. The öocyst divides to form sporoblasts, and eventually sporozoites, approximately 10 to 21 days after ingestion depending ambient temperature. Transmission is continued when the mosquito deposits between 1 and 100 infective sporozoites into the dermis of the next potential host in the next blood meal (Rosenberg, Wirtz *et al*. 1990).

b) Asexual stage

The asexual stage of the parasite development takes place in the vertebrate host. After the infective sporozoites are inoculated into the dermis, these parasite forms migrate into the blood stream (Rosenberg, Wirtz *et al*. 1990). Video microscopy shows that some sporozoites remain in the dermis before phagocytocis; 20-30% sporozoites are degraded in lymph nodes (Amino, Thiberge *et al*. 2007). For the remaining sporozoites to invade hepatocytes they have to cross the sinusoidal barrier with Kupffer cells (Pradel 2007, Thiberge, Blazquez *et al*. 2007). The invasive long and slender sporozoites travel to the liver, where 5-10% successfully invade hepatocytes (Ferreira, Enea *et al*. 1986). In the hepatocytes, the sporozoites multiply without causing any symptoms for up to two weeks, after which hepatocytes rupture and release up to 30,000 merozoites into systemic

circulation (Rodrigues, Prudencio *et al*. 2012). The merozoites enter the blood stream where they invade erythrocytes very rapidly. Invasion of erythrocytes by merozoites is organised into three steps including attachment to the erythrocyte surface, re-orientation, so that the apical end is positioned next to the erythrocyte membrane, formation of a tight junction between the membrane of the erythrocyte and the merozoite and finally invasion powered by an actin-myosin motor (Keeley and Soldati 2004). The act of invasion pushes a section of the erythrocyte membrane into the erythrocyte thus creating the vacuole membrane that the parasite resides in. The inverted portion of the erythrocyte membrane fuses with at the point of entry to form the parasitophorous vacuole. During the intra-erythrocytic stage the parasite consumes amino acids derived from the erythrocyte haemoglobin (Pasvol and Wilson 1982). The invasive merozoites grow from rings through trophozoites to schizonts and undergo up to 6 rounds of mitotic DNA replication, ingesting and digesting haemoglobin. The mature trophozoite occupies up to 20% of the erythrocyte volume and extends a tubovescular network extending from the parasitophorous vacuole membrane into the erythrocyte cytoplasm (Elmendorf and Haldar 1993). Subsequently the erythrocyte loses shape and becomes less deformable developing protruding modules known as knobs (Kilejian, Rashid *et al*. 1991). At approximately 48 hours post-merozoite invasion, the erythrocyte ruptures and releases 16-32 merozoites and other parasite material into the blood stream some of which act as pyrogens (Sherry, Alava *et al*. 1995). The intermittent chills suffered by malaria patients coincide with erythrocyte egress explaining the periodicity of some malaria symptoms. A small proportion of the the merozoites develop into the sexual stage as male and female gametocytes, which are ingested by a female mosquito during a blood meal to continue with the parasite life cycle. Most of the merozoites reinvade fresh erythrocytes to repeat the invasion cycle.

1.2 Clinical presentation of malaria

In endemic areas, repeated malaria infections lead to a build-up of a non-sterile malaria-specific immunity characterized by reduced incidence of clinical malaria episodes with age (Snow, Craig *et al*. 1999). Malaria infections are mostly characterized by a mild febrile illness, which is easily treated with anti-malarial drugs. However, for reasons that are not clearly understood, a small percentage of infections develop to severe disease. Since the number of infections globally runs in hundreds of millions per year, the absolute number of resulting severe malaria cases is enormous.

a) Non-severe malaria

The initial symptoms of malaria infection are common to many other infections. Early malaria symptoms include general malaise, fever, headache, myalgia, coughing and a typical shaking chill accompanied by fever, and at times, diarrhoea, vomiting and abdominal pain (Warrell and Gilles 2002). Children born in endemic areas are largely protected from severe malaria in the first 6 months of life by the passive transfer of maternal immunoglobulins and by fetal haemoglobin. A definitive malaria diagnosis is made by careful examination of the symptoms accompanied by a parasitological identification of asexual stages of the parasites in blood. In the absence of treatment, the disease may progress to severe malaria.

b) Severe malaria (SM)

Severe malaria consists of several syndromes that are associated with high rates of mortality. Common features associated with high mortality in children (impaired consciousness, convulsions, acidosis and severe malaria anaemia) are different from those

associated with adult severe malaria in areas of low malaria transmission (adult respiratory distress, jaundice and renal failure) (Warrell and Gilles 2002). Severe malaria has been categorised into three overlapping groups; impaired consciousness, severe malaria anaemia (SMA) and respiratory distress (Marsh, Forster *et al*. 1995). Understanding the pathophysiology of malaria is particularly important as most malaria deaths occur within 24 hours of admission.

i) Cerebral malaria (CM)

Cerebral malaria is the most severe form of malaria characterized by unconsciousness, with or without epileptic fits, and an inability to localize painful stimuli (Warrell and Gilles 2002). Cerebral malaria is associated with a high rate of mortality (White, Warrell *et al*. 1985, Molyneux, Taylor *et al*. 1989, Kwiatkowski, Molyneux *et al*. 1993, Marsh, Forster *et al*. 1995). A significant minority of the survivors develop neurological sequelae (Brewster, Kwiatkowski *et al*. 1990, van Hensbroek, Palmer *et al*. 1997) but for those who recover the return to consciousness can be rapid (Turner 1997). As the majority of cerebral malaria patients do not suffer neurological sequelae, it is plausible that the pathological processes underlying coma do not cause extensive irreversible destruction of the host cells and tissues. The presentation of disease changes from severe anaemia in children aged between 1 and 3 years in areas of high transmission to cerebral malaria in older children in areas of lower transmission (Snow, Craig *et al.* 1999).

ii) Severe malaria anaemia (SMA)

Severe malaria anaemia (SMA) is defined as haemoglobin concentration of 5g/dl or less or a haematocrit of less than 33 % in the presence of *P. falciparum* parasites with or without

respiratory distress (WHO 2000). The etiology of SMA is not clearly understood but can include a number of distinct, as well as overlapping features, including lysis of infected and uninfected erythrocytes (Dondorp, Angus *et al*. 1999), splenic sequestration of erythrocytes (Buffet, Safeukui *et al*. 2011), dyserythropoiesis and bone marrow suppression (Abdalla, Weatherall *et al.* 1980).

The anaemia of *P. falciparum* malaria is typically normocytic and normochromic, with a notable absence of reticulocytes, although microcytosis and hypochromia may be present due to the very high frequency of alpha and beta thalassemia traits and/or iron deficiency in many endemic areas (Roberts, Casals-Pascual *et al.* 2005, Kai and Roberts 2008). The underlying causes of SMA in humans may include, the clearance and/or destruction of infected erythrocytes , the clearance of uninfected erythrocytes (Dondorp, Angus *et al.* 1999), erythropoietic suppression and dyserythropoiesis (Abdalla, Weatherall *et al.* 1980). Each of these mechanisms has been implicated in both human and mouse malarial anaemia, but their relative contribution between the species is likely to differ.

iii) Respiratory distress (RD)

Respiratory distress is characterised by rapid breathing and use of auxiliary muscles for respiration and difficulty in breathing caused by metabolic acidosis (Marsh, Forster *et al*. 1995). Metabolic acidosis is the single most important predictor of death in clinical malaria and in children may be due to hypovolemia, reduced cardiac output, reduced oxygen carrying capacity of the blood, microcirculatory obstruction and/or impaired cellular metabolisms (Marsh, Forster *et al*. 1995)(Mackintosh, Beeson et al. 2004). In adults, severe malaria, respiratory distress may be caused by pulmonary oedema but in African children this is unusual (Taylor, Molyneux *et al*. 1988, Molyneux, Taylor *et al*.

1989). The capillary system in the lungs constitutes a large proportion of the body's total capillary system. Therefore IE binding to the lungs in adult vessels may lead to endothelial damage, resulting in reduced perfusion and possibly contribute to acidosis that is so prevalent in malaria. Poor peripheral perfusion leads to impaired oxygen delivery and acidosis while impaired pulmonary perfusion leads to hypoxia.

1.3 Cytoadherence of IEs

Mature stage-infected erythrocytes adhere to host endothelial cells, a phenomenon thought to contribute to malaria disease pathology. Cytoadhesion occurs in three different forms, namely, adhesion of IEs to endothelial cells in the post-capillary venules or other organs, rosetting of uninfected erythrocytes on IEs and platelet-mediated clumping. The adhesion of IEs to endothelia in post-capillary venules, formation of rosettes with uninfected erythrocytes and or aggregating of IE clumps with platelets may lead to obstruction of blood vessels that may result in tissue hypoxia, anaerobic respiration, metabolic acidosis and organ dysfunction (Rowe, Claessens *et al*. 2009). As the parasite matures IEs exhibit reduced deformability and sequester from circulation disrupting blood flow and causing localized endothelial dysfunction (Francischetti, Seydel *et al*. 2008, Moxon, Grau *et al*. 2011).

1.3.1 Cytoadhesion

Endothelial cell receptors are reported to play a role in the adhesion of IEs although the specific role of individual receptors in pathogenesis is not clearly understood. Sequestration of IEs to endothelial cells may involve multiple receptors. For some of them the parasite ligand has been identified while for others it is yet to be identified. The host receptors known to date for endothelial cell adhesion of IEs include:

a) CD36

CD36 is an 88kDa glycoprotein expressed on diverse host cells including platelets, endothelial cells, erythroid precursors and monocytes and has a correspondingly wide

range of functions. Virtually all malaria clinical isolates bind to CD36 with high avidity and this interaction is usually stable (Barnwell 1989, Cooke, Berendt *et al*. 1994). CD36-binding by IEs is associated with non-severe malaria, a phenomenon attributed to the ubiquity of CD36 in most part of the body except the brain, therefore it is thought that parasites are distributed throughout the body and are not concentrated in areas such as the brain that would potentially lead to severe disease (Newbold, Warn *et al*. 1997). Further evidence for the importance of CD36 is suggested from the prevalence of mutations in African populations in the regions that code for CD36 that either confers susceptibility (Aitman, Cooper et al. 2000) or protection (Aitman, Cooper *et al*. 2000, Pain, Urban *et al*. 2001) to clinical malaria episodes for people that carry these alleles.

b) Thrombospondin

Thrombospondin (TSP) is a 450kDa membrane receptor expressed by endothelial cells, platelets and monocytes. Physiological ligands associated with TSP are numerous and include: CD36, fibronectin, fibrinogen, plasminogen, collagen and calcium. Although TSP was the first receptor to be shown to bind to infected erythrocytes (Roberts, Sherwood *et al*. 1985), this phenotype is not as well characterised as others. The epitope on TSP that interacts with parasite ligands is thought to lie in the type 3 repeats on TSP (Eda, Lawler *et al*. 1999). Most wild isolates have been shown to be capable of binding to TSP but the parasite ligand on the IE surface that binds to TSP is still controversial. They include PfEMP-1 (Baruch, Gormely *et al*. 1996) and phosphatidyl serine (Eda and Sherman 2002). Thrombospondin binding is weak *in vitro* and no specific disease syndrome has been associated with isolates that bind TSP (Cooke, Berendt *et al*. 1994).

c) ICAM-1

Intercellular adhesion molecule 1 (ICAM-1) is a 80-115 kDa member of the immunoglobulin superfamily expressed by some ECs and leukocytes. Expression of ICAM-1 on endothelial cells is upregulated by inflammatory cytokines TNF-α and IFN-γ and is known to be elevated in acute malaria (Berendt, Simmons *et al*. 1989). Increased expression of ICAM-1 has been shown on endothelial cells of the brains of children that died from cerebral malaria (Turner, Morrison *et al*. 1994). Parasite isolates from patients with severe malaria exhibit a higher affinity for ICAM-1 binding than isolates from patients with non-severe malaria (Newbold, Warn *et al*. 1997). Binding of IEs to purified recombinant ICAM-1 *in vitro* is weak and needs to be stabilized by synergistic adhesion to other receptors (Craig, Pinches *et al*. 1997). The binding site on ICAM-1 for the IE ligands was mapped and was found to be different from the known LFA-1 binding site (Berendt, McDowall *et al*. 1992). Reasoning that selection pressure by malaria would lead to polymorphisms in the host ICAM-1, Newbold and colleagues sequenced the N-terminal domain of ICAM-1 and found a high frequency mutation that was associated with the severity of clinical malaria such that individuals homozygous for the mutation showed increased susceptibility to cerebral malaria (Fernandez-Reyes, Craig *et al*. 1997).

d) PECAM-1

Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) is a member of the immunoglobulin superfamily, has a molecular weight of 130 kDa, and is expressed on ECs, platelets, macrophages, some subsets of T-cells and NK cells. PECAM-1 is encoded by a gene covering 75 kb on the long arm of chromosome 17 in humans. *P. falciparum* binding to PECAM-1 is reported in both laboratory parasite lines and diverse patient

isolates (Newbold, Warn *et al*. 1997, Treutiger, Heddini *et al*. 1997, Heddini, Chen *et al*. 2001, Heddini, Pettersson *et al*. 2001). Although some studies did not find a correlation between binding of PECAM-1 and severe disease (Newbold, Warn *et al*. 1997, Heddini, Chen *et al*. 2001), polymorphisms were found to be associated with susceptibility to cerebral malaria in Thailand (Kikuchi, Looareesuwan *et al*. 2001). A different polymorphism found in Papua New Guinea was not associated with malaria outcome (Casals-Pascual, Allen *et al*. 2001). The binding site on PECAM-1 for parasite ligands was mapped to the first four immunoglobulin-like domains (Treutiger, Heddini *et al*. 1997). The parasite ligand for PECAM-1 is thought to be a PfEMP-1 (Chen, Heddini *et al*. 2000, Joergensen, Bengtsson *et al*. 2010, Berger, Turner *et al*. 2013).

e) P-Selectin

P-Selectin is a 140-kDa protein found in Weibel-Palade bodies of ECs and the alpha granules of platelets. Inflammatory mediators activate ECs and platelets leading to translocation of intracellular P-Selectin to the plasma membrane. The putative physiological ligands for P-Selectin include CD24 and P-Selectin Glycoprotein Ligand-1 (PSGL-1) found on all leucocytes. *P. falciparum*-infected erythrocytes use P-Selectin to roll on activated endothelium and immobilized platelets using a parasite ligand thought to be a PfEMP-1 (Ho, Schollaardt *et al*. 1998, Yipp, Anand *et al*. 2000, Senczuk, Reeder *et al*. 2001). The specific variant of PfEMP-1 that mediates P-Selectin adhesion has not been identified. P-Selectin is also a platelet receptor for platelet-mediated clumping. P-Selectin is a marker for platelet activation and using this criteria, platelets are activated on co-incubation with IEs (Srivastava, Cockburn *et al*. 2008). However, so far there is no published field study comparing P-Selectin binding comparing mild and severe malaria.

f) gC1qR/HABP (Hyaluronan binding protein)

gC1qR/HABP is a 33 KDa protein present in the mitochondria and expressed by endothelial cells and platelets. The main ligand for gC1qR is C1q on B-cells, neutrophils and mast cells. In its mature form, gC1qR/HABP has 209 amino acids. It is highly acidic with 28 glutamine and 20 asparagine residues. gC1qR was shown to bind to IEs as well as mediate platelet-mediated clumping (Biswas, Hafiz *et al*. 2007). Cytoadherence of IEs to gC1qR/HABP has been associated with prostration in severe malaria in Mozambique (Biswas, Hafiz *et al*. 2007, Mayor, Hafiz *et al*. 2011). The parasite ligand for binding to gC1qR is yet to be identified.

g) E-Selectin

E-Selectin is also known as the Endothelial Cell Adhesion Molecule (E-LAM). When parasite-binding to on cytokine-activated endothelial cells was enriched by *in vitro* selection, E-Selectin expression was upregulated on the endothelial cells and hence part of this binding was attributed to E-Selectin (Ockenhouse, Tegoshi *et al*. 1992). Field isolates from Asia and Africa have also been shown to bind to E-Selectin although none of these studies showed a relationship with malaria severity (Udomsangpetch, Taylor *et al*. 1996, Newbold, Warn *et al*. 1997, Udomsangpetch, Reinhardt *et al*. 1997). The parasite ligand for binding to E-Selectin is yet to be identified.

h) Vascular Cell Adhesion Molecule-1 (VCAM-1)

VCAM (CD106) is a member of the immunoglobulin superfamily that is up-regulated on endothelial cells upon cytokine activation. VCAM has six extracellular domains and the

part that interacts with IEs is thought to lie in domains 1 to 4 (Udomsangpetch, Reinhardt *et al*. 1997). Clinical isolates of infected erythrocytes rolled on VCAM-1 and other adhesion molecules but failed to bind VCAM-1 in static assays (Udomsangpetch, Reinhardt *et al*. 1997). There are reports of static binding of malaria field isolates to purified soluble VCAM (Ockenhouse, Tegoshi *et al*. 1992). The binding of a subset of parasites has been reported in various subsequent studies (Newbold, Craig *et al*. 1999). However so far, no polymorphisms have so far reported to be associated with malaria disease susceptibility. It is one of the least studied receptors for IE binding on human endothelial cells.

i) Neural Cell Adhesion Molecule (NCAM)

Neural Cell Adhesion Molecule (NCAM) is a member of the immunoglobulin superfamily encoded by a single gene that is very well conserved across vertebrate species. Pouvelle and colleagues showed that selection of IEs on NCAM lead to enrichment of NCAM-binding by the IEs (Pouvelle, Matarazzo *et al*. 2007). Clinical studies have not been conducted to study the association of NCAM-binding with clinical disease. Furthermore the parasite ligand that is used to bind NCAM is unknown.

j) αvβ3 integrin

Integrins are a family of transmembrane heterodimeric adhesion receptors with α and β subunits. The α_v-subunit has 125 kDa while the β_3 subunit has 105 kDa. The $\alpha_v\beta_3$ integrin exists in both a relaxed and in a flexed conformation. Indirect evidence implicates $\alpha_v\beta_3$ integrin as an IE-binding receptor on Human Dermal Endothelial Cells (HDMECs) (Siano, Grady *et al*. 1998). Monoclonal antibodies for $\alpha_v\beta_3$ integrin failed to inhibit platelet-

mediated clumping (Pain, Ferguson *et al*. 2001). No field study has been conducted to determine the relationship of $\alpha_v\beta_3$ integrin binding to different malaria disease syndromes and polymorphisms of the gene encoding $\alpha_v\beta_3$ integrin have not been investigated in relation to resistance or susceptibility to the various forms of malaria. Finally, the parasite ligand used by $\alpha_v\beta_3$ integrin to bind to the *P.falciparum*-infected erythrocytes has not yet been identified.

k) Chondroitin Sulphate A (CSA)

The binding of *P. falciparum* IEs to the placenta was first reported in 1979 (Bray and Sinden 1979). Adhesion of IEs to CSA is a major part of the pathogenesis of placental malaria (Fried and Duffy 1996, Beeson, Brown *et al*. 1999). IEs collected from *P. falciparum*-infected pregnant women, but not from children and other non-pregnant adults, have a preference for binding CSA *in vitro* (Fried and Duffy 1996, Beeson, Brown *et al*. 1999, Gysin, Pouvelle *et al*. 1999). Furthermore IEs recovered from the placenta of *P. falciparum*-infected pregnant women do not show any adherence to the commonly bound endothelial receptors such as CD36 or ICAM-1. However, IEs from peripheral blood of pregnant women bind to both CSA and CD36 suggesting these circulating ring-stage parasites have arisen from both placentally-bound parasites and parasites sequestered elsewhere in the vasculature (Fried and Duffy 1996, Beeson, Brown *et al*. 1999).

l) Endothelial Protein C Receptor (EPCR)

EPCR is encoded by the *PROCR* gene and is expressed on endothelial cells. EPCR is an important component of the protein C anticoagulant pathway (Simmonds and Lane 1999).

Protein C binds to EPCR promoting its conversion to activated protein C (Stearns-Kurosawa, Kurosawa *et al*. 1996). EPCR was shown to be an important receptor for *P. falciparum*-infected cells to bind to brain endothelial cells in a study that also identified the parasite ligand for this receptor to be a PfEMP-1 (Turner, Lavstsen *et al*. 2013). A different study showed that loss of EPCR was linked to severe malaria (Moxon, Wassmer *et al*. 2013).

1.3.2 Rosetting

Rosetting refers to the ability of *P. falciparum*-infected erythrocytes to agglutinate uninfected erythrocytes, a characteristic phenotype of a subset of parasites. Rosetting has been associated with severe malaria in several studies through direct and indirect evidence in Africa (Carlson, Helmby *et al*. 1990, Treutiger, Hedlund *et al*. 1992, Rowe, Obeiro *et al*. 1995), but not in Asia (al-Yaman, Genton *et al*. 1995). Rosetting is thought to contribute to malaria pathology by enhancing obstruction of post-capillary venules (Kaul, Roth *et al*. 1991). Rosetting involves several erythrocyte ligands including heparan sulphate (HS), complement receptor 1 (CR1), IgM and blood group antigens (Carlson, Ekre *et al*. 1992, Rowe, Moulds *et al*. 1997, Chen, Barragan *et al*. 1998, Barragan, Kremsner *et al*. 2000, Vogt, Barragan *et al*. 2003, Kyriacou, Steen *et al*. 2007, Ghumra, Semblat *et al*. 2008). The parasite ligands on *P. falciparum*-infected IEs that mediate resetting have been found to be of a specific sub-type of the PfEMP-1 family that are associated with severe malaria known as the group A type and with a specific DBL-alpha domain (Rowe, Moulds *et al*. 1997, Chen, Heddini *et al*. 2000, Ghumra, Semblat *et al*. 2008). (The classification of PfEMP-1 is discussed below section 1.4).

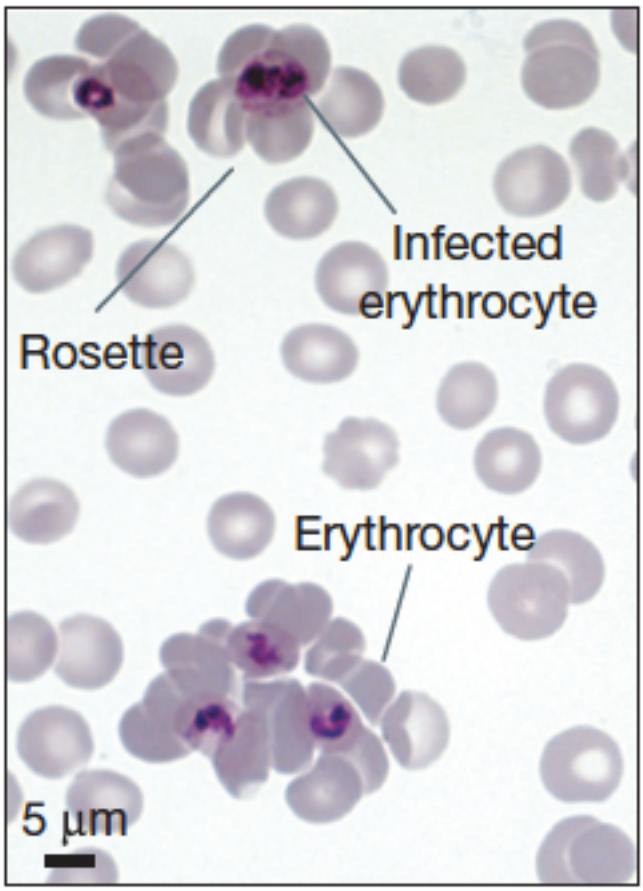


Figure 1.2 Rosetting

Rosettes detected in *in vitro P. falciparum* cultures observed after preparation of Giemsa-stained thin smears and light microscopy (Rowe, Claessens *et al*. 2009).

1.4 Antigenic variation and parasite adhesion ligands

Antigenic variation is the process by which several pathogens are able to prolong infection and therefore maximize chances of transmission among hosts by escaping antigen-specific immune responses of the host. This is achieved by varying the dominant epitopes that are recognized and targeted by the host immune system thus providing a means for escape from previous variant-specific immune responses mounted by the host. This can be in the form of clonal antigenic variation –the expression of functionally similar but antigenically distinct forms of a protein within a population of cells derived from a clone. Pathogenic microorganisms that exhibit antigenic variation include bacteria (*Neisseria, Borrelia*, and *Anaplasma*), a fungus (*Candida*) and protozoa (*Plasmodium, Trypanosoma, Babesia*, and *Giardia*) (Borst and Cross 1982, Kyes, Horrocks *et al*. 2001, Nash 2002, Allred and Al-Khedery 2004, Guizetti and Scherf 2013).

Antigenic variation in *P. falciparum* was alluded to in early work in bird, rodent and non-human primate studies by testing the ability of sera from infected animals to specifically agglutinate IEs but not uninfected erythrocytes (Eaton 1938, Cox 1959, Voller and Rossan 1969, Brown 1977, Wery, Weyn *et al*. 1979, McLean, Pearson *et al*. 1982). Brown showed that the agglutinating antibody responsible for this agglutination in *P. knowlesi* infections was specific to each recrudescent parasite population (Brown 1973). The variant antigenic types resulting from a clonal *P. knowlesi* infection of Rhesus monkeys appeared in sequential order during the natural *P. knowlesi* infection (Barnwell, Howard *et al*. 1983). The definitive biochemical identification of the variant surface antigen for *P. knowlesi* showed that the antigens were high-molecular-weight, parasite-encoded proteins distributed evenly across the IE surface, were sensitive to trypsin-treatment, were exposed

to surface radioiodination, and insoluble in non-ionic detergents (Howard, Barnwell *et al*. 1983).

Antigenic variation in *P. falciparum* was linked to the binding phenotype of a single laboratory adapted parasite line *in vitro* (Roberts, Craig *et al*. 1992). In 1995 the *var* gene family that encodes *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP-1) protein was reported to be responsible for both endothelial cell binding of IEs and antigenic variation (Baruch, Pasloske *et al*. 1995, Smith, Chitnis *et al*. 1995, Su, Heatwole *et al*. 1995).

P. falciparum parasites have a *var* gene repertoire of around 60 genes (Guizetti and Scherf 2013). *In vitro* cloning showed that parasites switch away from the parental variant type at a rate of up to 2% per generation in the absence of immune pressure (Biggs, Anders *et al*. 1992, Roberts, Craig *et al*. 1992). Indeed, mosquito-induced experimental infections in human volunteers highlighted a switching rate away from the initial dominant expressed *var* gene at a rate of 16% per generation, much higher than the 2% described *in vitro* while subsequent switching occurred at a slower rate (Roberts, Craig *et al*. 1992, Peters, Fowler *et al*. 2002). The pattern of subsequent transcription, at least early on in the infection seemed to be an intrinsic property of the set of *var* genes present within the infecting parasite as the same switch to an alternative expressed *var* was seen in more than one human volunteer (Peters, Fowler *et al*. 2002) A subsequent study found that that on and off rates for a given variant are dissimilar and that isogenic clones expressing the same *var* gene had constant on and off rates showing that they were intrinsic properties of that particular gene (Horrocks, Pinches *et al*. 2004). More recently Noble and colleagues have

shown that antigenic switching is governed by a global activation hierarchy that appears to favour short and highly diverse genes located in central chromosomal regions whereas long and conserved genes associated with severe disease are rarely activated (Noble, Christodoulou *et al*. 2013).

1.4.1 PfEMP-1 protein structure and function

PfEMP-1 is a multi-domain protein expressed on the *P. falciparum*-infected erythrocytes in clusters of knob-like protrusions. In the 1980s a strain-specific component of parasite origin was identified on the IE surface (Leech, Barnwel *et al*. 1984). This strain-specific component on the IE was later shown to be involved in the adhesion of IEs and to be accessible to host immune system (Roberts, Craig *et al*. 1992).

The PfEMP-1 protein has an extracellular and intracellular domain. The extracellular component is highly variable in both amino acid sequence composition and length among various PfEMP-1 variants. The extracellular component of the protein has a short N-terminal sequence that is required for export of PfEMP-1 out of the parasitophorous vacuole (Kriek, Tilley *et al*. 2003). The rest of the extracellular component is made up of Duffy Binding-Like (DBL) domains and Cysteine Rich Inter-Domain Regions (CIDR) (Smith, Gamain *et al*. 2001). These domains have a conserved structure, although the *var* genes encoding them are highly variable in sequence identity. A typical PfEMP-1 is made up of 4 to 7 DBL domains and 2 to 4 CIDR domains. The intracellular component of the PfEMP-1 is short and conserved.

The extreme diversity of PfEMP-1 led to doubts on whether studying the PfEMP-1 expressed by clinical isolates would yield any meaningful information on understanding

malaria disease pathogenesis due to the high rate of recombination (Ward, Clottey *et al*. 1999, Freitas-Junior, Bottius *et al*. 2000, Taylor, Kyes *et al*. 2000). However, it was later shown that PfEMP-1 variants can be classified into specific groups hence implying a functional specialization in the expressed *var* gene family from clinical isolates (Kraemer and Smith 2003). PfEMP-1 from parasites isolated from children with severe malaria expressed fewer cysteine residues than those with non-severe malaria (Kirchgatter and Portillo 2002). Another study showed that isolates from severe disease patients expressed longer *var* genes than those from mild disease (Bian and Wang 2000). Taken together, this evidence shows that despite the extreme diversity in PfEMP-1 proteins, a limited set of expressed *var* genes is associated with severe disease. Therefore, it is possible that a vaccine could be designed that induces antibodies against the PfEMP-1 proteins that are associated with severe disease.

Interestingly, parasite surface proteins of parasites from children with severe malaria are more commonly recognized by immune sera as compared to parasites from children with mild malaria (Bull, Kortok *et al*. 2000). Such surface proteins from children suffering from severe disease are more prevalent in younger children hence supporting the theory that expression of a subset of *var* genes is associated with the development of severe disease and are more likely to be expressed in children who have not developed immunity for the PfEMP-1 allotypes encoded by these *var* genes. Cerebral malaria patient isolates also express commonly recognized *var* genes (Lindenthal, Kremsner *et al*. 2003). Furthermore, IEs from blood samples taken from severe malaria patients are more commonly identified by sera even from geographically distant regions (Nielsen, Vestergaard *et al*. 2004).

In *P. falciparum* malaria, PfEMP-1 contributes to immune escape by mediating cytoadherence to host receptors on endothelial cells in deep microvasculature. By sequestering to endothelial cells in post-capillary venules, *P. falciparum*-infected erythrocytes avoid clearance from circulation by the spleen. However, other malaria parasites that do not appear to exhibit substantial cytoadherence phenotypes also insert parasite proteins in the host erythrocyte membrane.

The *Plasmodium falciparum* parasite uses the antigenically variable PfEMP-1 to inhibit maturation and activation of dendritic cells therefore compromising the host immune response to malaria infection and possibly prolonging the infection (Urban, Ferguson *et al*. 1999).

1.4.2 Var genes

PfEMP-1 is encoded by a large family of approximately 60 *var* genes per haploid genome. *Var* genes are 6 to 13kb long with a two-exon structure. The first exon encodes a variable extracellular region that is diverse and has the protein-binding regions. The second exon encodes an intracellular conserved region referred to as the Acid Terminal Sequence (ATS) (Reviewed by (Kraemer and Smith 2006)).

The majority of published data discussed below on the expression of *var* genes support a mutually exclusive expression of *var* genes. However many *var* transcripts are expressed in the ring stages as assayed by reverse-transcriptase PCR and sequencing but only a single PfEMP-1 protein is detected in the later stages of parasite maturity (Chen, Fernandez *et al*. 1998, Scherf, Hernandez-Rivas *et al*. 1998, Fernandez, Chen *et al*. 2002). The current understanding of such data is that although many *var* mRNA species are detected in the initial stages of parasite development, ultimately only one PfEMP-1 protein from the 60 *var* gene repertoire is expressed as the surface antigen on the IEs, hence limiting the exposure of antigens to the host immune system. Although Northern blots, nuclear run-on assays and single-cell RT-PCR experiments showed several *var* genes were transcribed at the ring stages (Chen, Fernandez *et al*. 1998, Scherf, Hernandez-Rivas *et al*. 1998, Horrocks, Pinches *et al*. 2004), this does not simply reflect the use of mRNA extracted from cell populations rather than individual cells. As the parasite develops from the ring to mature stages, the ‘‘loose’’ multi-*var* transcription decreases, and a single mRNA *var* becomes predominant; this dominant transcript becomes the sole translated and exported PfEMP-1 (Scherf, Lopez-Rubio *et al*. 2008). However, more recently it has been reported that some parasites may simultaneously express two different PfEMP-1

proteins on a single IE and therefore could further explain why some IEs have an ability to bind to more than one endothelial cell receptors (Joergensen, Bengtsson *et al*. 2010).

Var genes have previously been classified into subgroups according to their chromosomal location and direction of transcription (Kraemer and Smith 2003, Lavstsen, Salanti *et al*. 2003). The *var* genes were grouped into three major groups (group A, B and C) and two intermediate groups B/A and B/C representing transitions between the three major groups. In this classification it appeared that genes of the different subgroups evolved separately with limited recombination across groups. The best-defined *var* group, group A, comprised of sub-telomeric genes transcribed towards the telomere. Group A *var* genes encode PfEMP-1s with complex domain structures that are different from groups B and C. In the genome of laboratory parasite line 3D7, two sequences belonging to the *var1* and *var2* sub-families formed independent groups found in the sub-telomeric region of the chromosomes. They are functionally similar since they all have a similar CIDR region that does not bind to CD36. The majority of *var* genes fall in Group B; they are also sub-telomeric but are transcribed to the opposite direction as Group A genes. Functionally, most of Group B *var* genes have a CD36 binding CIDR region. Group C *var* genes are not sub-telomeric, they are found in the central regions of the chromosomes and are transcribed in the opposite direction of Group B *var* genes (for review see (Kraemer and Smith 2006)).

Bull and colleagues developed an alternative new way of classifying *var* genes using the length of the the first DBL domain and the number of cysteine residues on the first DBL domain resulting in six groups (Bull, Kyes *et al*. 2007). In this method it was found that

the resulting groups correlated with Group A to Group E classification that was initially described both in the 3D7 genome and in field isolates (Bull, Berriman *et al*. 2005, Bull, Kyes *et al*. 2007). Group A genes corresponded with Cys2 genes that were associated with severe disease and rosetting but were not associated with CD36 binding. Subsequent studies have since validated this classification to show that shorter *var* genes are less likely to bind to CD36 and more likely to be associated with severe malaria than Cys4 *var* genes that will bind to CD36 and will be associated with non-severe malaria.

Rask et al recently reclassified the PfEMP-1 domains and introduced fewer large and several smaller new subclasses (Rask, Hansen *et al*. 2010). This classification was done by sequence alignment, distance tree analysis and definition of homology blocks. DBL and CIDR domains appeared to be inherited within four domain components that appear to be functionally conserved. More importantly the established division of group A, B and C was confirmed.

Genomic structure and organization of *var* genes as shown by Fluorescent In Situ Hybridization (FISH) analysis implies that there is an order to the chromosomal location of the *var* genes. Sub-telomeric *var* genes would recombine since between four and seven chromosome ends are aligned near the nuclear periphery in such a way to permit recombination among homologous regions (Kraemer and Smith 2006). Most interesting is the finding that even central *var* genes are tethered to the perinuclear space hence supporting the idea that the chromosomal orientation may form recombination hotspots (Freitas-Junior, Bottius *et al*. 2000).

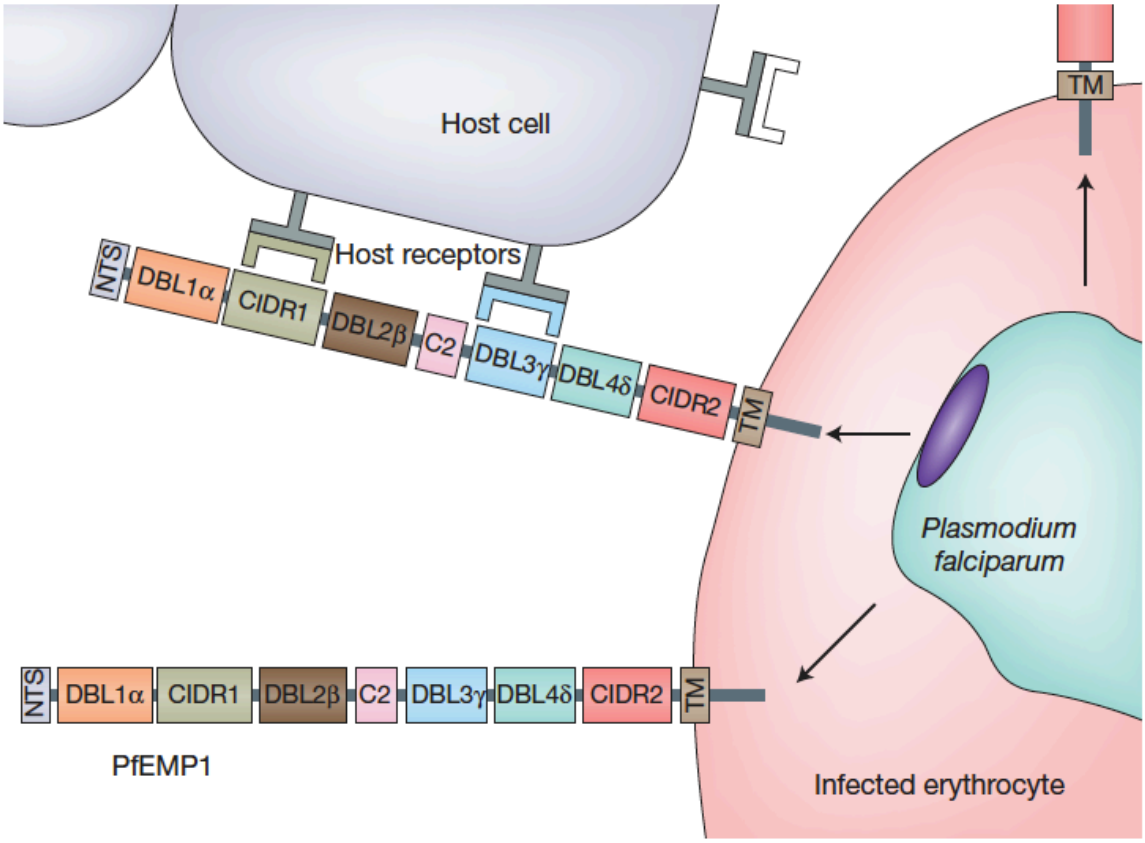


Figure 1.3 Schematic representation of PfEMP-1 on the surface of an infected erythrocyte

The extracellular region of PfEMP1 has an N-terminal segment (NTS) followed by several cysteine-rich domains known as DBL (duffy-binding-like) and CIDR (cystein-rich interdomain regions) that can be classified into distinct types based upon sequence similarity. There are six DBL types, (α, β, γ, δ, ε, ζ and X) and three CIDR types (α, β and γ). The number, location and type of DBL and CIDR domains vary among PfEMP1 variants, and this variable domain composition and extensive sequence polymorphism is thought to provide great flexibility in binding properties (Rowe, Claessens *et al.* 2009).

1.4.3 Other variable surface adhesive ligands

Besides the antigenically variable PfEMP-1, other parasite-derived proteins are expressed on the surface of the *P. falciparum*-infected erythrocyte. Some of these are thought to mediate adhesion or may have functions that are yet to be defined. Rifin (Cheng, Cloonan *et al*. 1998, Fernandez, Hommel *et al*. 1999, Kyes, Rowe *et al*. 1999), Stevor (Limpaiboon, Taylor *et al*. 1990, Cheng, Cloonan *et al*. 1998, Blythe, Surentheran *et al*. 2004, Lavazec, Sanyal *et al*. 2006, Niang, Yan Yam *et al*. 2009) and PfMC-2TM (Sam-Yellowe, Florens *et al*. 2004) have been identified as potential variable surface antigens of P. *falciparum*-infected erythrocyte. They are encoded by the *rif, stevor* and *pfmc-2tm* multigene families respectively. These genes belong to the two-transmembrane (2TM) family, which share a two-exon gene architecture (Cheng, Cloonan *et al*. 1998, Sam-Yellowe, Florens *et al*. 2004, Lavazec, Sanyal *et al*. 2006). The first exon encodes a signal peptide and the second a semi-conserved N-terminal region, separated from a highly polymorphic region by a putative transmembrane domain, with a second predicted transmembrane domain occurring before the conserved highly charged C-terminal region. All members have the Plasmodium export element (PEXEL)/vacuolar transport signal (VTS) motif (Hiller, Bhattacharjee *et al*. 2004, Marti, Good *et al*. 2004) near the start of exon 2, targeting them for translocation into the host cell. Recent data challenge the existence of the first N-terminal putative transmembrane domain (TM1), at least for some RIFIN and STEVOR family members, and instead support a topology where both the semi-conserved N-terminal region and the hypervariable region are exposed on the IE surface and anchored to the membrane only by the second C-terminal transmembrane domain (TM2) (Bultrini, Brick *et al*. 2009, Niang, Yan Yam *et al*. 2009). Regardless of the exact membrane topology of the 2TM proteins, most of the data suggest an extracellular hypervariable

region that is hypothesized to be subject to antibody-mediated immune selection leading to its diversification (Sam-Yellowe, Florens *et al*. 2004, Przyborski, Miller *et al*. 2005, Dzikowski, Templeton *et al*. 2006, Lavazec, Sanyal *et al*. 2006, Templeton 2009).

1.4.4 Knob-Associated Histidine Rich Protein (KAHRP)

Knobs are protrusions on the IE surface that are required for IEs to adhere to endothelial surfaces. The absence of knobs on IEs inhibits adhesion of IEs *in vitro*. *In vivo,* knobless parasites are not observed unless the patient is splenectomized hence implying a strong functional selection for knobs in spleen-intact patients (Langreth and Peterson 1985, Pongponratn, Viriyavejakul *et al*. 2000). Knobless IEs are generated in *in vitro* culture through the loss of one end of chromosome 2 which deletes sub-telomeric genes including *kahrp* (Lanzer, Wertheimer *et al*. 1994).

KAHRP is a major protein in knobs hence the best candidate in examining the role of knobs in cytoadhesion. The expression of KAHRP is essential for the formation of knobs on *P. falciprum*-infected erythrocytes (Crabb, Cooke *et al*. 1997). KAHRP is thought to contribute to the export of PfEMP-1 and hence could explain why knobless parasites express lower levels of PfEMP-1 on the IE surface (Knuepfer, Rug *et al*. 2005). The C-terminal region of the *KAHRP* gene is required for the development of knobs and adhesion as determined using 3D7 parasites expressing mutated forms of KAHRP (Rug, Prescott *et al*. 2006). Isogenic parasite clones expressing the same PfEMP-1 type but differing in expression of knobs have different adhesion profiles (Horrocks, Pinches *et al*. 2005). PfEMP-1 is enriched at the surface of knob structures (Baruch, Pasloske *et al*. 1995), implying that the cytoplasmic tail of PfEMP-1 interacts with some component of the knob.

The most likely candidate protein for this interaction is KAHRP which itself is then linked to spectrin in the red cell cytoskeleton (Kilejian, Rashid *et al*. 1991). There are different alleles of the KAHRP gene expressed by parasite isolates from distinct geographical regions though their structure is conserved (Kant and Sharma 1996, Mayor, Trujillo *et al*. 2000).

1.5 Platelets in malaria infections

The involvement of platelets in malaria is apparent by the almost universal occurrence of thrombocytopenia in malaria infections, reported platelet activation and platelet-mediated clumping. In this section I review published literature on the involvement of platelets in malaria infections as a background to the specific research area of this study: clumping adhesive phenotypes of *P. falciparum*-infected erythrocytes mediated by platelets.

1.5.1 Thrombocytopenia

Thrombocytopenia refers to a reduction of peripheral platelet count below 150 x 10^9 L^{-1}, a common feature in asymptomatic malaria infections, clinical malaria patients and other infections (Kelton, Keystone *et al*. 1983, Seka-Seka, Brouh *et al*. 2004, Casals-Pascual, Kai *et al*. 2006). Thrombocytopenia is so common in malaria that some researchers have proposed its use as a diagnostic and prognostic tool (Gerardin, Rogier *et al*. 2002). It is not clear what causes thrombocytopenia in malaria, but platelet numbers are likely to be reduced by platelet consumption rather than decreased platelet production. One study reported normal thrombopoetin and an increase in young platelet fraction in peripheral circulation of children with thrombocytopenic malaria indicating that platelet production may not be compromised in malaria (Casals-Pascual, Kai *et al*. 2006). There are many possible causes of thrombocytopenia in malaria. Firstly, immune mediated platelet consumption has been proposed to explain the occurrence of thrombocytopenia in both human and murine malaria (Beale, Cormack *et al*. 1972, Seka-Seka, Brouh *et al*. 2004). Interestingly, the fall in platelet counts are reported around 7-9 days after infection, which coincides with of the beginning of the erythrocytic infection (Essien 1989, Srivastava, Cockburn *et al*. 2008). This fall in platelet count is seen when the body starts to develop

antibodies for malaria infection both in murine malaria and in experimental human malaria (McMorran, Marshall *et al*. 2009). One study associated thrombocytopenia with platelet-associated antibodies (Kelton, Keystone *et al*. 1983) while others showed thatle levels of malaria-specific IgE (Seka-Seka, Brouh *et al*. 2004) and IgM levels (Beale, Cormack *et al*. 1972) were associated with thrombocytopenia and severe disease. Secondly, malaria-associated thrombocytopenia could be caused by accumulation of platelets in different parts of the body including the spleen and brain microvessels although data for this is controversial (Ezekowitz, Sim *et al*. 1985, Grau, Mackenzie *et al*. 2003, Pongporatn, Day *et al*. 2003, Karanikas, Zedwitz-Liebenstein *et al*. 2004).

1.5.2 Platelet activation

Platelets are anucleate cells traditionally known for their role in haemostasis. The platelet membrane surface expresses many receptors that bind to adhesive proteins upon stimulation besides recruiting other platelets for aggregation. Platelet activation refers to the excitation or stimulation of platelets by an agonist leading to changes in the platelets including new expression profile of surface receptors for adhesive proteins and release of granular contents (Michelson 2002). The granular contents that platelets release upon stimulation contain over 300 proteins and other bioactive substances such as Adenosine diphosphate (ADP) and serotonin (Coppinger, Cagney *et al*. 2004). Acute malaria infection is an inflammatory condition in which pyrogens and inflammatory cytokines are released into circulation. Platelets are activated by *P. falciparum*-infected erythrocytes *in vitro* (Polack, Peyron *et al*. 1990). Activation of platelets by IEs is inhibitable by trypsinization of IEs and is contact-dependent suggesting that the activation is caused by a parasite ligand possibly one of the PfEMP-1 proteins (Polack, Peyron et al. 1990).

Activation of platelets by IEs can also be inhibited by blocking platelet CD36 with monoclonal antibodies (Srivastava, Cockburn *et al*. 2008).

Platelet microparticles are sub-micron elements that are produced after membrane remodelling following platelet activation. Platelet microparticles, prevalent in plasma from patients with acute malaria infections, are a marker for platelet activation and are implicated in the pathogenesis of cerebral malaria (Combes, Coltel *et al*. 2006). It is worth noting that in one study co-incubation of platelets with *P. falciparum*-infected erythrocytes failed to activate platelets and did not lead to agglutination but lead to a release of soluble glycoprotein Ib (GpIb) in experimental malaria (De Mast, De Groot *et al*. 2010).

1.5.3 Anti-parasitic effect of platelets

In *in vitro* experiments, purified platelets inhibited growth of *P. falciparum* parasites through platelet receptor CD36 (Polack, Peyron *et al*. 1990) and recently a mechanism of parasite-killing that is dependent on metabotrophic puronergic receptor P2Y1 receptor has been described (McMorran, Marshall *et al*. 2009, McMorran, Wieczorski *et al*. 2012). Furthermore, in a mouse knock-out model, mice that lacked the *cmpl-1*gene that codes for a thrombopoetin receptor and had only 10% normal circulating platelet count were found to be more susceptible to death from malaria compared to wild type mice (McMorran, Marshall *et al*. 2009). Activated platelets release immunologically active substances into systemic circulation. The fall in platelet numbers in circulation in acute malaria may be protective from the severe disease by decreasing the number of available platelets to form clumps with IEs (Wassmer, Taylor *et al*. 2008). Platelet antagonists aspirin, prostaglandin E1 and sodium nitroprusside inhibited the platelets-killing activity against infected

parasites *in vitro* (McMorran, Marshall *et al*. 2009). This indicates that platelet activation is an important mechanism by which platelets inhibit parasite growth and therefore protect the host. More recently, it has been shown that the platelet molecule platelet factor 4 and the erythrocyte Duffy-antigen receptor are necessary for platelet-mediated killing of *P. falciparum* parasites, a protective function afforded by platelets during a malarial infection (McMorran, Wieczorski *et al*. 2012). It is not clear if this platelet-killing effect has any role to play in Africa since the protective function afforded by platelets would be lost in Duffy-negative individuals. In the later stages of malaria infection, platelets appear to be detrimental to the host by contributing to the pathogenesis of the disease, although the mechanisms by which platelets contribute to disease outcome are unclear. Platelet accumulation was reported in the brain microvessels of children who died from cerebral malaria but not those who died from non-malarial encephalopathies (Grau, Mackenzie *et al*. 2003).

Other researchers have associated thrombocytopenia in malaria with excessive platelet pooling in the spleen although results from another study failed to show enhanced platelet sequestration in the spleen using imaging techniques and labelled platelets (Karanikas, Zedwitz-Liebenstein *et al*. 2004). Although Kreil and co-workers did not find significant differences in platelet count among individuals with malaria, by comparing individuals with symptomatic and those with asymptomatic infections; they found that thrombopoetin levels were inversely proportional to platelet count (Kreil, Wenisch *et al*. 2000).

1.5.4 Platelet-mediated clumping

Platelet-mediated clumping refers to the aggregation of mature *P. falciparum* IEs in close contact with platelets (Pain, Ferguson *et al*. 2001). Apparent autoagglutination of IEs was initially reported in a laboratory parasite line and later in patient field isolates (Roberts, Craig *et al*. 1992, Roberts, Pain *et al*. 2000). Subsequent work showed that this autoagglutination was mediated by platelets and was dependent on surface-expressed platelet receptor CD36 (Pain, Ferguson *et al*. 2001). The requirement of CD36 for the platelet-mediated clumping phenotype has been reaffirmed in more studies both in field isolates and in laboratory parasite lines (Chotivanich, Sritabal *et al*. 2004, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011, Arman, Adams *et al*. 2013). Two more receptors have been reported to mediate platelet-mediated clumping, namely, P-Selectin and gC1qR (Biswas, Hafiz *et al*. 2007, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011). Curiously, the parasite ligand that interacts with the above platelet receptors to cause clumping is yet to be identified.

Platelet-mediated clumping of a given parasite isolate is determined *in vitro* by counting the number of IEs in clumps as a proportion of the total number of IEs. A clump is defined as an aggregation of three or more IEs. The level of platelet-mediated clumping of isolates varies from parasite to parasite. Platelet-mediated clumping has been associated with severe disease in four clinical studies thus far (Pain, Ferguson *et al*. 2001, Chotivanich, Sritabal *et al*. 2004, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011) but one study associated it with high parasitaemia and not disease severity (Arman, Raza *et al*. 2007, Kyriacou, Steen *et al*. 2007). Interestingly, platelet-mediated clumping was associated with severe disease in Thailand, a study that, unlike the others, was conducted in an area

of unstable malaria transmission and in adults (Chotivanich, Sritabal *et al*. 2004). The remainder of the studies were conducted with isolates from children from sub-Saharan Africa where malaria is endemic (Arman, Raza *et al*. 2007, Wassmer, Taylor *et al*. 2008, Arman and Rowe 2009, Mayor, Hafiz *et al*. 2011). In multivariate analysis it was found that both parasitaemia and clumping were independently associated with severe disease, so to investigate this further, Arman and colleagues enrolled children with non-severe hyperparasitaemia as controls to investigate the association of platelet-mediated clumping and disease severity (Arman, Raza *et al*. 2007). This led to the recommendation of using standardized parasitaemia in clinical studies investigating the platelet-mediated clumping phenotype (Arman and Rowe 2008). In the Mozambican case-control study, platelet-mediated clumping was associated with severe disease both with standardised and admission parasitaemia. This study further associated platelet-mediated clumping with prostration (Mayor, Hafiz *et al*. 2011). Table 1.1 summarizes the results from the studies on platelet-mediated clumping.

The cellular and molecular interactions that contribute to the development of platelet-mediated clumping are not entirely understood. Although the parasite ligand that mediates platelet-mediate clumping has not been identified, the asexual stage of the parasite that exhibits the platelet-mediated clumping phenotype also adheres to endothelial cell receptors (Arman and Rowe 2008). It is plausible that the parasite ligand for platelet-mediated clumping is the same or is similar to parasite ligands for other endothelial receptors.

An interesting paradox exists in the relationship of the platelet-mediated clumping phenotype and severe disease. On one hand, platelet mediated clumping is associated with severe disease while on the other hand platelet-mediated clumping is dependent on CD36, which on the endothelium is associated with non-severe malaria causing IEs (Pain, Ferguson *et al*. 2001, Robinson, Welch *et al*. 2003, Ghumra, Semblat *et al*. 2008, Pleass 2009). Furthermore, virtually all field isolates bind to CD36 whereas not all of them exhibit the platelet-mediated clumping phenotype. There are two probable explanations for this paradox, first platelet-mediated clumping may not be associated with severe disease, secondly the parasite clumping ligand may bind to different domains on CD36 from the one that is used in endothelial binding.

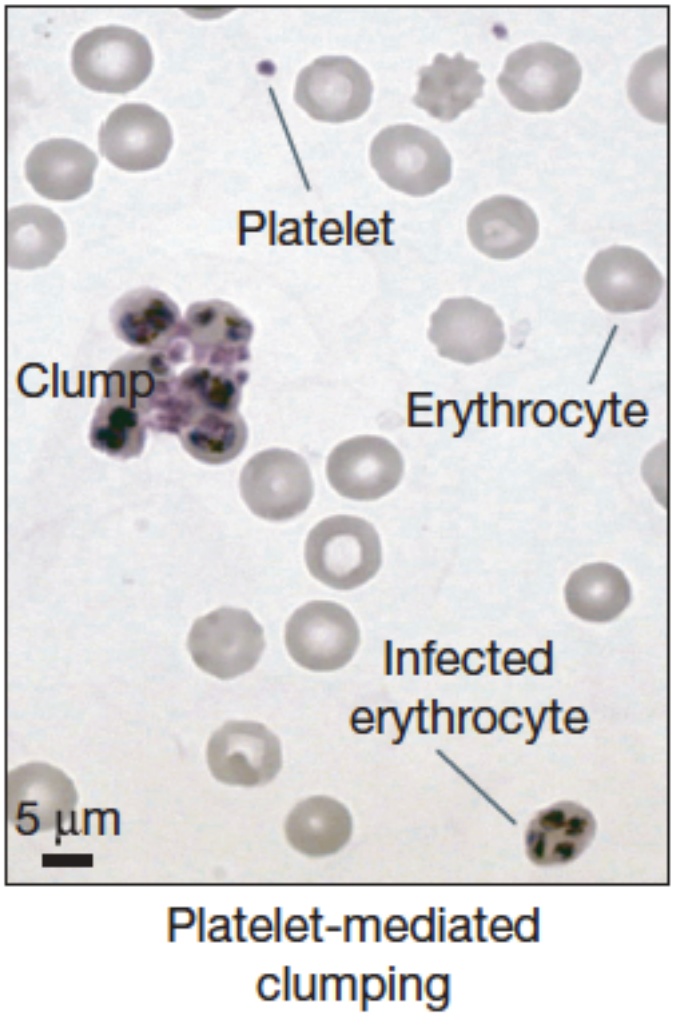


Figure 1.4 Platelet-mediated clump

Platelet-mediated clumps detected in *in vitro P. falciparum* cultures observed after preparation of Giemsa-stained thin smears and light microscopy (Rowe, Claessens *et al*. 2009).

Table 1.1 Summary of clinical studies on platelet-mediated clumping

Study	Subjects	Finding
Pain et al 2001 (Kenya)	57 Severe 64 Non-severe	Autoagglutination requires platelets CD36 required Clumping was associated with severe disease (p= 0.01, Mann–Whitney Test)
Chotivanich et al 2004 (Thailand)	104 Severe 15 Cerebral 63 Non-severe	All cerebral malaria patients had clumping (p=0.001) Proportion of patients with clumping between severe and non-severe malaria was similar (43% vs 41%)
Arman et al 2007 (Mali)	51 Severe 51 Non-severe 29 Non-severe*	Clumping was associated with admission parasitaemia (p<0.001) Clumping was not associated with disease (p=0.17)
Wassmer et al 2008 (Malawi)	22 CM 12 Non-severe 15 SMA	Clumping was associated with severe disease Thrombocytopenia was associated with severe disease Clumping was associated with cerebral malaria
Mayor et al 2011 (Mozambique)	46 Severe 46 Non-severe	Clumping prevalence was higher in cases (p=0.19) Clumping prevalence was higher in children with prostration (p = 0.49)

* Asymptomatic patients with high parasitaemia

1.6 Previous work and current study

1.6.1 Previous work

Platelet mediated clumping has been studied in both laboratory parasite lines and in field isolates as discussed above and is thought to be important in the pathogenesis of severe disease in diverse clinical and geographical settings. Although not all studies thus far that investigated the platelet-mediated clumping phenotype agree on its importance and relevance in malaria pathogenesis. The dissenting results have been explained by the sensitivity of the clumping assay to experimental conditions and it has been noted that earlier studies were conducted under different experimental conditions. A further challenge was that the studies were conducted in a relatively small number of samples.

Pain and colleagues showed that platelets are required for platelet-mediated clumping to take place. They further showed that platelet receptor CD36 was the most important receptor for the platelet-mediated clumping phenotype. The next logical step is to investigate the parasite ligand that mediates this phenomenon. Previous unpublished work in our lab by Dr Taco Kooij attempted to identify the parasite ligand involved in platelet-mediated clumping. At the same time George Warimwe, a PhD student of Dr Peter Bull, screened the platelet-mediated clumping phenotype of clinical isolates in Kilifi, Kenya.

1.6.2 Current study

This study has continued on the work of studying the interactions involved in the platelet-mediated clumping of *P. falciparum*-infected erythrocytes. The objectives of the study were

a) To optimize the experimental conditions for the clumping assay
b) To investigate *var* gene expression of laboratory isolates with the clumping phenotype
c) To investigate platelet receptors and mechanisms involved in platelet-mediated clumping
d) To study the clumping phenotypes of clinical isolates from Kilifi, Kenya.

Chapter 2

Materials and Methods

The work presented in this thesis was conducted at NHS Blood and Transfusion Laboratories in Oxford and at KEMRI/Wellcome Trust Research Laboratories in Kilifi Kenya. This chapter describes general materials and methods. Each results chapter has a description of specific materials and methods relevant to work in that chapter.

2.1 Materials

All chemicals and materials were supplied by Sigma unless stated otherwise (Sigma, Poole, UK).

2.2 Parasite Culture

P. falciparum parasites were cultured as previously described with modifications (Trager and Jensen 1976). All preparations and manipulations of media and cell culture procedures were conducted in a sterile environment and the contaminated apparatus were disinfected in 1% Virkon™ before processing for incineration. The surfaces were cleaned with 70% Industrial Methylated Spirit (IMS) solution regularly.

2.2.1 Culture Media

Parasites were grown in RPMI-1640 (RPMI) supplemented with 37.5mM HEPES (Gibco), 20mM glucose, 100μM hypoxanthine, 2mM glutamine, 25μg/ml gentamicin sulphate (Gibco), 10% (v/v) pooled human serum (NHS Blood and Transplantation, Filton, UK) to referred to as complete culture medium. Sodium hydroxide (1M solution) was added to adjust to pH 7.2.

2.2.2 Preparation of erythrocytes

Fresh human erythrocytes of blood group O were obtained from a *P. falciparum*-naïve donor from the NHS Blood and Transplant (NHSBT). In brief, blood was washed to remove serum and leucocytes. Whole blood was suspended in RPMI to 40% haematocrit and layered on 0.5 volume of Lymphoprep™ before the suspension was centrifuged at 3,000g for 15 minutes at 4°C. Erythrocytes were centrifuged to the bottom of the tube, leaving leucocytes at the interface between the Lymphoprep™ and the RPMI thus making it possible for them to be aspirated off. The remaining pellet of erythrocytes were washed twice in 10 volumes of incomplete medium, centrifuging at 3,000g for 5 minutes at 4°C after each wash. The final erythrocyte pellet was resuspended in incomplete medium to 25% haematocrit, and this suspension was stored at 4°C for up to 1 month.

2.2.3 Growing parasites in culture

P. falciparum-infected erythrocytes were grown in complete culture medium, gassed with a gas mixture of 96% nitrogen, 3% carbon dioxide and 1% oxygen in an incubator at 37ºC. The parasitaemia of the parasite cultures was maintained at 1-10%. To change the culture medium, the parasite cultures were transferred into a suitable centrifuging tube and centrifuged for 5 minutes at 2,000g. The spent medium was aspirated off and the parasite culture was washed in incomplete culture medium by centrifuging for 5 minutes at 2,000g. The resulting pellet from the washes was re-suspended in fresh pre-warmed culture medium and diluted with fresh erythrocytes to a desirable parasitaemia. The parasites cultures were grown at 1-2% haematocrit and suitable flasks were used to keep the depth of the culture medium to less than 1cm. The amount of culture medium and erythrocytes used for culture was determined by the following formula:

Final haematocrit (packed cell volume) x parasitaemia (%) x 5 = volume medium required per day

2.2.4 Examining parasite growth

A thin blood smear was used to assess the stage of parasite development and the state of the culture. A small aliquot (500µl) of the parasite culture was centrifuged briefly in a microfuge. The supernatant was discarded to leave the pellet sufficient supernatant to leave 50% haematocrit. The red cells were resuspended before making a drop on a clean glass slide and spreading it using another clean slide to make a smear on a blood film. The blood film was air dried then fixed with methanol and again air-dried. The slide was stained with Giemsa stain (10% (v/v) in buffer, (21mM sodium dihydrogen phosphate, 4.4mM potassium dihydrogen phosphate, pH 7.35) for 20-30 minutes. The slide was rinsed with

tap water before it was examined under an oil immersion microscope at x100 objective magnification. Parasitaemia was determined by counting the number of parasites seen in at least 500 red blood cells.

2.2.5 Preservation and storage of IE

P. falciparum stabilates were cryopreserved in glycerolyte following previously described methods (Diggs, Joseph *et al*. 1975). The parasite culture to be preserved was centrifuged at 2,000g for 5 minutes and the supernatant was aspirated. Glycerolyte (57g glycerol, 1.6g sodium lactate, 30mg potassium chloride and 1.38g sodium dihydrogen phosphate made up to 100ml in dH_2O and adjusted to pH 6.8 with sodium hydroxide) was added drop-wise to the parasite pellet to obtain a ratio of 3 cell pellet volumes: 5 glycerolyte volumes. The suspension was incubated for 5 minutes at room temperature before further addition. The suspension was frozen at -80°C in 500μl aliquots overnight before being moved to liquid nitrogen (-196°C) for long-term storage.

2.2.6 Thawing of glycerolyte-frozen parasites

To thaw parasites, frozen stabilates were thawed on ice before they were transferred to sterile 50mL centrifuge tube (Falcon™; 50 ml) then 0.2mls of 12% (w/v) sodium chloride solution was added drop wise while gently swirling the tube. The suspension was incubated at room temperature for 5 minutes before adding 10 volumes of 1.6% (w/v) sodium chloride suspension drop-wise with gentle agitation over 5 minutes. The cells were pelleted by centrifugation at 2,000g for 5 minutes. Finally the cells were washed with 10ml incomplete culture medium, centrifuged at 2,000g for 5 minutes. The

supernatant was discarded and the pellet was re-suspended and re-cultured in complete *P. falciparum* culture medium and fresh erythrocytes.

2.2.7 Parasites

The experiments for the work described in this thesis were conducted using well-characterized laboratory-adapted parasite lines that have been described before including IT/A4, IT/C10, and 3D7 (See below for a description of their derivation). Parasite clone refers to a parasite culture developed from a single infected erythrocyte. IT/A4 and IT/C10 are progeny developed from a Brazilian parasite line known as IT4/25/5 by sequential cloning of (Roberts, Craig *et al*. 1992). 3D7 is a derivative of the parasite isolate NF54 that was isolated from a patient in Amsterdam, The Netherlands (Rosario 1981). Parasite lines FCR-PECAM and FCR-gC1qR were derived by selection on PECAM-1 and gC1qR recombinant proteins were gifts from Prof Mats Wahlgren and Dr Artur Scherf respectively. Parasite line FCR was initially isolated from a Gambian parasite line (Jensen and Trager 1978).

2.2.8 Synchronization of cultures by sorbitol lysis

P. falciparum-infected erythrocytes with ring-stage parasites are more resistant to osmotic stress than those infected with mature-stage parasites. The sorbitol method for synchronization takes advantage of this difference to preferentially lyse IEs with older forms of the parasite (Lambros and Vanderberg 1979). A mixed parasite culture was pelleted by centrifugation at 2,000g for 5 minutes and the supernatant was discarded. The pellet was resuspended in 10 pellet volumes of pre-warmed 5% sorbitol (w/v) in distilled water and incubated at 37°C for 10 minutes. After incubation the cells were pelleted and

washed in incomplete culture medium by centrifuging at 2,000g for 5 minutes. Giemsa-stained thin films were prepared to confirm the enrichment of ring-stage parasites of the cell pellet.

2.2.9 Enrichment of mature stage by Plasmagel™ flotation

Plasmagel™ was used to enrich cultures for knob-positive mature-stage IEs following the method previously described (Pasvol, Wilson *et al*. 1978). Uninfected erythrocytes and ring-stage IEs form rouleaux in gelatin solutions such as Plasmagel™. Mature-stage IEs sediment more slowly in gelatin solutions and are separated from uninfected erythrocytes. Parasite cultures were pelleted by centrifugation at 2,000g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1.5 pellet volumes of incomplete culture medium. Then 2.5 pellet volumes were added and gently mixed. The suspension was incubated at 37°C for 15-20 minutes. After incubation the top layer containing the non-rosetting trophozoites and schizonts were aspirated, transferred to a clean centrifuge tube and washed in incomplete culture medium. The cells were resuspended in complete culture medium and returned to culture.

2.2.10 Mycoplasma detection and treatment of cultures

Mycoplasma are the smallest known free-living prokaryotes that can infect parasite cultures leading to impaired growth (Rowe, Scragg *et al*. 1998). Parasite cultures were routinely tested for Mycoplasma infection by a biochemical test following the manufacturer's proprietary instructions (Lonza, USA). Spent cell-free *P. falciparum* culture medium (1ml) was obtained from culture by centrifugation. All reagents were brought to room temperature. Mycoalert™ sample assay buffer (10ml) was added to a

lyophilized Mycoalert™ reagent and allowed to equilibrate to room temperature for 15 minutes.

A small aliquot of supernatant (2ml) was centrifuged in a microfuge briefly to pellet the cells before 100µl of the clear supernatant were transferred into a cuvette. The luminometer was programmed to take a 1 second integrated reading before 100µl Mycoalert reagent was added to each sample and incubated at room temperature for 5 minutes. The cuvette was placed in the luminometer to take reading A. Mycoalert substrate (100µl) were added to the cuvette and incubated for 10 minutes before the luminometer was used to take reading B. The ratio Reading B/Reading A was calculated. Uninfected culture gave readings over 1 whereas infected cultures gave a reading of less than 1.

2.2.11 Treatment of Mycoplasma contamination

Cultures that were shown positive were treated for 7 days with 0.5µg/ml of Mycoplasma removal agent (4-oxo-quinoline-3-carboxylic acid derivative in water) and later retested to ensure that the treatment was effective (Rowe, Scragg *et al*. 1998).

2.2 Platelet-mediated clumping assays

2.2.1 Preparation of platelet rich plasma (PRP)

Blood from a *P. falciparum* naïve blood group AB volunteer was collected into citrate, EDTA or Heparin anticoagulant tubes. The blood was centrifuged for 20 minutes at 250g to obtain Platelet Rich Plasma (PRP). PRP was transferred to a clean tube and centrifuged further at 800g for 15 minutes to obtain Platelet Poor Plasma (PPP). Some of the PPP was

transferred to a new tube while the remaining was used to dilute the platelet pellet to required suspensions containing 10% plasma in PBS.

2.2.2 Platelet-mediated clumping assays

Clumping assays were performed following the methods described by Pain et al (Pain, Ferguson *et al*. 2001). To make a clumping assay suspension, mature-stage IEs were stained with ethidium bromide to a final concentration of 20μg/ml for 30 minutes at room temperature. The stained trophozoite-infected erythrocytes were washed thrice in RPMI and re-suspended in 10% PRP to make a clumping assay suspension at the desired haematocrit. Ten microlitres aliquots of the clumping assay suspension were placed in individual wells of a 96-well-round-bottomed plate and incubated on a bench-top rotor for up to 2 hours rotating at 10rpm. A truncated pipette tip was used to transfer the suspension to make slides. The wet preparation of cells was examined under a greased cover slip to prevent leakage of cells. The slides were analysed by a Nikon E600 fluorescent microscope with a TRITC filter in a dark background (Nikon, Tokyo, Japan). Three slides were prepared at each time point. The entire slide was scanned using a systematic castellated pattern to determine the distribution of cells and ensure the counted cells were representative of the whole slide. At least 1,000 IEs were counted on each slide. Clumping frequency was scored as the number of IEs in clumps per 1,000 IEs. All assays are the average of clumping frequency determined by counting three slides.

2.3 Binding assays

I conducted binding assays with trophozoite-infected erythrocytes by panning on purified immobilized receptors. Here 3µl of 100μg/ml recombinant proteins CD36 (R & D

Systems, UK), P-Selectin (R & D Systems) and PECAM-1 (R & D Systems) and gC1qR (gift of Dr Artur Scherf) were spotted on plastic plates in triplicate and incubated overnight at 4ºC in a moist environment. The next day, the spots were aspirated and the plate rinsed with washing buffer (RPMI-1640 without bicarbonate). The remaining free sites on the plates were saturated by incubating for 1 hour with 1% BSA in PBS at 37ºC. The blocking buffer was aspirated and the plates washed 5 times with washing buffer. A suspension of mature IEs was prepared in binding medium (RPMI-1640 with 1% BSA) at 2% haematocrit and added to the plates. The plates were incubated at 37ºC for one hour with gentle agitation every 10 minutes. After incubation the plates were washed at least 6 times with washing buffer until no more cells could be seen adherent to the blocked areas of the dish through an inverted microscope. The remaining bound cells were fixed with 2% glutaraldehyde for 10 minutes at room temperature. The plates were washed once in washing buffer before adding 10% Giemsa solution for 30 minutes. The plates were washed in tap water by gently dipping in a beaker to avoid dislodging the bound cells. The binding phenotype was determined by counting the number of bound IEs per mm^2.

2.4 PCR amplification of specific DNA sequences

Polymerase Chain Reaction (PCR) was performed using specific primers to amplify target genomic regions. To set up PCR reactions, approximately 100ng of genomic DNA (gDNA) or complementary DNA (cDNA) were mixed in 1x PCR buffer (10mM tris pH 8.3, 50mM potassium chloride), 0.3mM each of dATP, dCTP, dGTP, and dTTP, 1.5-3.5mM magnesium chloride, 1μM of each primer and 1.25 Units of Taq polymerase. Reactions were performed in 20-25μl volumes.

2.5 Separation of DNA fragments

Plasmid DNA extracted from bacterial cells or DNA fragments amplified from genomic DNA was separated and sized by agarose gel electrophoresis. Gels were poured using between 1-2% weight/volume (w/v) agarose in Tris/Borate/EDTA buffer (1x TBE – 6g TRIS, 5.5g orthoboric acid and 4ml 0.5M EDTA pH 8.0 made up to 1 litre with deionised water. Samples were mixed with equal volumes of loading buffer (30% volume/volume (v/v) glycerol, 10mM EDTA pH 8.0, 0.25% (w/v) xylene cyanol and 0.25% (w/v) bromophenol blue) immediately prior to loading. Gels were run in 1x TBE for approximately 1 hour until the first dye front was within 2-3cm of the end of the gel. On completion, gels were stained for 20 minutes in 0.5μg/ml ethidium bromide in distilled water before ultraviolet (UV) visualisation. Fragment sizes were estimated by comparison to appropriate molecular weight markers run in adjacent lanes.

2.6 Bacterial transformation

2.6.1 Bacteriological Media

Bacterial cultures were grown in Luria-Bertani (LB) medium (10g tryptone, 5g yeast extract and 5g sodium chloride in 1 litre of distilled water, pH 7.0 with sodium hydroxide) and LB-agar plates (LB medium supplemented with 15g agarose per litre). All media were autoclaved after preparation. Agar plates were stored at 4ºC and used within a month of preparation, whereas the culture medium was stored at room temperature. Ampicillin or kanamycin was added to make a final concentration of 50μg/ml from a stock solution of 50mg/ml in water. All cultures were grown in a shaking incubator 37ºC.

2.6.2 Bacterial Strains Used

In our experiments two strains of *Escherichia coli* bacteria were used. Strain DH5α or TOP10 competent bacterial cells were used in the cloning and transformation of the PCR products.

Strain	**Genotype**
DH5α	SupE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1 deoR
TOP10	F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG

2.6.3 Transformation of competent bacterial cells

DH5α or TOP10 were used in the transformation and replication of recombinant plasmids cells were used for initial transformation and replication of recombinant plasmids. The proprietary manufacturer's protocol (Invitrogen) was followed. A vial of competent cells (50µl) was thawed on ice. Approximately 20ng of PCR product containing the region of interest was added to each transformation reaction and mixed gently without pipetting up and down. The reactions were then incubated on ice for 5-30 minutes. Each transformation reaction was heat pulsed in a 42ºC water bath for precisely 30 seconds (45 seconds for DH5α cells) before being transferred onto ice again for 2 minutes. Pre-heated SOC medium (150µl) was added to each reaction before incubating them at 37ºC for 1 hour while shaking at 225rpm. Using a sterile spreader, 50µl or 100µl of transformed cells was plated onto LB-agarose plates containing ampicillin or kanamycin. The plates were cultured overnight at 37ºC. Transformed bacterial cells appeared in colonies following

overnight incubation at 37ºC. The colonies were either used immediately or were stored at 4ºC for up to a month.

2.6.4 Plasmid Isolation and Purification

Single clones of transformed bacterial were picked from LB-agarose plates with a sterile pipette tip and inoculated into 3ml of LB medium containing ampicillin or kanamycin. The culture was grown overnight at 37ºC in a shaking incubator at 200 rpm. The next day the culture was harvested by centrifugation for 5 minutes at 10,000g using a tabletop centrifuge. The supernatant was aspirated before 250µl of cell resuspension solution P1 (50mM Tris-HCl, pH 7.5, 10mM EDTA, 100µg/ml RNAse A) was added to the pellet and the cells resuspended thoroughly by vortexing. Buffer N3 (350µl) was then added and the solution was mixed gently by repeated inversion of the tube. The lysate was centrifuged at 8,000g for 10 minutes. The supernatant (750µl) was transferred to Qiagen™ column and centrifuged for 1 minute at 14,000g. The flow-through was discarded and the column was reinserted into a clean collection tube. Wash solution (750µl) (162.8mM potassium acetate, 22.6mM Tris-HCl, pH 7.5, 0.109mM EDTA, 95% Ethanol) was added to the column and centrifugation was repeated. To remove the residual wash buffer the column was centrifuged for one minute at 8,000g into an empty collection tube. The column was reinserted into a clean microfuge tube before adding 50-100µl nuclease-free water or elution buffer. The tubes were left to stand for 1 minute at room temperature before centrifuging at maximum speed for one minute. The purified DNA was stored at –20ºC. DNA yields were determined by a Nanodrop™ spectrophotometer.

2.7 Nucleotide sequencing

The extracted purified DNA was sent to the Weatherall Institute of Molecular Medicine (Oxford University, UK) for sequencing. An automatic sequencer using the ABI Prism Dye Terminator Cycle sequencing kit was used for sequencing. Plasmid DNA (500ng) was mixed with reaction buffer and 3.2pmol M13 forward primer CGCCAGGGTTTTCCCAGTCACGAC specific to insert boundaries spanning the 5' and 3' end of the inserted construct. Reactions were performed in a thermocycler then extension products were purified by ethanol precipitation, whereby 2 volumes of 0.3M sodium acetate were added. The reaction tubes were incubated at 0ºC for 10 minutes the were centrifuged at 20,000g for 15 minutes. The DNA pellet was washed once in 70% (v/v) then they were re-suspended in ethanol. Finally the precipitated DNA was resuspended in loading buffer and separated by an automated sequencing gel.

Further methods used in this study are described in the appropriate chapters.

Chapter 3

Optimizing platelet-mediated clumping assays

3.1 Introduction

Agglutination of malaria-infected erythrocytes in non-immune serum for parasite line IT/C10 was reported in 1992 (Roberts, Craig *et al*. 1992). A decade later a similar agglutinating phenotype that relied on the presence of platelets was reported for this parasite line (Roberts, Pain *et al*. 2000). Electron micrographs of the clumps were used to show platelets intertwined among malaria-infected erythrocytes (Pain, Ferguson *et al*. 2001). Platelet-mediated clumping of malaria-infected erythrocytes now refers to the agglutination of mature stage (trophozoite or schizont) -infected erythrocytes through direct contact of infected erythrocytes (IEs) and platelets. Platelet-mediated clumping has since been studied in field isolates from various regions.

The importance of platelet-mediated clumping in malaria pathogenesis was highlighted in field studies where IEs from children suffering from severe malaria exhibited significantly higher clumping frequencies *in vitro* than isolates from children with mild forms of malaria (Pain, Ferguson *et al*. 2001, Chotivanich, Sritabal *et al*. 2004, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011). It has been suggested that the giant clumps formed in *in vitro* experiments would occlude small capillaries if they formed *in vivo*. Furthermore, in a post mortem study, platelet accumulation in brain microvessels of children that succumbed to cerebral malaria has been reported but not in those that died from non-malarial encephalopathies (Grau, Mackenzie *et al*. 2003). Taken together, these findings

support the hypothesis that platelet-mediated clumping plays a significant role in pathogenesis of severe or cerebral malaria.

To date, five field studies have been conducted on platelet-mediated clumping. Findings from four of these clinical studies (Pain, Ferguson *et al*. 2001, Chotivanich, Sritabal *et al*. 2004, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011) associated platelet-mediated clumping with severe malaria, however one study (Arman, Raza *et al*. 2007), showed platelet-mediated clumping was associated with parasitaemia but not with severe disease.

The absolute proportion of IEs that form clumps in a clumping assay is determined by the experimental conditions employed (Arman and Rowe 2008). Previous studies were conducted under varying clumping protocols making comparison of findings from different laboratories difficult. Some investigators have overcome this discrepancy by enrolling age-matched uncomplicated malaria case controls with similar parasitaemia (Arman, Raza *et al*. 2007), enriching mature stage parasites and standardizing parasitaemia by Plasmion™ flotation (Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011) or by multivariate analysis to account for the varying admission parasitaemia (Pain, Ferguson *et al*. 2001).

The overriding objective of this thesis is to characterise platelet-mediated clumping of malaria-infected erythrocytes by identifying the mechanisms by which platelets agglutinate a subset of *P. falciparum* infected-erythrocytes (IEs) to form clumps. In this chapter, I begin by setting up optimal conditions using the parasite line IT/C10 for an accurate and sensitive assay to allow for the screening of the clumping phenotype and to

fully characterize the clumping phenotype by testing the effect of various clumping assay conditions.

3.2 Specific methods

3.2.1 Sources of parasites

Parasite line IT/C10 is a sub-clone derived from the laboratory parasite line IT/A4 (Roberts, Craig *et al*. 1992), itself a derivative of the Ituxi isolate of *P. falciparum* that was initially isolated in Brazil (Berendt, Simmons *et al*. 1989). Infected erythrocytes of the IT/A4 line show strong host cell receptor binding characteristics even after multiple passages in culture hence its use in cytoadherence studies. IT/3C3 was cloned from IT/A4 parasites (Horrocks, Pinches *et al*. 2005). 3D7 was originally obtained by limiting dilution of the NF54 line (Walliker, Quakyi *et al*. 1987).

3.2.2 Adjustment of parasitaemia and haematocrit

Parasite cultures were maintained in erythrocyte suspensions at 1-2% haematocrit. To obtain the desired haematocrit an aliquot of the culture was centrifuged in a microfuge tube, the spent culture medium was discarded. To a 10 μL infected erythrocyte pellet was added 90μL of PRP to obtain a haematocrit of 10% unless stated otherwise. The required amount of PRP was determined and added to get the final desired haematocrit. Different parasitaemia was obtained by diluting a culture at 1% haematocrit with uninfected erythrocytes suspended at a similar haematocrit. Parasitaemia was determined by counting up to 500 cells in a Giemsa-stained slide.

3.2.3 Platelet-mediated clumping assays

Clumping assays were conducted as described in Chapter 2 (Clumping assays). Briefly, synchronised trophozooite-infected erythrocytes were stained with ethidium bromide to a final concentration of 20μg/ml for 30 minutes at room temperature. The stained trophozoite-infected IEs were washed three times in incomplete culture medium and re-suspended in 10% PRP to make the clumping assay suspension at the desired haematocrit. Aliquots (10 μL) of the clumping assay suspension were placed in individual wells of a 96-well round-bottomed plate and incubated on a bench-top rotor for 15 minutes, 30 minutes, 1 hour or 2 hours rotating at 10 rpm. Pipette tips with their ends cut off were used to transfer the parasites from the wells to the glass slide. Coverslips used to make wet preparation slides were greased at their edges and placed on the parasite samples to prevent leakage of cells. The slides were analysed using a Nikon E600 fluorescent microscope with a TRITC filter in a dark background (Nikon, Tokyo, Japan). The entire slide was scanned using a systematic castellated pattern to determine the distribution of cells and ensure that the counted cells were representative of the whole slide. Clumps consisting of 3-100 IEs were included when a total of at least 1000 IEs were counted on each slide to determine the clumping frequency. Clumping frequency was scored as the proportion of IEs in clumps out of 1000 IEs. All assays are the average of clumping frequency determined by counting three slides.

3.2.4 Immunofluorescence assays with anti-platelet-antibodies

PRP was incubated with 30µg/ml anti-CD61-FITC for 30 minutes. The PRP was washed twice and resuspended in PBS to adjust the platelet concentration to approximately 150 x 10^9 /L. Platelet concentration was determined by a cell counter Coulter (Coulter

Corporation, USA). Clumping assays were conducted with ethidium bromide-stained trophozoite-infected parasites at 10% haematocrit in darkness. A wet preparation slide of the clumping mixture was prepared after 2 hours incubation and analysed by a fluorescent microscope (Nikon, Tokyo, Japan).

3.2.5 Modified Plasmion™ method

The Plasmion™ method for enriching knobby mature-stage parasites described in Chapter 2 was modified to enhance platelet-mediated clumping. A trophozoite-stage infected culture was washed and the pellet suspended in 3 pellet volumes of incomplete medium and 4 pellet volumes of Plasmion™ (*Laboratoire Fresenius Kabi, France*). A PBS-washed platelet concentrate was added to a final concentration of 100 x 10^9/L. The suspension was gently mixed then incubated at 37ºC for 30 minutes. After 30 minutes the parasites were gently withdrawn using a pipette and transferred to a fresh tube and washed in incomplete medium. Aliquots were immediately diluted with fresh uninfected erythrocytes and used in clumping assays.

3.3 Results

3.3.1 Platelet-mediated clumping

a) Platelet-mediated clumping of common laboratory parasite lines

To find the appropriate parasite line for further study of the platelet-mediated clumping, common laboratory parasite lines IT/A4, IT/3C3, 3D7 and IT/C10 were tested under similar assay conditions. The parasitaemia was adjusted to 5% and the IEs were suspended in PRP at 10% haematocrit. The mean clumping frequency of IT/C10 parasites was found to be 32.77$\pm$4.2%, which was significantly higher than parasite lines IT/A4 ($p<0.001$), IT/3C3 ($p<0.001$) and 3D7 ($p<0.001$) (Figure 3.1). Platelet-mediated clumping is a distinct characteristic of a subset of malaria-infected erythrocytes of which IT/C10 is an example. In subsequent experiments, parasites that exhibited an IT/C10-like clumping phenotype are referred to as clumpers whereas the parasite lines or clones that exhibited clumping frequencies of less than 100 IEs per 1000 IEs are referred to as non-clumpers. IT/C10 was used to study and characterize the platelet-mediated clumping phenotype.

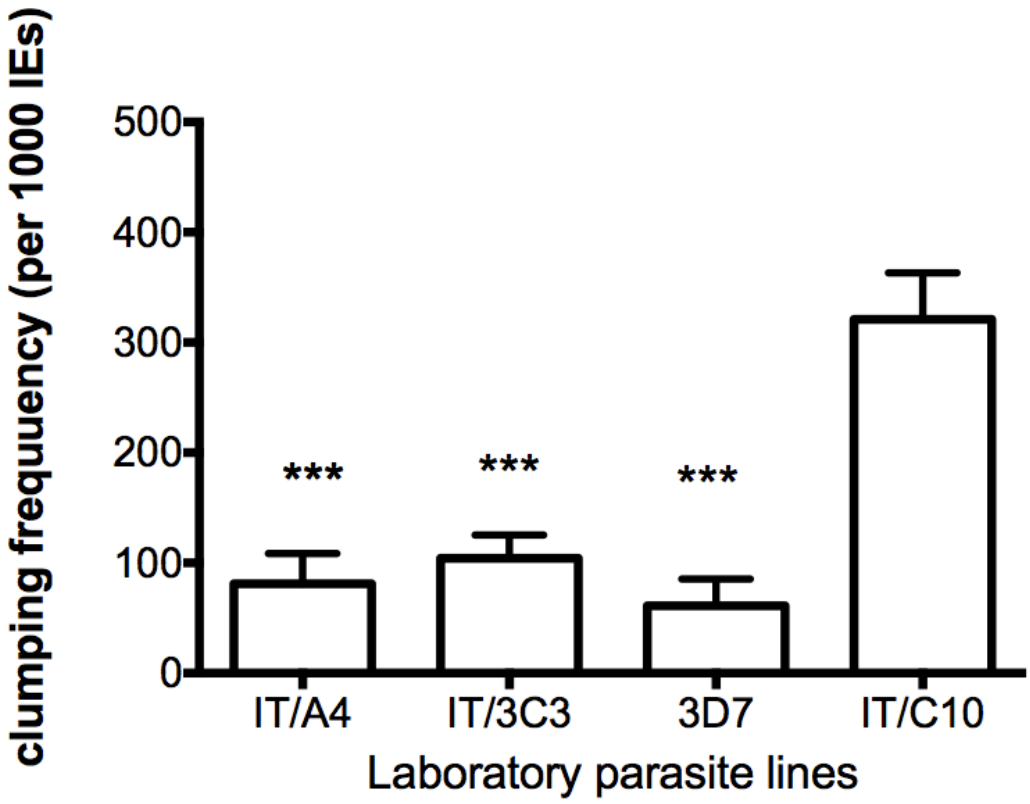


Figure 3.1 Platelet-mediated clumping of laboratory parasite lines

Platelet-mediated clumping assays were conducted with synchronized ethidium bromide-stained trophozoites of common laboratory lines IT/C10, IT/3C3, 3D7 and IT/C10. Three slides were prepared after incubation in PRP. Clumping frequency of IT/C10 parasites was significantly higher than other tested laboratory lines (IT/A4, IT/3C3 and 3D7) under similar experimental conditions. Error bars represent standard deviation of three experiments (ANOVA $P < 0.0001$).

b) Representation of platelet-mediated clumps in images

In a typical clumping assay, ethidium bromide-stained IEs are mixed with PRP or PPP and examined under a fluorescent microscope. Clumpers are defined by a greater proportion of infected erythrocytes expressing the clumping phenotype in PRP than in PPP (Figure 3.2A and 2B). On the other hand platelet-mediated clumping is absent or minimal for non-clumpers in the presence of platelets (PRP) or in their absence (PPP) (Figure 3.2C and 3.2D). To further determine the localization of platelets in the IE-platelet clumps, clumping assays were conducted with CD61-FITC-stained platelets and ethidium bromide-stained trophozoite infected erythrocytes. CD61 is an integrin subunit that is abundant on platelets. FITC-stained platelets were intertwined among IEs consistent with platelets cross-linking IEs together in clumps (Figure 3.2).

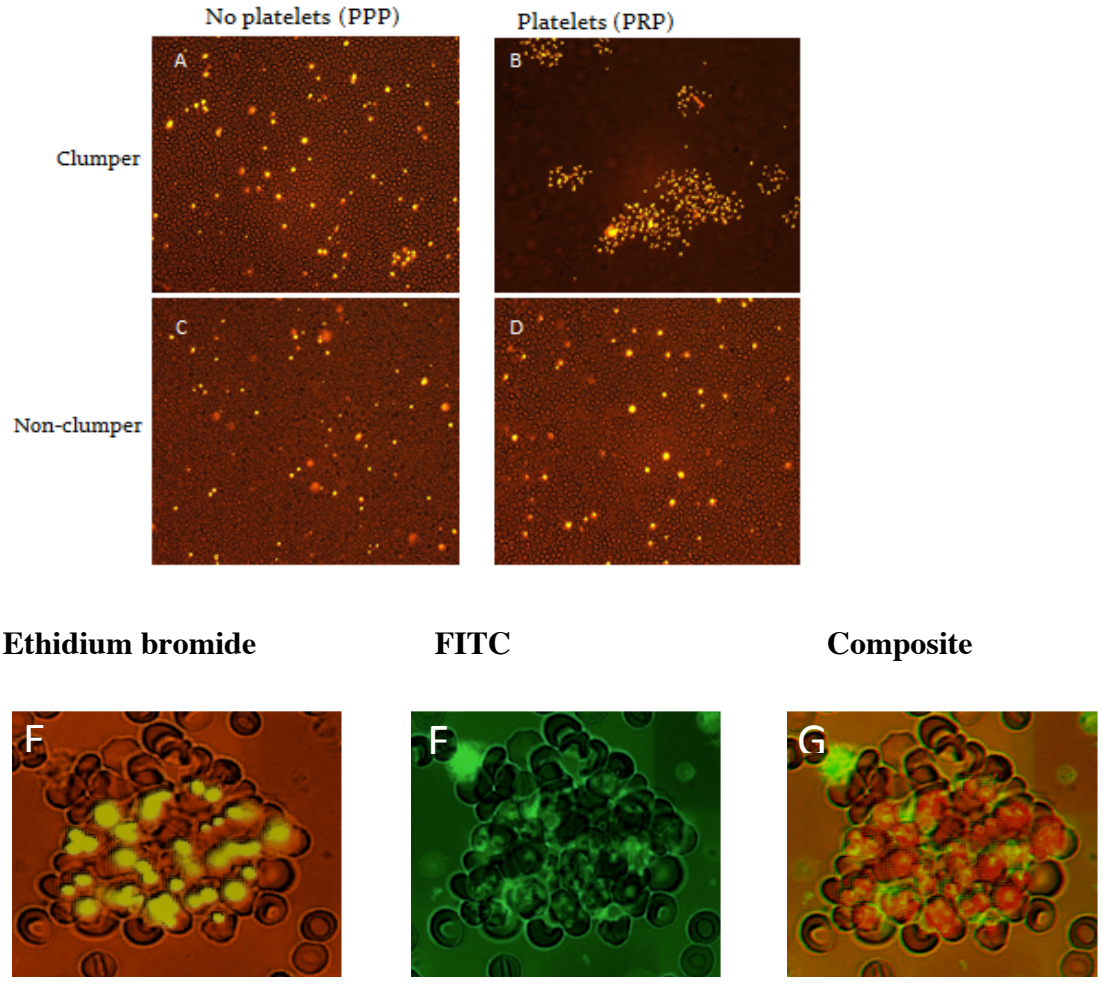


Figure 3.2 Clumping assay images

Typical images of wet preparation slides of ethidium bromide-stained trophozoite-infected erythrocytes incubated in PRP at x400 magnification. (A) In the presence of PPP parasites appear to be homogenously distributed on the slide. (B) In the presence of platelets, clumpers agglutinate to form clumps (C) Non-clumpers do not form clumps in PPP (D) Clumps are not formed by non-clumpers in PRP. Clumping assays were conducted in the presence of CD61-FITC-stained platelets and images taken with TRITC (ethidium bromide) and FITC filters. The parasites are highlighted in (E) platelets are highlighted in

(F). The composite of this image shows that platelets are intertwined between infected erythrocytes (G).

c) Cryopreserved mature stage parasites

In a field setting, availability of samples is spread over different times of the day making planning and execution of experiments difficult. To overcome this, IEs can be frozen at the ring-stage to be grown later to mature stage (trophozoite) for clumping assays. However, cryopreserved parasites do not always grow and out of those that grow a proportion of IEs are lost therefore modifying the overall adhesive phenotype (Forsyth, Philip *et al*. 1989, Reeder, Rogerson *et al*. 1994). However, Kinyanjui and colleagues showed that the antigenic phenotype of glycerolyte-frozen trophozoites is unaltered as determined by flow cytometry and antibody agglutination (Kinyanjui, Howard *et al*. 2004). I therefore sought to determine the feasibility of using glycerolyte-frozen mature-stage IEs for clumping assays. To test this, I compared the clumping frequency of glycerolyte-frozen trophozoites with fresh trophozoites of parasite lines IT/C10 and 3D7. An aliquot of the cultures was frozen in glycerolyte and stored at -80^{0}C. Clumping assays were performed as described above comparing the clumping frequency of fresh (straight from culture) with glycerolyte-frozen IEs under identical experimental conditions.

The clumping frequency of IT/C10 fresh trophozoites was comparable to comparable IT/C10 frozen trophozoites. Although frozen trophozoites had slightly higher clumping frequencies, the differences were statistically insignificant by t-test IT/C10 ($p=0.2$) and 3D7 ($p=0.2$) (Figure 3.3).

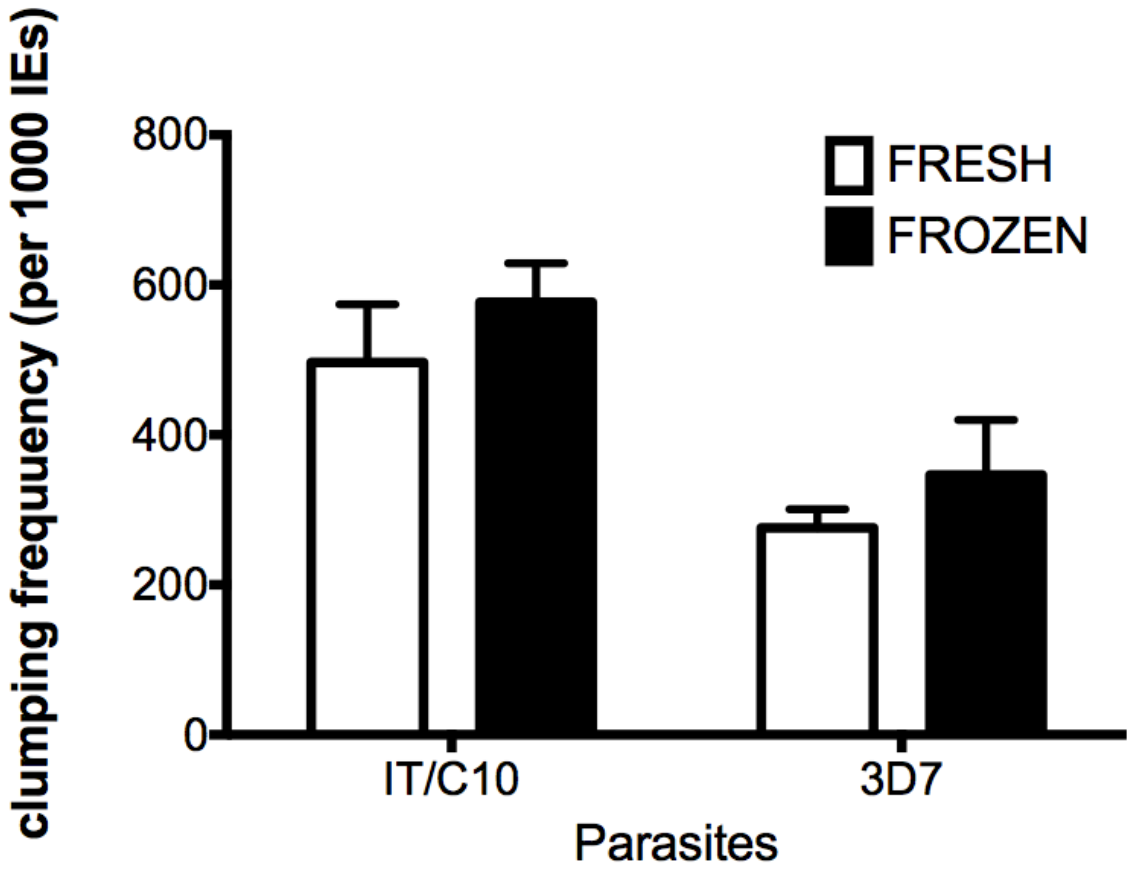


Figure 3.3 Comparison of clumping in fresh and freeze-thawed trophozoites

Clumping assays were conducted to compare clumping frequency between freeze-thawed IEs and fresh-from-culture IEs using parasite lines IT/C10 (5% trophozoites) and 3D7 (10% trophozoites) and slides were prepared after incubation in PRP. Error bars represent standard deviation of two experiments. No significant difference in phenotype was observed between fresh and frozen IEs using either IT/C10 ($p=0.2$, paired t-test) or 3D7 ($p=0.2$, paired t-test).

3.3.2 Optimizing the platelet-mediated clumping assay

In the classical clumping assay, trophozoite-stage IEs are stained using fluorescent DNA-intercalating dyes and incubated in PRP before wet slides are prepared and analysed by a fluorescent microscope. In experiments conducted with parasite line HB3, it has been reported that the experimental conditions such as parasitaemia, haematocrit and pH influenced the outcome of clumping assays (Arman and Rowe 2008). Since previous field studies on platelet-mediated clumping were conducted in varying clumping assay conditions, it is plausible that some of the differences in the reported findings could be explained by differences in experimental clumping assay protocols. To determine the optimal assay conditions for an accurate and sensitive clumping assay to use in characterizing this phenotype, I tested clumping of IT/C10 parasites under different conditions by modifying the classical clumping assay protocol and testing parasitaemia, haematocrit, duration of incubation, platelet concentration, the duration of platelet storage, type of anticoagulants and presence of calcium ions.

a) Parasitaemia

Due to wide variation in the admission parasitaemia of malaria patients, it is important to investigate the contribution of parasite density of the sample when determining the platelet-mediated clumping phenotype of a given parasite isolate. To identify the optimal parasitaemia for further experiments, clumping assays were conducted with a fixed haematocrit of 10% and varying parasitaemia for IT/C10 (2.5 to 10%) and 3D7 (0.63 to 5%) trophozoites. The clumping mixture was incubated for 2 hours before slides were prepared and read by a fluorescent microscope within 3 hours of preparation. For IT/C10 in general the clumping frequency increased correspondingly with parasitaemia from 2.5% to 10%. The differences in clumping frequency were statistically significant between low (2.5-5%) and high (7.5-10%) parasitaemia as compared by ANOVA ($p=0.0015$) (Figure 3.4A). Notably larger clumps were formed at 5-10% parasitaemia than at 2.5% parasitaemia, which could be explained by the availability of more IEs in close proximity that readily interacted to form clumps. In the non-clumping parasite line 3D7, a clumping frequency at 5% parasitaemia was significantly higher than at lower parasitaemia (ANOVA, $p= 0.0021$) (Figure 3.4B). However the clumping frequency in 3D7 remained below 10% and therefore was still considered a non-clumper.

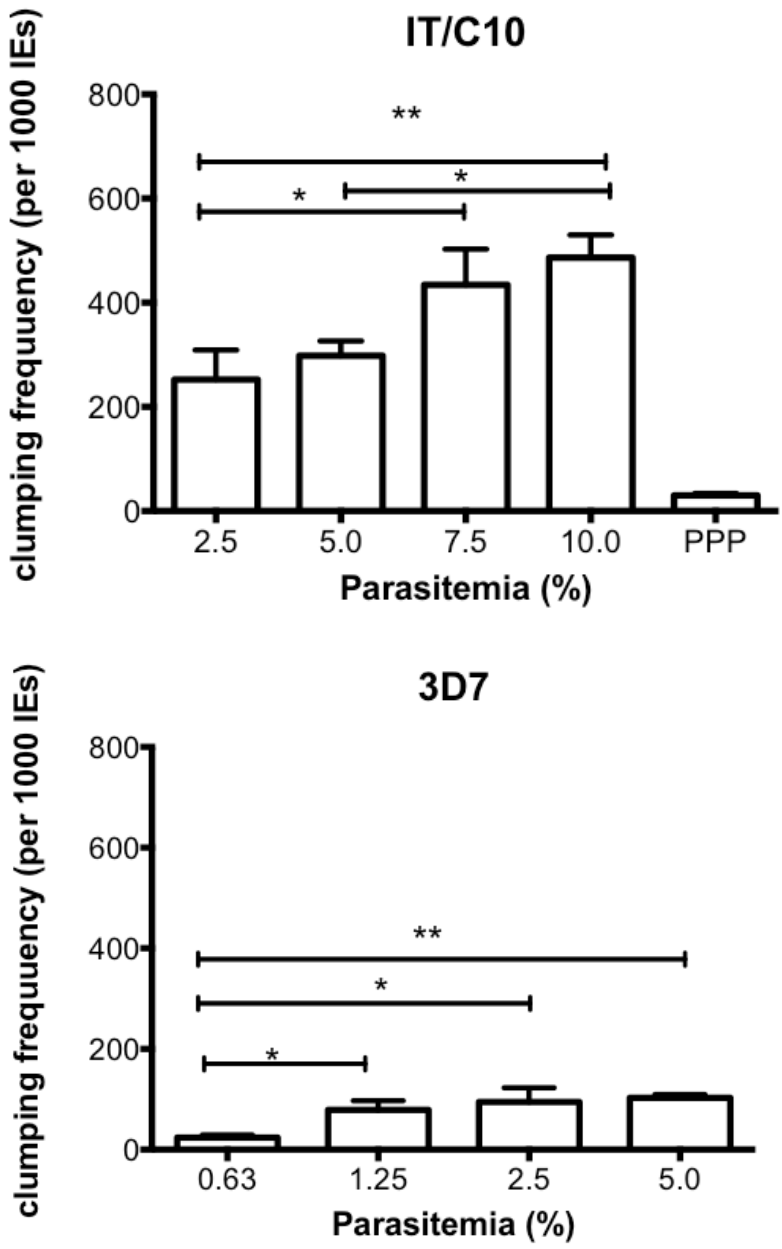


Figure 3.4 Relationship between parasitaemia and platelet-mediated clumping

Platelet-mediated clumping assays for parasite line IT/C10 and 3D7 were conducted with fixed haematocrit (10%) and by varying parasitaemia (2.5 to 10%). Parallel slides in PPP were prepared at 10% parasitaemia. (B) Platelet-mediated clumping assays for parasite line 3D7 at fixed haematocrit (10%) and varying parasitaemia (0.63% to 5%). Error bars

represent standard deviation of three experiments. ANOVA analysis (IT/C10 p=0.0015, 3D7 p=0.0021).

b) Haematocrit

Previous data for parasite line HB3 showed that the haematocrit of the clumping assay had a significant effect on the outcome of the clumping assay especially at low parasitaemia (Arman and Rowe 2008). As part of optimizing the clumping assay, I determined the extent by which haematocrit influenced clumping assay outcomes by conducting clumping assays with a fixed parasitaemia of 5% while haematocrit was varied (2.5%, 5% and 10%). Clumping frequency was significantly influenced by the haematocrit of the suspension with differences reaching statistical significance between 2.5% and 5% or 10% by ANOVA analysis ($p=0.0165$) (Figure 3.5). However, the clumping frequency was similar for assays at 5 and 10% haematocrit.

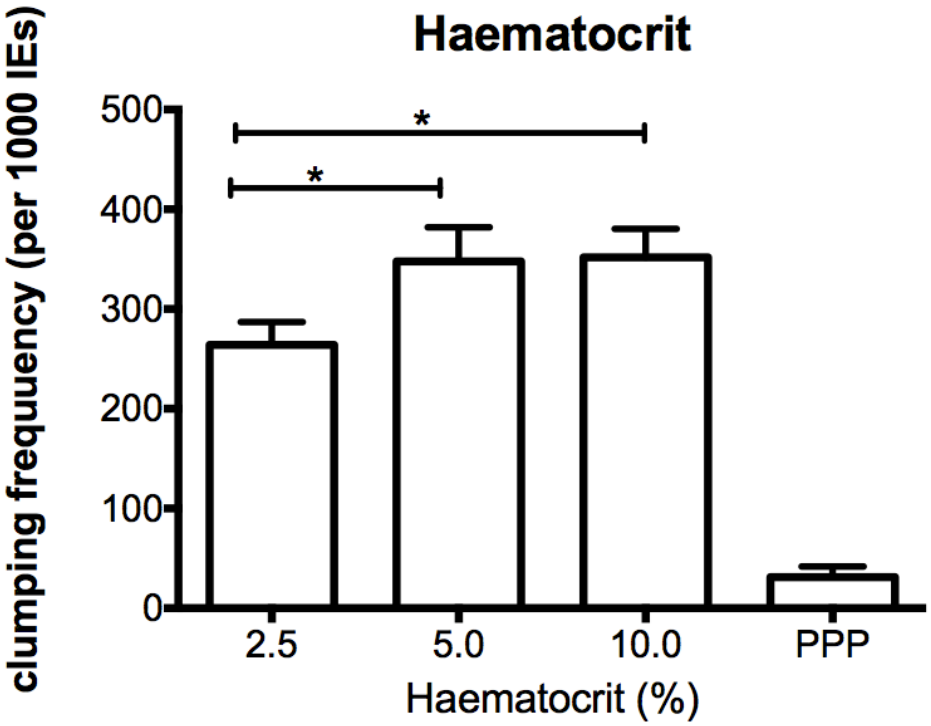


Figure 3.5 Haematocrit in clumping assays for IT/C10

Platelet-mediated clumping assays were conducted with IT/C10 trophozoite IEs with fixed parasitaemia of 5% and varying haematocrit of 2.5%, 5%, and 10% and wet preparation slides prepared after 2h incubation. Error bars represent standard deviations of three experiments with three replicates per experiment. Haematocrit influences the platelet-mediated clumping phenotype but the differences between 5% and 10% are not statistically significant by ANOVA (p=0.0165)

c) Duration of incubation

In previous studies, clumping assay slides were prepared after incubation for varying lengths of time. To investigate the time required for IEs and platelets to assemble into clumps and also determine the stability of the clumps while rotating on a 96-well-plate, slides for the haematocrit experiments described above were prepared at different time points (15 minutes, 30 minutes, 60 minutes and 120 minutes. Virtually no clumping took place immediately after preparing the clumping suspension, clumps started to form after 15 minutes incubation (Figure 3.6). There was an increase in clumping frequency counted with prolonged incubation, and was greatest after 2 hours of incubation.

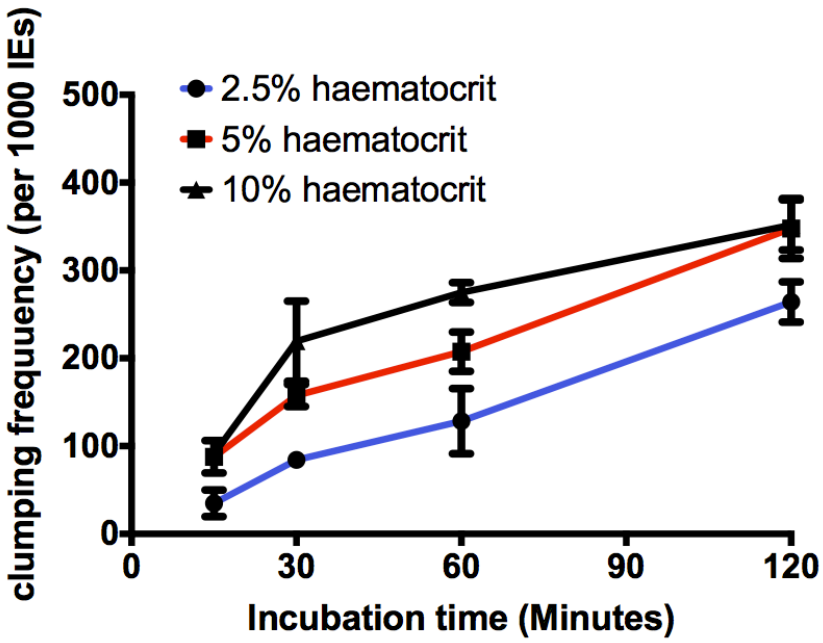


Figure 3.6 Clumping is influenced by the duration of incubation

Three slides for haematocrit experiment were prepared at different time points after incubation with PRP 15, 30, 60 and 120 minutes. Error bars represent standard deviations of three experiments (with three replicates each) Platelet-mediated clumping increased with increased time of incubation with highest clumping observed after 120 minutes incubation

d) Plasma is not required in platelet-mediated clumping

Platelet-mediated clumping is dramatically enhanced in PRP as clumping did not occur in PPP indicating that platelets play a key role (Figure 3.1). Previous studies on the clumping phenotype used platelets suspended in 10% plasma. It is therefore plausible that plasma components contribute to the observed clumping phenotype at least in part. To determine the importance of plasma proteins and other soluble factors in the clumping phenotype I compared clumping frequencies of IT/C10 parasites with and without plasma. I found that the clumping frequency in PRP was comparable to that of washed platelets suspended in PBS. The differences were not statistically significant ($p=0.74$). On the other hand clumping frequency was minimal in the absence of platelets (both in PPP and in PBS) where clumping frequency peaked at 6.5% and 4.9% respectively (Figure 3.7). Platelets without plasma are required for platelet-mediated clumping to occur in parasite line IT/C10 trophozoites.

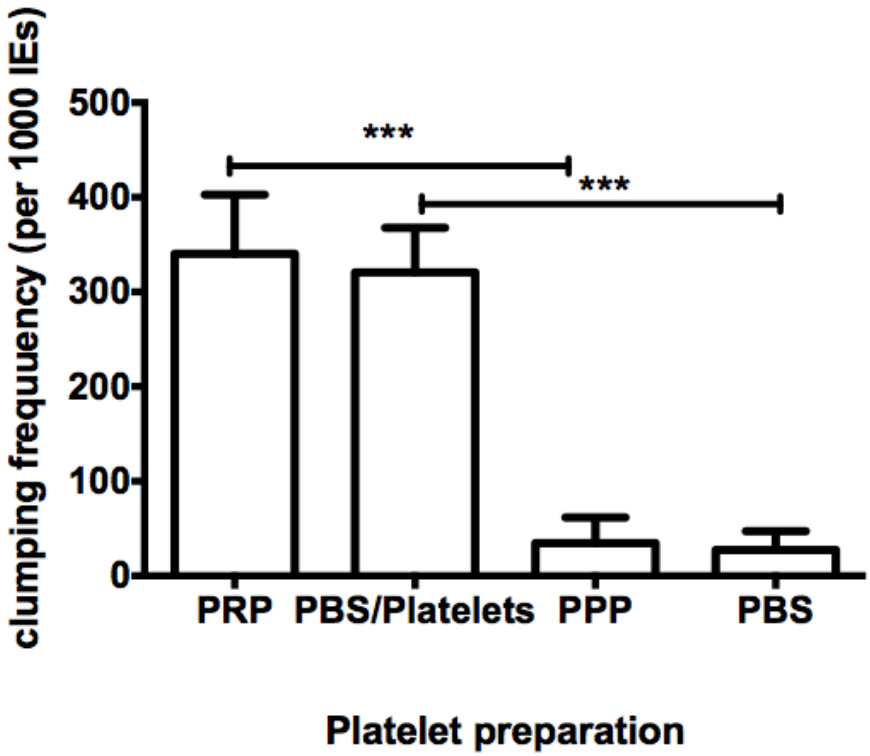


Figure 3.7 IT/C10 clumping requires platelets

Platelet-mediated clumping assays were conducted with IT/C10 parasites in 10% PRP or in a plasma-free platelet concentrate. Three slides were prepared after 120 minutes incubation. Parallel slides for PPP and PBS were prepared. Error bars represent standard deviations of three experiments. Platelet mediated clumping does not require plasma.

e) Platelet concentration

Clumping frequency of isolates from thrombocytopenic malaria patients increased when platelet concentrations were adjusted showing there is a threshold of platelet concentration required for platelet-mediated clumping to occur (Wassmer, Taylor *et al*. 2008). Having shown that platelets are required for platelet-mediated clumping to occur and plasma components are not required, I sought to determine the amount of platelets required for clumping to occur in IT/C10 parasites. PRP was prepared from citrate-anticoagulated blood and re-suspended in 10% plasma. The PRP was then serially diluted before clumping assays with IT/C10 IEs at 5% trophozoites and 10% haematocrit and three slides were prepared after 120 minutes incubation on a bench top rotor. A cell counter was used to determine platelet concentrations. The clumping frequency of IT/C10 parasites was unchanged at concentrations above 70 x 10^9 platelets per L ($p=0.25$) but declined exponentially as platelet counts decreased (Figure 3.8). Platelet mediated clumping occurred in platelet concentrations that would be considered thrombocytopenic in malaria patients implying that platelet-mediated clumping could still occur *in vivo* for thrombocytopenic patients.

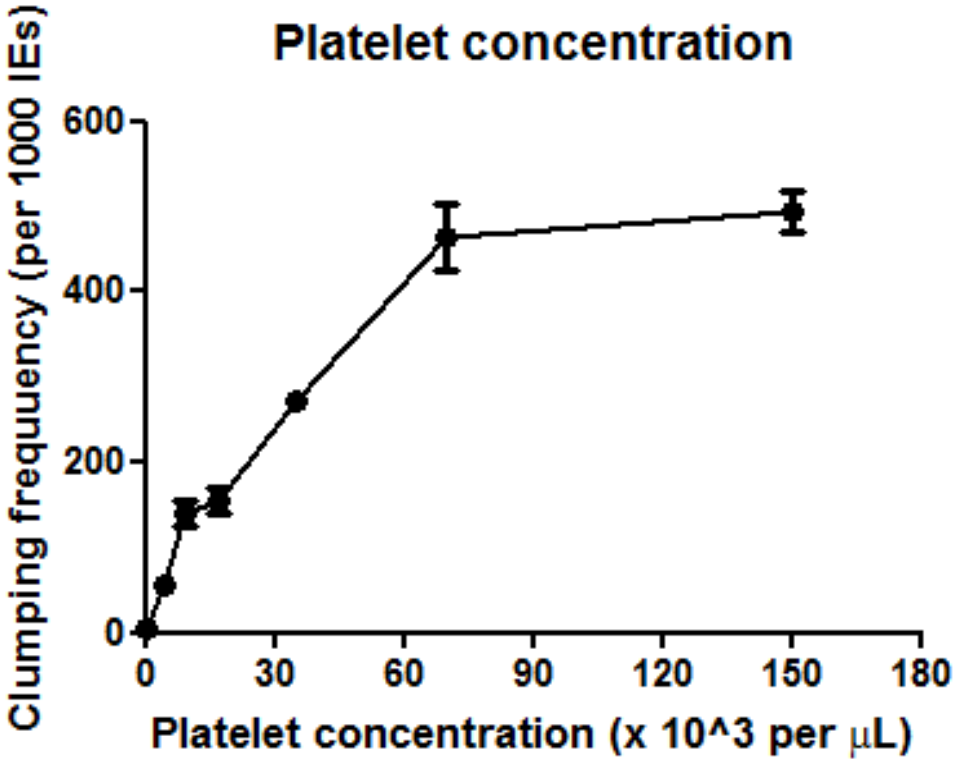


Figure 3.8 Relationship between clumping and platelet concentration

Platelet-mediated clumping assays were conducted with IT/C10 IEs in serially diluted PRP from a single donor. Three slides were prepared after 120 minutes of incubation. Error bars represent standard deviations of three experiments. Platelet mediated clumping for parasite line IT/C10 depends on the concentration of platelets in the assay mix.

f) Platelet storage

In blood centres platelets are stored at 22ºC for up to of usually for up to 5 days but can be 7 days in some circumstances with mild agitation or stored at 4ºC without agitation whereas in platelet research platelets are used within two weeks of bleeding. In previous clumping studies, platelets were chilled at 4ºC and used within two weeks of bleeding. The way platelets are prepared and stored influences platelet function, I therefore sought to determine the effect of prolonged platelet storage on their ability to induce platelet-mediated clumping. PRP was prepared from a single donor at different time points and chilled at 4ºC for up to 2 months. Clumping assays were conducted with IT/C10 parasites with platelets for stored for 1 day, 2 weeks, 1 month and 2 months. PRP was stored in neat concentration and the dilutions were only adjusted in PBS on the day of the experiment. Interestingly, platelets maintained their ability to induce platelet-mediated clumping of IEs after prolonged storage. However there was increased variability in the clumping frequency recorded between the replicates after prolonged storage of PRP (Figure 3.9). The differences in clumping frequency for the different platelets were statistically insignificant ($p=0.81$, ANOVA).

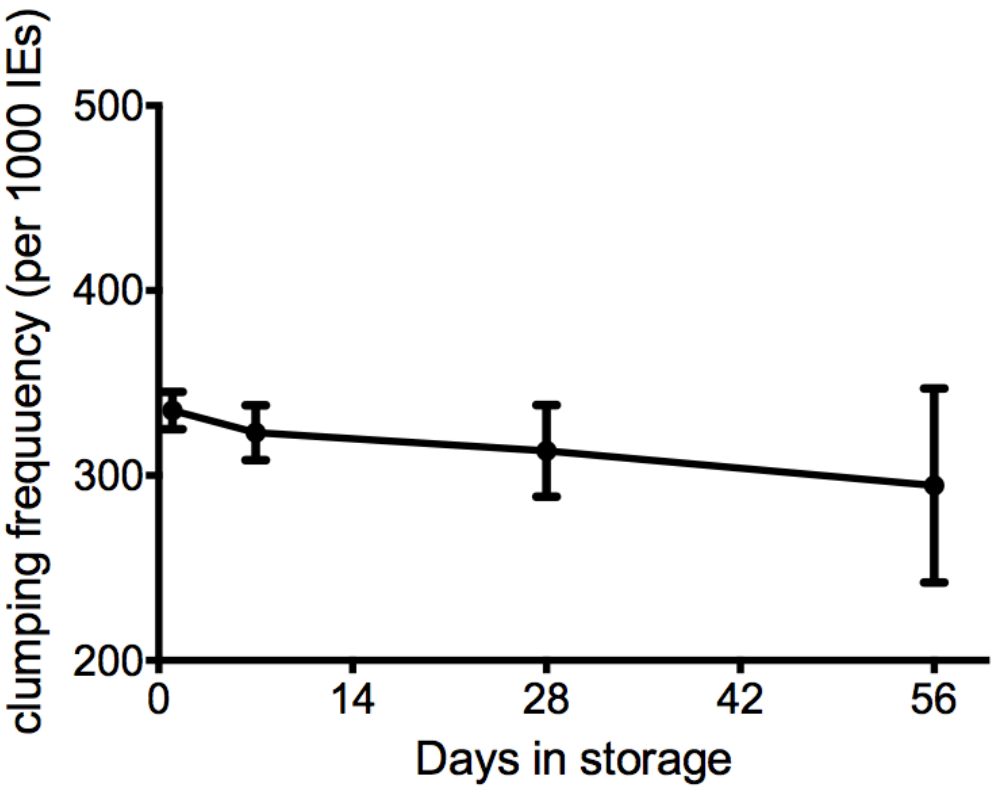


Figure 3.9 Relationship between clumping and platelet storage

Platelet-mediated clumping assays were conducted with platelets from a single donor collected at different times and chilled at 4ºC for up to 56 days. Platelet concentrations were adjusted and clumping assays conducted in triplicate. Error bars represent standard deviations of two experiments. Three slides were prepared after 120 minutes incubation. Platelets maintained the ability to support platelet-mediated clumping even after 56 days in cold storage.

g) Anticoagulants for phlebotomy

Specific anticoagulants may interfere with specific pathways of platelet activation and thus platelet function. Sodium citrate is preferred in platelet research because compared to heparin and EDTA it modifies platelet functions the least. In order to determine the effect of common anticoagulants on the ability of platelets to induce platelet-mediated clumping, a healthy donor was bled into tubes containing heparin, EDTA or sodium citrate anticoagulants. PRP was prepared using identical methods and platelet concentration was adjusted in PBS before clumping assays were conducted with 5% parasitaemia and 10% haematocrit. Three slides were prepared after 2 hours of incubation. EDTA and citrate anticoagulated platelets supported platelet-mediated clumping although clumping in EDTA was significantly higher than it was in citrate $p<0.01$. Platelet-mediated clumping was almost virtually inhibited in heparinised PRP. Interestingly the highest clumping frequency was reported in calcium depleted platelets $p=0.0001$ when compared with heparinized platelets (Figure 3.10).

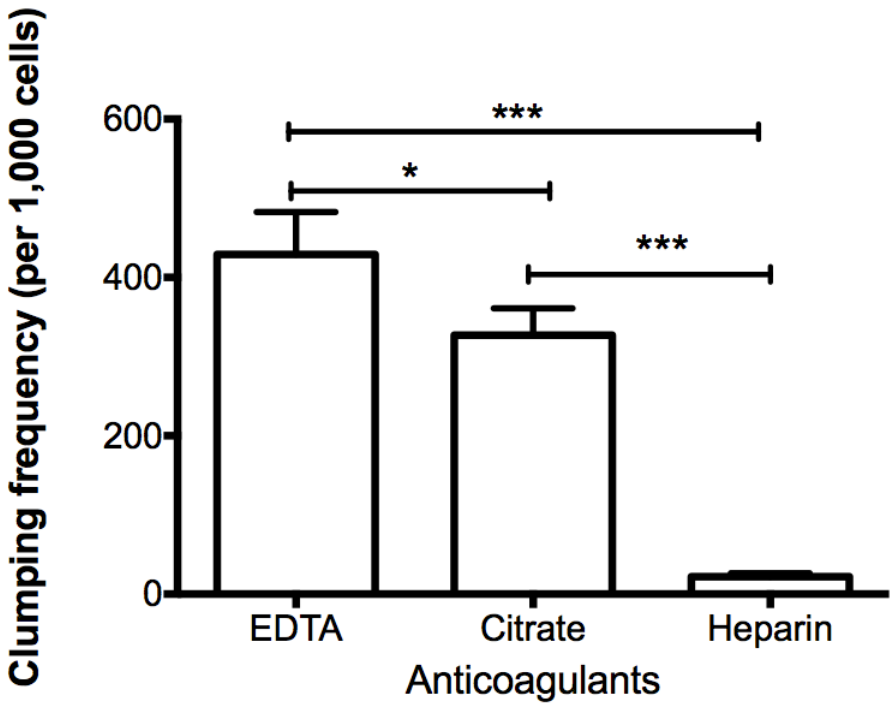


Figure 3.10 Clumping in different anticoagulants

Clumping assays were conducted with PRP collected from a single donor into EDTA, sodium citrate or heparin anticoagulants. Three slides were prepares after 120 minutes incubation, error bars represent standard deviations of two experiments. Clumping frequency of IT/C10 was significantly inhibited in heparin but clumping occurred in EDTA and citrate anticoagulated PRP.

h) Calcium ions

Calcium ions are required for signalling pathways involved in platelet activation and also the structure and function of glycoproteins. EGTA, a strong cation chelator inhibited platelet-mediated clumping although clumping was maintained in the presence of a similar chelating solution - EDTA (Chotivanich, Sritabal *et al*. 2004). To differentiate the effect of the two chelators on the ability of the platelets to support platelet-mediated clumping, PRP was prepared from citrate anticoagulant before washing in EDTA, EGTA or PBS. The platelets were then suspended in PBS to a desired platelet concentration. Clumping assays were then conducted with the three different platelet-preparations. Platelet-mediated clumping was significantly inhibited in the platelet suspensions that were treated with EDTA ($p=0.03$) and EGTA ($p=0.02$) as compared with PBS showing that calcium is important element in the interactions that are involved in platelet-mediated clumping but is not required for platelet-mediated clumping to occur (Figure 3.11). It is noteworthy that the platelets washed in PBS had very little Ca2+ since the cells were washed in PBS.

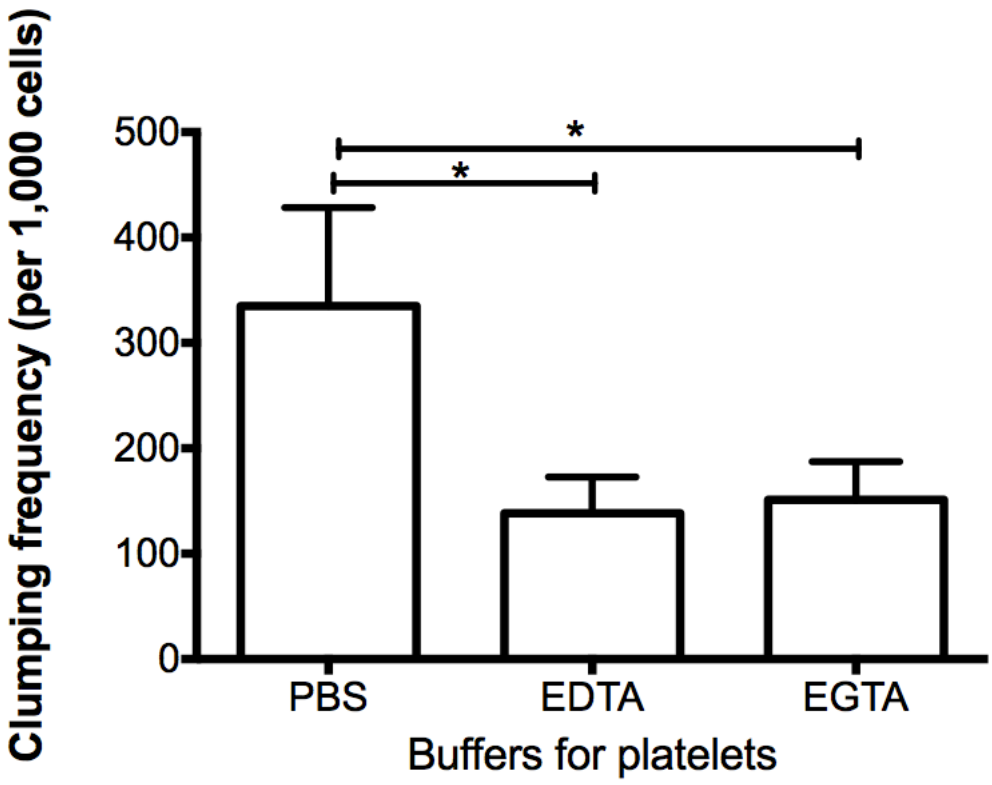


Figure 3.11 Inhibition of clumping by calcium chelation

Platelet-mediated clumping assays were conducted with IT/C10 trophozoites with platelets suspended in PBS, EGTA or EDTA buffers. Three slides were prepared after 120 minutes incubation in PRP. Error bars represent standard deviations of three experiments. Chelation of calcium led to a significant inhibition of the platelet-mediated clumping.

3.4 Discussion

Here I have shown that platelet-mediated clumping is a specific adhesion phenotype that is characteristic of a subset of malaria parasites. I determined the optimal experimental conditions required for a reproducible and accurate assay to be used in screening parasites for the platelet-mediated clumping phenotype. In the process I have described the platelet-mediated clumping phenotype for parasite line IT/C10 in considerable detail in preparation for a more detailed clumping study.

Agglutination of mature-stage IEs and platelets referred to as platelet-mediated clumping displays platelets interposed next to several IEs (Pain, Ferguson *et al*. 2001). In the platelet-mediated clumps, I showed that platelets were juxtaposed with IEs (Figure 3.2G), which is consistent with previous findings (Pain, Ferguson *et al*. 2001, Biswas, Hafiz *et al*. 2007). IEs are held together by platelets in a way that makes them appear to lie side by side to make distinct clumps that are easily discernible by a fluorescent microscope. The clumps are held together robustly since they remain attached even after flattening of the clumping suspension with a sealed glass coverslip.

A subset of IEs appear to be selected for the platelet mediated clumping phenotype. Furthernore, among the clumpers it appears that the proportion of parasitized erythrocytes (parasitaemia) and how closely they are packed (haematocrit) determine the rate at which platelet-mediated clumping occurs. Unsurprisingly, freeze-thawing of mature parasite IEs did not diminish their ability to form clumps in the presence of platelets consistent with findings that reported the surface antigens of IEs are retained following freeze-thawing (Kinyanjui, Howard *et al*. 2004).

Platelets are required for platelet-mediated clumping to occur. I also showed that platelets rather than a plasma component in PRP is required for clumping to take place. Fresh platelets are better for conducting clumping assays than stored platelets. Platelets collected in heparin are less likely to support platelet mediated clumping than platelets collected in sodium citrate. There is a slight increase in clumping with EDTA platelets compared to citrate-anticoagulated platelets. Calcium ions are not required for platelet-mediated clumping to occur but their inclusion enhances the phenotype.

Platelet-mediated clumping is associated to severe disease both in adults and children. I have clearly shown that platelet-mediated clumping occurs in conditions that are relevant clinically, i.e, thrombocytopenic malaria patients can also exhibit platelet mediated clumping.

This study employs an *in vitro* method to test a clinical phenomenon which has not been proven to occur *in vivo* although platelet deposits have been reported in brain microvesels of children deceased from cerebral malaria (Grau, Mackenzie *et al*. 2003). The platelet-mediated clumping assay is labour intensive and intricate and the examination and counting of IEs in clumps can be subjective. The use of pipette tips to pick clumps could lead to break up of clumps but this was overcome by truncation of pipette tips to make wider mouths.

In summary, I have described optimal assay conditions for screening a given parasite culture for the platelet-mediated clumping phenotype. I recommend conducting the

clumping assay at a high haematocrit of 10%, which ensures that the clumping phenotype is detected, but also to control the parasitaemia to ensure that the clumps formed are limited assay at high parasitaemia.

Chapter 4

Study of parasite-encoded ligand for host receptors in platelet-mediated clumping

4.1 Introduction

Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP-1) is a family of parasite proteins expressed on the infected-erythrocyte (IE) surface that undergo clonal antigenic variation. PfEMP-1 is a major adhesive ligand of IEs to host cell receptors on endothelial cell receptors and for rosetting of uninfected erythrocytes. Specific host cell receptors that interact with PfEMP-1 include CD36 (Ockenhouse, Tandon *et al*. 1989), P-Selectin (Ho, Schollaardt *et al*. 1998), PECAM-1 (Treutiger, Heddini *et al*. 1997) and Complement Receptor-1-(CR1) (Rowe, Moulds *et al*. 1997) and others described in the Introduction chapter (section 1.3). Furthermore, specific disease syndromes in malaria and adhesion phenotypes have been linked to subsets of PfEMP-1 including rosetting, endothelial cell binding and platelet-mediated clumping therefore underscoring the functional importance of these parasite proteins (Rowe, Obeiro *et al*. 1995, Newbold, Craig *et al*. 1999).

Platelet-mediated clumping refers to the aggregation of mature-stage IEs in contact with platelets, an adhesive phenomenon linked to severe malaria in clinical studies (Pain, Ferguson *et al*. 2001, Chotivanich, Sritabal *et al*. 2004, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011). The platelet receptors that mediate platelet-mediated clumping include CD36, P-Selectin and gC1qR but the parasite ligands that interact with these

platelet receptors to bring about clumping are unknown (Pain, Ferguson *et al*. 2001, Biswas, Hafiz *et al*. 2007, Wassmer, Taylor *et al*. 2008). PfEMP-1 is hypothesized to be a candidate ligand that mediates platelet-induced clumping of IEs because it mediates binding to CD36 (Baruch, Ma *et al*. 1997) and P-Selectin (Senczuk, Reeder *et al*. 2001) both of which are known receptors in the platelet-mediated clumping phenotype. Platelets also express PECAM-1 (Treutiger, Heddini *et al*. 1997) and $\alpha_v\beta_3$ integrins (Siano, Grady *et al*. 1998) which mediate adhesion of malaria-infected erythrocytes to endothelial cells.

The extracellular erythrocyte surface is modified by the intra-erythrocytic parasite through insertion of parasite-encoded proteins resulting in insertion of parasite proteins. *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1), RIFIN and STEVOR encoded by *var, rifin* and *stevor* multigene families respectively have been shown to be expressed at the surface of the infected erythrocyte and are implicated in host-parasite interactions (Reviewed by (Kraemer and Smith 2006)) PfEMP-1 is presented on elevations on the erythrocyte plasma membrane known as knobs which are underlied by electron-dense structures predominantly comprised of parasite-encoded Knob Associated Histidine Rich Protein (KAHRP) (Crabb, Cooke *et al*. 1997). Interestingly Horrocks and colleagues showed that among isogenic parasites, knobless parasites presented less PfEMP-1 on the surface which subsequently lead to lower adhesion as compared to knobbed parasites (Horrocks, Pinches *et al*. 2005).

Approximately 50–60 different *var* genes encode PfEMP-1 proteins. *Var* genes vary in length (6–13 kb) with a two-exon structure. Exon-1 is extremely diverse and encodes a binding region while exon-2 encodes a conserved cytoplasm region. The domains

responsible for the binding properties of PfEMP-1 named Duffy Binding Like (DBL) and Cysteine inter-Doman Region (CIDR) are located exon 1 (Smith, Gamain *et al*. 2001). DBL and CIDR domains can be divided into different sequence types according to sequence similarity (Smith, Subramanian *et al*. 2000). In addition to domain categories, the upstream non-coding sequences of *var* genes group into 5 distinct families: UpsA-E (Voss, Thompson *et al*. 2000, Gardner, Hall *et al*. 2002, Kraemer, Gupta *et al*. 2003). These classifications also differentiate *var* genes with respect to chromosomal location and transcriptional orientation, with a high degree of correspondence to the domain groupings.

Knobs are protrusions on the IE surface that are required for IEs to adhere to endothelial surfaces. The absence of knobs on IEs inhibits adhesion of IEs *in vitro*. *In vivo,* knobless parasites are not observed unless the patient is splenectomized hence implying a strong functional selection for knobs in spleen-intact patients (Langreth and Peterson 1985, Pongponratn, Viriyavejakul *et al*. 2000). Knobless IEs are generated in *in vitro* culture through the loss of one end of chromosome 2 which deletes sub-telomeric genes including *kahrp* (Lanzer, Wertheimer *et al*. 1994).

Knob Associated Histidine Rich Protein (KAHRP) is a major protein in knobs whose expression of is essential for the formation of knobs on *P. falciprum*-infected erythrocytes (Crabb, Cooke *et al*. 1997) (Section 1.4.4). The C-terminal region of the *KAHRP* gene is required for the development of knobs and adhesion as determined using 3D7 parasites expressing mutated forms of KAHRP (Rug, Prescott *et al*. 2006). I sought to investigate the role of PfEMP-1 and KAHRP in platelet-mediated clumping of parasite line IT/C10. To determine if the platelet-mediated clumping phenotype is linked to specific sequence features of PfEMP-1 I developed a panel of parasite clones and determined their adhesion

phenotypes to immobilized purified receptors, clumping frequency in Platelet Rich Plasma (PRP) and sequenced the semi-conserved region of the *var* genes known as the Duffy Binding-Like domain (DBL-1). I hypothesized that expression of specific PfEMP-1 proteins and/or KAHRP is associated with platelet-mediated clumping. I therefore derived a panel of *Plasmodium falciparum* parasite sub-clones from a well-defined clumping clone (IT/C10).

Specific aims

a) To develop a panel of isogenic IT/C10 clones
b) To study the clumping phenotype in the IT/C10 clones in relation to PfEMP-1 expressed
c) To study the adhesion phenotypes of the IT/C10 clones

4.2 Specific methods

4.2.1 Source of parasites

Parasite line IT/A4 was obtained by cloning from the Brazilian line IT4/25/5 provided to Prof Chris Newbold by Dr R.J. Howard (Berendt, Simmons *et al*. 1989). IT/A4 was cloned to produce parasite lines IT/C10 (Roberts, Craig *et al*. 1992) and IT/3C3 (Horrocks, Pinches *et al*. 2005). Infected erythrocytes of the IT/A4 line show strong host cell receptor binding characteristics even after multiple passages in culture hence its use in cytoadhesion studies. 3D7 was originally obtained by limiting dilution of the NF54 line (Walliker, Quakyi *et al*. 1987). Parasite lines FCR-PECAM (gift of Prof M. Wahlgren, Karolinka Institute, Sweden) and FCR-gC1qR (gift of Dr A. Scherf) parasites are derived from the same South American Ituxi parasite line that were selected on PECAM-1 and gC1qR respectively. T996 is a knobless parasite line that was originally isolated in Thailand.

4.2.2 Cloning IT/C10 by limiting dilution

To develop a panel of suitable clones for the study and characterization the platelet-mediated clumping phenotype, IT/C10 parasites were grown up to 12% parasitaemia. The parasite culture was serially diluted with uninfected erythrocytes at 1% haematocrit in serum-free culture medium to a final concentration of approximately 100 infected erythrocytes in 30ml culture medium. One hundred µl aliquots were transferred to two 96-well-round-bottomed plates giving a probability of finding approximately 0.3 IEs per well. The plates were gassed and cultured at 37ºC as previously described (Roberts, Craig *et al*. 1992). A culture schedule was devised to ensure replacement of fresh erythrocytes was not required until several cycles of parasite growth had elapsed. Fresh erythrocytes were therefore added at defined intervals (see Table 1). Thick films prepared from a sample from each well, stained with Giemsa and examined by microscopy to determine which of the wells were positive with parasite clones. The positive wells were then transferred to small flasks for expansion to be grown and for storage and subsequent phenotyping. A majority of the IT/C10 clones were expected to be derived from a single parasite and therefore would be clonal.

Table 1: Cloning IT/C10 by limiting dilution

Day	Expected parasite Stage	cycle	Culture volume (µl)	Fresh erythrocytes added (µl)	Screened by Giemsa stain
1	T		100		No
2	R				No
3	T	1	150	50	No
4	R				No
5	T	2	200	50	No
6	R				No
7	T	3			No
8	R				No
9	T	4	250	50	No
10	R				No
11	T	5	250	replace 100	No
12	R				No
13	T	6	250		No
14	R				No
15	T	7	250	replace 100	No
16	R				No
17	T	8	250	replace 100	No
18	R				No
19	T	9			No
20	R				No
21	T	10			Yes
22	R				Yes
23	T	11			Yes
24	R				Yes
25	T	12			Yes
26	R				Yes
27	T	13			Yes
28	R				Yes

T- Trophozoite stage

R- Ring stage

Replace refers to adding fresh erythrocytes after sampling for thick smears

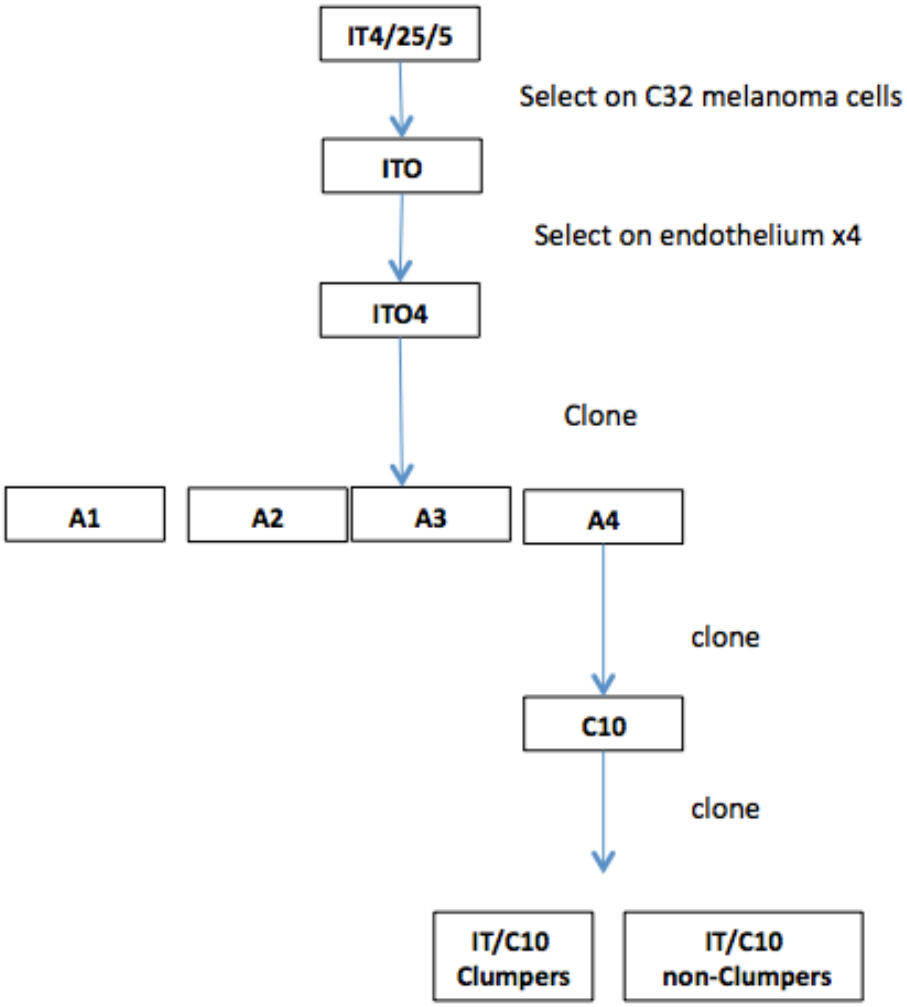


Figure 4.1. IT/4/25 clone tree

IT/4/25 parasites were derived from South American Ituxi parasite line by selection on C32 melanoma cells to make ITO. ITO was selected on HUVEC cells to make ITO4, which was cloned to make the four A clones. Clone A4 was cloned to produce 21C clones. In this study one of the C clones (C10) was cloned to make IT/C10 clones. In this study. Adapted from (Roberts, Craig *et al*. 1992).

4.2.3 Binding assays

Binding assays were performed with mature stage IEs by panning on spots of purified immobilized receptors. Here 3µl of 100μg/ml recombinant proteins CD36 (R & D Systems, UK), P-Selectin (R & D Systems) and PECAM-1 (R & D Systems) and gC1qR (gift of Dr Artur Scherf) were spotted on 60 x 15 mm bacteriological plastic petri dishes (Falcon 1007; Becton Dickinson, Oxford, UK) in triplicate and incubated overnight at 4ºC in an air-tight plastic container and covered with moist paper towels. The next day, the spots were aspirated with a suction pump; the plates were washed with washing buffer (RPMI-1640 without bicarbonate). The uncoated surface on the plates was saturated by incubating with 1% BSA in PBS at 37ºC for 1 hour. The blocking buffer was aspirated and the plates washed 5 times with washing buffer. A suspension of mature stage IEs was prepared in binding medium (RPMI-1640 with 1% BSA) at 2% haematocrit and added to the plates. The plates were incubated at 37ºC for one hour with gentle agitation every 10 minutes at approximately 10rpm. Unbound cells were washed off at least 6 times with washing buffer. To confirm that all unbound cells were removed the plates were examined using an inverted microscope with the final washing solution. The bound cells were fixed with 2% glutaraldehyde for 10 minutes at room temperature and stained with 10% Giemsa for 30 minutes. The plates were then rinsed using tap water. The binding phenotype of the parasites was determined by counting the number of bound IEs per mm^2 under a light microscope with x400 magnification.

4.2.4 Selecting IT/A4 parasites for P-Selectin binding

Two protocols were used to enrich P-Selectin binding of IT/A4 parasites. In the first protocol 60 x 15 mm bacteriological plastic petri dishes were coated with 50μg/ml P-

Selectin (R & D Systems) in PBS overnight at 4ºC. The next morning the unbound protein was aspirated and recycled. The dishes were washed twice using wash medium (RPMI-1640 with 1% BSA). The unbound sites on the dishes were saturated by incubation for 1 hour with 1% BSA in PBS at 37ºC. The plates were gently and quickly rinsed once with binding medium and then incubated with enriched trophozoite stage-infected erythrocytes at 2% haematocrit for 1 hour with gentle agitation every 10 minutes. Unbound cells were washed off with washing medium before culture medium (1.5mL) was added and fresh erythrocytes (10-20μL) were added. The plates were cultured overnight and gassed as described in chapter 2. The culture was transferred to a culture flask the next day and culture media added to make a total of 5mL. After a parasitaemia of approximately 3% was reached the whole process was repeated to enrich P-Selectin binding. The recovery of parasites selected by this method took several weeks therefore I tried a second method.

In the second protocol I used Protein-A-coated magnetic Dynabeads (Invitrogen Dynal, Oslo, Norway). On the first day a 50µl aliquot of beads was washed thrice in 500µl of 1% BSA in PBS and the beads isolated using a magnetic holder designed for holding microfuge tubes (Invitrogen). To the washed beads three micrograms of soluble P-Selectin (R&D systems) was added and made up to 100μl with 1% BSA (PBS). The beads were incubated at room temperature on a rotor at 10 rpm for 45 minutes before being washed twice in 1% BSA and each time using a magnet to retain the beads. The beads were suspended in 200μl 1% BSA and incubated overnight at 4ºC to block non-specific binding. On the second day a parasite culture with a majority of mature stage IEs was Plasmagel^{TM}-treated to enrich for mature stage parasites. The Plasmagel^{TM} -enriched mature stage IEs (approximately 50% parasitaemia) were added to the P-Selectin coated

Dynabeads at 5% haematocrit and incubated for 45 minutes at room temperature on a rotor rotating at 10 rpm. A magnet was used to retain Selectin-bound IEs whereas unbound cells were washed off. Fresh erythrocytes were then added to the P-Selectin-bound IEs and recultured. On the third day the magnetic beads were removed from the IE culture with the magnet. The process was repeated at the next trophozoite stage to enrich further for P-Selectin binding parasites. By combining the two selection methods IT/A4 parasites were selected to produce the A4-P-Selectin parasites.

4.2.5 Trypsin treatment of IEs

IEs were washed twice in incomplete medium by centrifuging for 5 minutes at 1000g at 25ºC. The parasite pellet was suspended in 10 pellet volumes of incomplete malaria culture medium supplemented with Trypsin enzyme (Sigma, Poole, UK) at desired concentrations (10-100μg/mL) and incubated for 30 minutes at 37ºC. Trypsin activity was inhibited by addition of trypsin inhibitor (Sigma, UK) at a final concentration of 2mg/mL and incubating for a further 5 minutes. The parasite pellet was washed twice in incomplete culture medium then used in platelet-mediated clumping assays as previously described.

4.2.6 Molecular biology

a) Trizol™ RNA extraction

RNA was extracted in Trizol™ reagent according to the manufacturer's instructions as described before (Kyes, Pinches *et al*. 2000). Parasite cultures were synchronized at the early ring stage with 5% sorbitol. Ten pellet volumes of pre-warmed Trizol™ reagent were added to the parasite pellet, gently mixed and incubated for 5 minutes at 37ºC. The Trizol™ lysates were then stored at -80ºC.

On the day of extraction the Trizol™ lyaste was thawed then 0.2 volumes of chloroform added, mixed gently and centrifuged for 30 minutes (4500 rpm at 4ºC). The aqueous clear top layer was transferred to a clean tube and 0.2 Trizol™ volumes of isopropanol were added and mixed briefly then split into 1ml aliquots that were placed on ice to precipitate the RNA for at least 3 hours. The tubes were spun at 13000 rpm and 4ºC for 30 minutes in a bench-top centrifuge to pellet the RNA. The supernatant was aspirated and the pellet air-dried. The RNA was suspended in 50μL formamide and heat denatured in a 60ºC water bath for 10 minutes. The RNA yield was determined by Nanodrop™ analyzer and stored at -80ºC.

b) DNase treatment of RNA

A total of 4.5µg of RNA was suspended in 100µl DNase free water, 10µl 3M sodium acetate and 250µl ethanol and incubated at -80ºC overnight to precipitate RNA from the formamide. The frozen RNA was placed on ice before centrifugation at 13500 rpm for 30 minutes at 4ºC. The supernatant was removed by aspiration and the pellets were air-dried. RNA was treated with DNase to remove contaminating genomic DNA by adding the following: 17µl DNase free water, 2µl 10x DNA-free buffer (Ambion) and adding a further 1μl DNase solution (Ambion). The reaction tube was incubated at 16ºC for 20 minutes before adding 1µl DNase and incubating at 16ºC for another 20 minutes. DNase was subsequently inactivated by adding inactivation reagent (2μl) (Ambion) and incubating at room temperature for 2 minutes. The reaction mix was centrifuged at 13500 rpm for 2 minutes before transferring the RNA supernatant to a new tube. The effectiveness of DNase treatment was tested by PCR using 2μl of the DNase-treated RNA

to amplify a housekeeping gene and resolving on agarose gel. The treatment was repeated if any DNA was detected.

c) cDNA synthesis

cDNA was synthesized from DNase-treated RNA by reverse transcription. To 2µl of DNase-treated RNA 100ng random hexamers (Invitrogen) and 4.75µl nuclease-free water were added. The mixture was then incubated at 70ºC for 5 minutes to denature the RNA. The denatured RNA was placed on ice for 2 minutes before adding 5µl 10x Bioscript buffer (Bioline), 0.25µl Bioscript (Bioline), 1µl of 10mM dNTPs (Invitrogen). The mixture was incubated for 40 minutes at 40ºC to make cDNA. The cDNA was stored in 10µl aliquots at -80ºC that were only thawed once for use.

d) Genomic DNA extraction

Parasite genomic DNA was extracted using a proprietary kit (Qiagen miniprep kit). All manipulations were performed at room temperature unless stated otherwise. Parasite cultures were centrifuged for 5 minutes at 1000g at room temperature. The culture pellet (40µl) was washed twice in incomplete culture medium before it was suspended in 200µl PBS and 20µl Proteinase K. Two hundred microlitres of ethanol were added before vortexing and incubating for 20 minutes in a 56ºC water-bath. The mixture was transferred into a DNeasy mini-spin column and centrifuged at 600g for 1 minute. The flow through was discarded and the column washed with 500µl of buffer AW1 and centrifuged at 600g for 1 minute. The column was then washed with buffer AW2 by centrifuging at 2000g and discarding the flow through. Finally the column was placed in a microfuge tube and 50µl elution buffer was added before centrifuging at 600g for 2 minutes. A Nanodrop™

analyzer was used to quantify DNA by measuring absorbance of UV-visibility light of the sample.

e) Cloning PCR products in E. coli and sequencing

The semi-conserved region (DBL-1) of the *var* genes was amplified with generic primers described by Bull and colleagues (Bull, Berriman *et al*. 2005). The fresh PCR products were cloned into plasmid vector PCR4-TOPO of the TA cloning system (Invitrogen). The PCR4-TOPO plasmid vector has a single 3' thymidine overhang and a topoisomerase covalently bound to the vector. Manufacturer's instructions were followed to transform the TOP10 *E. Coli* cells.

To make the ligation mix, between 0.5 and 4µl fresh PCR product was added to 1µl TOPO vector, 1µl of dilute salt solution and made up to a total of 6µl with water. The mixture was incubated at room temperature for 5 minutes then placed on ice. Two types of cells were transformed, namely TOP10 or DH5α E. Coli cells. An aliquot of the TOPO Cloning reaction mix (2µl) was gently added gently to a vial of TOP10 cells and incubated on ice for 5 minutes. TOP10 cells were heat shocked for 30 seconds at 42ºC (45 seconds DH5α cells) before adding 250µl pre-warmed Super Optimal Culture (S.O.C) medium. The vial was tightly screw-capped and incubated horizontally in a shaking incubator at 37ºC for 1 hour. Bacterial cells were grown using Luria-Bertani (LB) medium (10g tryptone, 5g yeast extract and 5g sodium chloride in 1 litre of distilled water, pH 7.0 with sodium hydroxide) and LB-agarose plates (LB medium supplemented with 15g agarose per litre). Fifty or 200µl of the transformation mix was spread on a pre-warmed LB Agar plate with 50µg/ml ampicillin then incubated overnight at 37ºC. The next day individual colonies were picked and inoculated in 3ml LB medium supplemented with 50µg/ml

ampicillin in a shaking incubator for 24 hours. The culture was harvested by centrifugation for 5 minutes at 1000g in a bench-top centrifuge.

f) Northern blot

i) Electrophoresis of RNA for Northern blots

Northern blot analysis was conducted as described before (Kyes, Pinches *et al*. 2000). The gel apparatus was soaked in 0.5M sodium hydroxide for two hours to remove RNase enzymes and rinsed with distilled water. Agarose was dissolved in 1x TBE buffer (1 x TBE – 6g TRIS, 5.5g orthoboric acid and 4ml 0.5M EDTA pH 8.0 made up to 1 litre with deionised water) and heated in a microwave. The agarose was let to cool to approximately 55ºC before adding 0.5µg/ml ethidium bromide and 5mM guanidium thiocynate to deactivate RNases. The gel was poured and left to set for 1 hour. RNA samples were prepared by heating at 60ºC for 5 minutes. Approximately 10µg RNA was run in each lane and one lane was loaded with bromophenol dye. The gel was run until the bromophenol dye had run for 8 to 10 cm. The gel was placed under UV light for approximately 1 minute before soaking the gel in 7.5mM sodium hydroxide for 15 minutes. RNA was then transferred to Hybond N+ membrane (Amersham, UK) in 7.5Mm sodium hydroxide overnight. After transfer to the Hybond membrane, the filter was neutralised for 5 minutes in 2x SSC (1 liter of 20× SSC = 175.3 g NaCl, 88.2 g sodium citrate, to pH 7 with HCl; dilute 1:10 for 2× SSC) and air-dried.

ii) Hybridization probes for Northern blot

Radioactive probes were prepared with Megaprime labelling kit (Amersham, UK). Briefly,

water and random hexamers were added to a 25ng aliquot of PCR product, before placing them in a boiling water bath to denature the DNA. The denatured PCR product was then added to, Labeling Buffer (10µl), mixture of the unlabeled dNTPs (2µl) 20µM each, 500ng/ml Nuclease-Free BSA (2µl), 400µg/ml, [α-32P]dNTP (5µl) AND 333nM DNA Polymerase I Large (Klenow) fragment. Nuclease free water was added to make up the total volume to 50µl. The reaction was terminated by heating the mixture at 95–100°C for 2 minutes and subsequently chilling in on ice. The mixture was used directly in a hybridization reaction. Completed probes were added to saturated blot membranes. The temperature of hybridization was varied according to the specificity required for a given probe. After overnight incubation the filter was washed in 0.5x SSC/0.1% SDS solution.

Filters were prehybridized 3 h in 7% SDS: 0.5 M Na-phosphate (NaH2PO4 , pH 7.2): 2% Dextran sulfate, then hybridized overnight in the same solution with ^{32}P-a -dATP labeled probes (Megaprime, Amersham), at 55°C. Probes were varC (complex *var* exon2 fragments amplified from A4 genomic DNA, primers in Duffy binding-like domain (dbl)-a fragment, amplified from A4 genomic DNA. Filters were washed twice at 60°C in 0.5 SSC: 0.1%SDS, and film developed after overnight exposure with intensifying screen at 80°C.

h) Bioinformatic analysis

To assess the nucleotide or amino acid similarity of sequences I used the Basic Local Alignment Similarity Tool (BLAST). BLAST is designed to identify regions of local alignment by detecting sequences that share only isolated regions of similarity. A score is calculated for each aligned residue based on a matrix of possible alignments. The algorithm finds the number of locally aligned high scoring pairs, which cannot be

improved if extended, and depending on the sequence database the query sequence is compared with, a value of statistical significance for the alignment I assigned called the E-value. The ClustalW algorithm also evaluates the similarity of sequences based on the substitution matrix as well as changeable score penalties for opening of gaps in the alignment.

4.3 Results

4.3.1 Characteristics of the platelet-mediated clumping ligand

a) The platelet-mediated clumping phenotype is expressed at the mature stage of asexual life cycle

During the asexual stage of parasite development the malaria parasite undergoes extensive morphological and transcriptional changes characterized by proteins expressed in a stage specific way. To determine the temporal onset of the clumping phenotype in the erythrocytic life cycle of IT/C10 parasites, a parasite culture was synchronized by treatment with 5% Sorbitol leaving trophozoites only. Aliquots from a tightly synchronized culture were collected at 6-hour intervals of the asexual life cycle from the early ring to late schizont stages and screened simultaneously for the clumping phenotype. A total of 7 time-points were collected.

Parasites at the early ring stage (0-12 hours post invasion) did not exhibit a clumping phenotype. Platelet-mediated clumping was developed between 12-18 hours post invasion at the early trophozoite-stage and was maintained up to the late schizont stage (Figure 4.2). The temporal expression of the clumping phenotype coincides with the time when IE surface protein PfEMP-1 is detected on the surface and the development of other cytoadhesion phenotypes (Bozdech and M. Linas 2003).

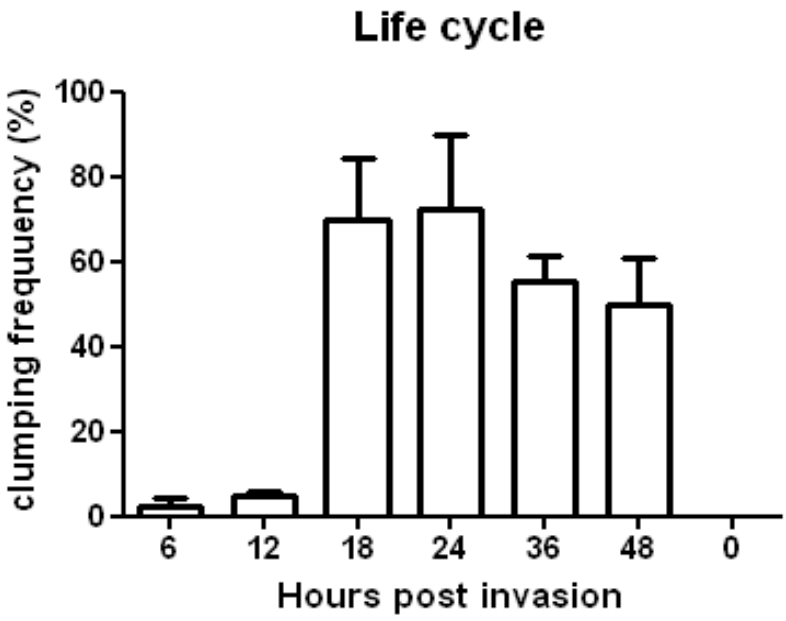


Figure 4.2. Platelet mediate clumping is developed at the trophozoite stage

Platelet-mediated clumping assays were performed with tightly synchronized IT/C10 IEs collected every 6 hours throughout the asexual lifecycle. Three slides were prepared after 2 hours incubation in PRP. Error bars represent standard deviations of three experiments.

b) Trypsin treatment of IEs inhibits platelet-mediated clumping

PfEMP-1 is known to be extremely sensitive to trypsin treatment at low concentrations (1-10 μg/ml) whereas rifins are trypsin resistant at concentrations below 100μg/ml (Leech, Barnwell *et al*. 1984, Fernandez, Treutiger *et al*. 1998, Kyes, Rowe *et al*. 1999). To delineate the biochemical properties of the clumping parasite ligand, trophozoite stage IEs were treated with varying concentrations of trypsin before clumping assays were conducted and compared to a PBS-treated control. IEs were treated with trypsin (10-100μg/ml) before clumping assays were conducted. Interestingly for IT/C10 parasites, platelet-mediated clumping phenotype was almost entirely abrogated at concentrations 10μg/ml of trypsin indicating that platelet-mediated clumping relies on an IE ligand that is sensitive to trypsin (Figure 4.3). The parasite ligand for platelet-mediated clumping appears to have the characteristics of a PfEMP-1. The timing of the onset of the platelet-mediated clumping phenotype by IEs and their biochemical property of sensitivity to trypsin (Figure 4.3) is consistent with a role for PfEMP-1 in the expression of the platelet-mediated clumping phenotype.

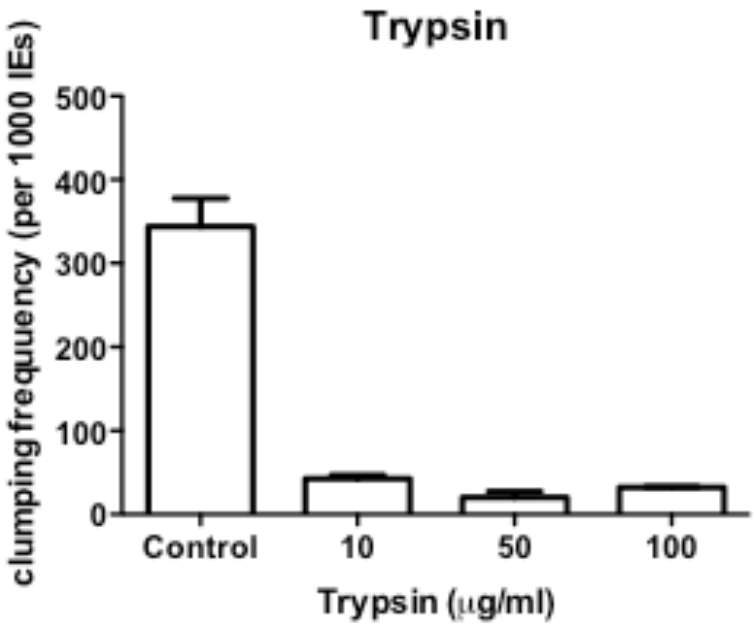


Figure 4.3. Trypsinization of IEs inhibits platelet-mediated clumping

Platelet-mediated clumping assays were conducted with trypsinized IT/C10 trophozoites and compared with mock-treated controls. Three slides were prepared after 2 hours incubation. Error bars represent standard deviations of two experiments.

4.3.2 Screening the phenotypes of the IT/C10 clones

The sensitivity of the clumping ligand to trypsin and temporal development of the platelet-mediated clumping phenotype suggests that the platelet-mediated clumping ligand present on the erythrocyte surface could be a PfEMP-1. To test this hypothesis the IT/C10 parasite line and the resulting sub-clones were screened for their clumping phenotypes in the presence of platelet rich plasma. The clones were further tested for binding to immobilized recombinant platelet receptors CD36, P-Selectin, PECAM-1 and gC1qR.

a) Clumping phenotypes of the IT/C10 clones

Autoagglutination was a novel property of IT/C2 and IT/C10 clones derived from the IT/A4 parasite line out of a total of 11 distinct antigenic phenotypes (Roberts, Craig *et al*. 1992) . To characterise the platelet-mediated clumping phenotype, sub-clones of IT/C10 parasite line were developed by limiting dilution. Eighteen clones were used in this study from a possible of 60 possible clones based on protocol described. Forty-three clones grew successfully but only 23 clones were bulked up for further experiment. Out of these 18 were selected for this study. To determine the precise clumping phenotype of the IT/C10 sub-clones, trophozoite-infected erythrocytes were tested within 30 cycles of the cloning cycle. Twelve out of 18 IT/C10 sub-clones exhibits a clumping frequency above 10% in PRP therefore are referred to as clumpers whereas six clones (clone 3, clone 10, clone 17, clone 32 and clone 44) exhibited significantly lower clumping frequencies (Figure 4.4).

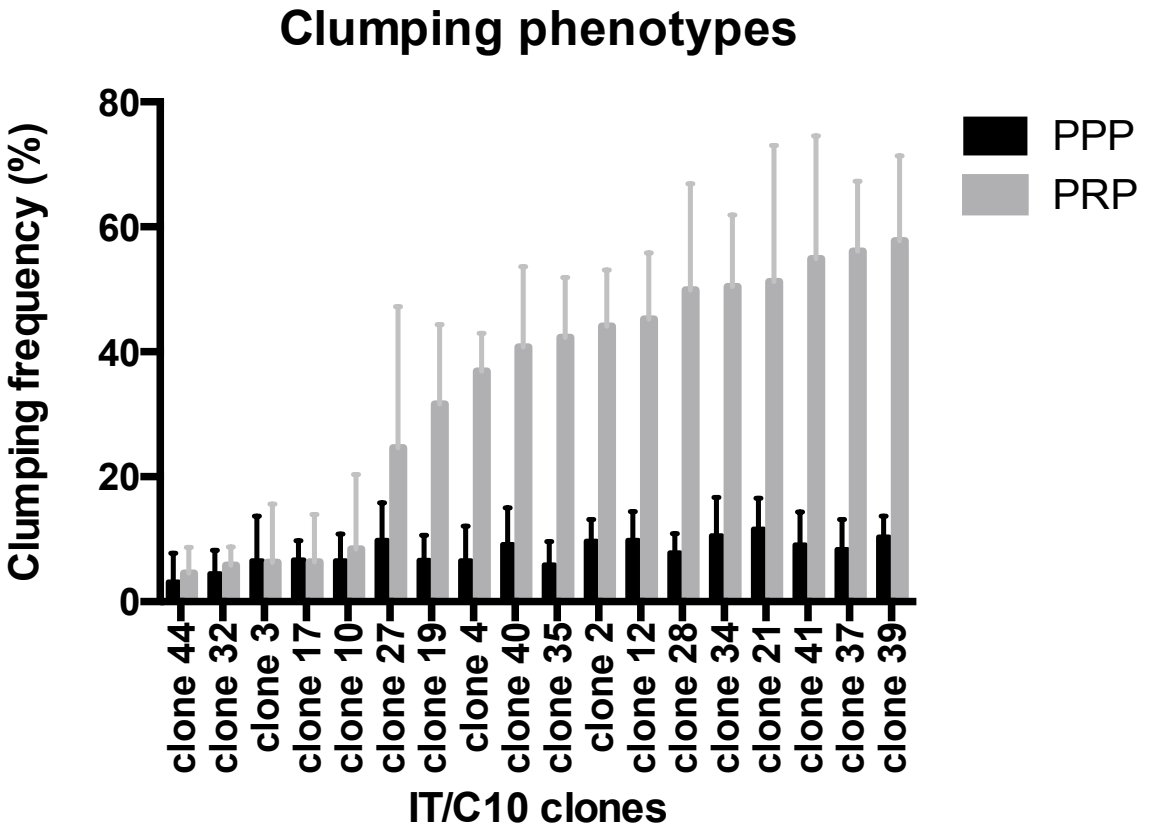


Figure 4.4 Clumping phenotypes of the IT/C10 clones

Parasites of the IT/C10 clone panel were screened for platelet-mediated clumping phenotypes by staining with ethidium bromide, suspending in PPP or PRP and 2h incubation on a bench-top rotor at 10rpm. Three slides were prepared and analysed with a fluorescent microscope. At least 1000 IEs were counted on each slide. Error bars represent standard deviations of three experiments. The clumping phenotype of a majority of the clones was dramatically enhanced by platelets. The clones are ordered by clumping frequency.

b) Adhesion to immobilized receptors

Malaria-infected erythrocytes bind to endothelial cells through host adhesion receptors interacting with parasite protein PfEMP-1. The timing of the development of platelet-mediated clumping in the asexual life cycle is consistent with PfEMP-1 expression (Figure 4.1) (Bozdech and M. Linas 2003). I sought to determine the relationship between parasite adhesion and platelet-mediated clumping in IT/C10 clone thus investigating if the adhesion phenotypes of a given parasite clone would predict its platelet-mediated clumping phenotype. I conducted static adhesion assays with recombinant proteins CD36, P-Selectin, PECAM-1 and gC1qR on plastic plates.

As expected all the IT/C10 clones showed a significant binding to CD36. There was variation in the binding with clumpers in general having similar binding rates to CD36 as non-clumpers. There was no correlation between the level of binding to CD36 and the level of clumping for the IT/C10 clones. Binding to P-Selectin was present in only 4 clones. There is a weakly positive correlation between P-Selectin binding and level of clumping in platelets. Finally no significant binding was observed for the IT/C10 clones with PECAM-1 and Gc1qR in our experiments.

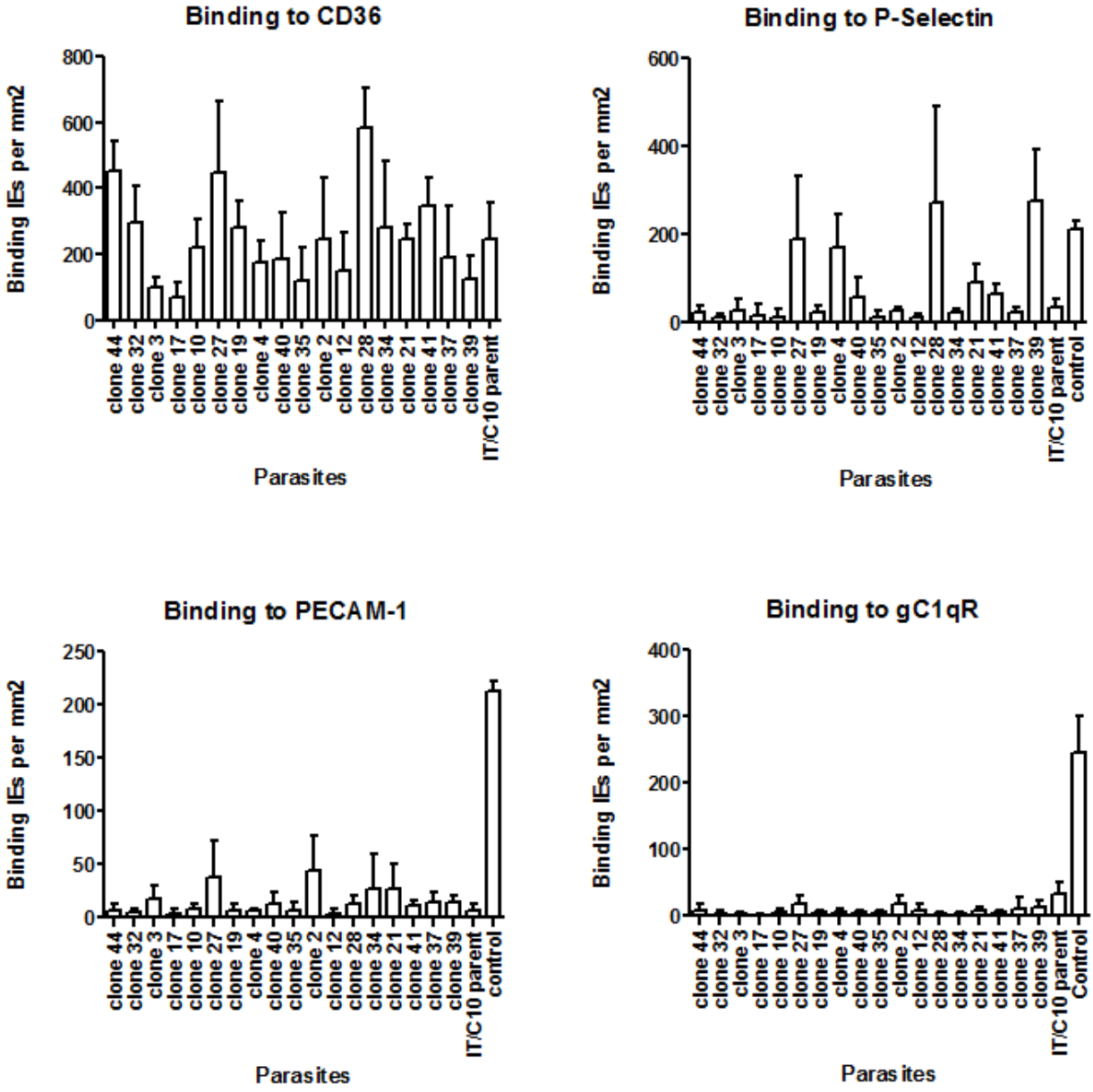


Figure 4.5. IT/C10 clones binding to CD36, P-Selectin, PECAM-1 and gC1qR

Parasites of the IT/C10 clones were tested for binding to immobilized receptors arranged in increasing clumping frequency (A) CD36 (B) P-Selectin, (C) PECAM-1 (D) gC1qR. Three spots were counted and the experiment was repeated twice. Error bars represent standard deviation of two experiments.

To determine the correlation between the binding phenotypes of the IT/C10 clones and the clumping phenotype I conducted correlations. There was no direct correlation between clumping frequency and the number of bound IEs. The correlations were statistically insignificant CD36 (R^2=0.001), P-Selectin (R^2=0.139), PECAM-1 (R^2=0.08) and gC1qR (R^2=0.06) (Figure 4.6A-J).

iii) Correlation of adhesion phenotypes of clumping phenotypes of IT/C10 cones

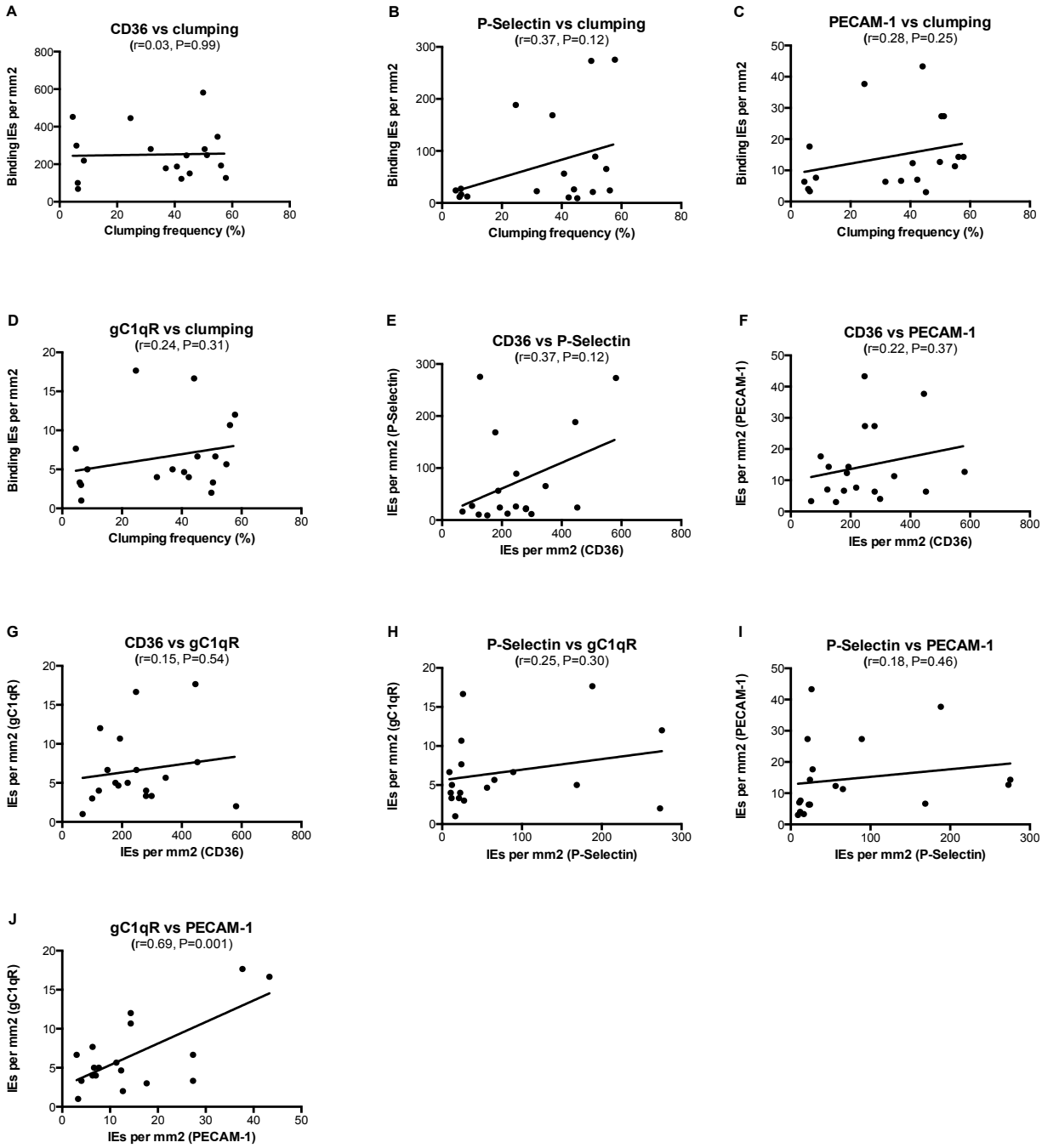


Figure 4.6 Correlation of binding phenotypes and clumping of IT/C10 clumping panel

Scatter plots here represent the correlation between clumping frequency of 3 experiments and binding phenotypes of the IT/C10 sub-clones.

4.3.3 Study of PfEMP-1 expression in IT/C10 clones

Platelet-mediated clumping develop approximately 12-18 hours post-merozoite invasion, which coincides with the development of other cytoadhesion phenotypes (Figure 2). To investigate the relationship between the platelet-mediated clumping phenotype in the IT/C10 sub-clones and the transcribed PfEMP-1 proteins (*var* gene transcripts), I extracted RNA from synchronized ring stage and profiled the *var* gene transcription using different approaches.

a) Northern blot

To study *var* gene transcription of the IT/C10 clones whose clumping phenotype is well defined (Pain, Ferguson *et al*. 2001). RNA was extracted from ring-stage infected-erythrocytes and transferred to a membrane. The membrane was first probed for a conserved *var* gene sequence domain of exon-2 that is expected to be present in all *var* genes. The exon-2 probe showed a considerable variation in the signal detected. The filter was re-probed with 6 *var* gene specific probes namely IT*var*30, IT*var*34, IT*var*27, IT*var*58, A4TRES and IT*var*46. Previous work by Dr Taco Koiij with the IT/C10 parent clone showed IT*var*58 as the majority *var* gene therefore it was included in the probes. Interestingly, a majority of the clones were positive for IT*var*30 a majority of which were clumpers except for clone 10, which was positive for both IT*var*30 and IT*var*58. Clumping clones 21, 41, 37, 27 and 38 were positive for IT*var*30 (Figure 8). Non-clumping clone 32 was positive for A4Tres. In general it appeared that IT*var*30 was the most common *var* gene among the clumping clones and could be linked to the clumping phenotype. Since the membrane was probed for only 6 *var* genes many more might have been missed. The Northern blot method is not very sensitive to *var* genes that could have

been expressed in very low levels. Therefore I sought to study *var* gene transcription in these parasites by sequencing the semi-conserved region of the *var* gene called the DBL-1 tag.

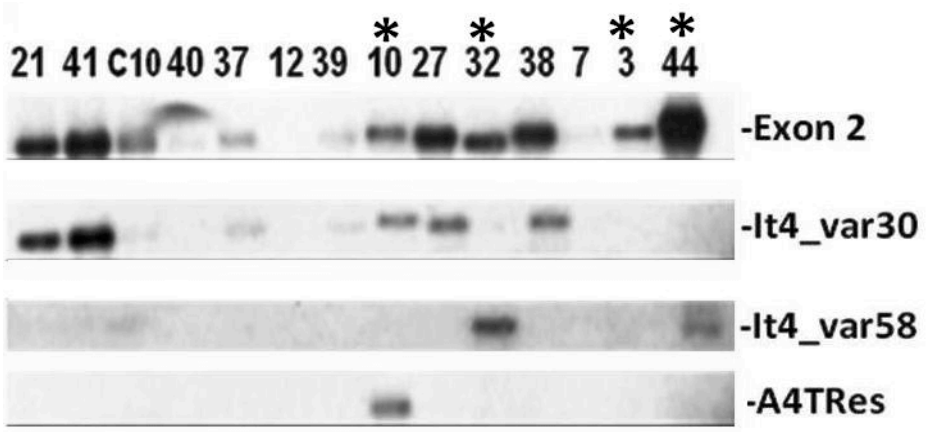


Figure 4.7. Northern blot analysis of IT/C10 clones

Ring-stage RNA was transferred to a membrane and probed with *var* gene specific probes. The filter was stripped after each probe and re-probed. Total ring-stage RNA from IT/C10 sub-clones was run on a gel, transferred to a Hybond membrane and probed for Exon-2 that is expressed across all var genes. The *var*-specific probes used to probe the blot were amplified by PCR from genomic DNA (* Denotes non-clumpers)

b) DBL-1 tag transcription in IT/C10 clone

The transcription of *var* genes can also be studied using generic primers that amplify a semi-conserved region of the gene then in a semi-quantitative manner determine the identity of the present transcripts by comparing the proportions in which they are expressed. The semi-conserved *var* gene domain DBL-1 was amplified from cDNA with generic primers AFBR (Bull, Berriman *et al*. 2005), the PCR products were cloned and 20 to 50 colonies were picked for sequencing. Frome sequencing I was able to determine the frequency of finding each unique sequence that represents the abundance of expression for that *var* gene variant.

Firstly, to determine if the primers had primer bias in the genetic background of the IT/C10 parasites, I compared the DBL-1 tags amplified from either genomic DNA or cDNA from clone 21 using the AFBR2009 primers (Bull, Berriman *et al*. 2005). Clone 21 was selected because of it binding and clumping profile. PCR products were cloned and amplified in TOPO cloning system then sequenced. The primers amplified a total of 48 unique DBL-1 tag sequences from the genomic DNA whereas 23 unique sequences were amplified from cDNA, three of which represented half the total DBL sequences present. The primer set was then used to analyse *var* gene transcription in the entire set of clones derived from the IT/C10 parent line whose clumping and binding phenotypes were described above.

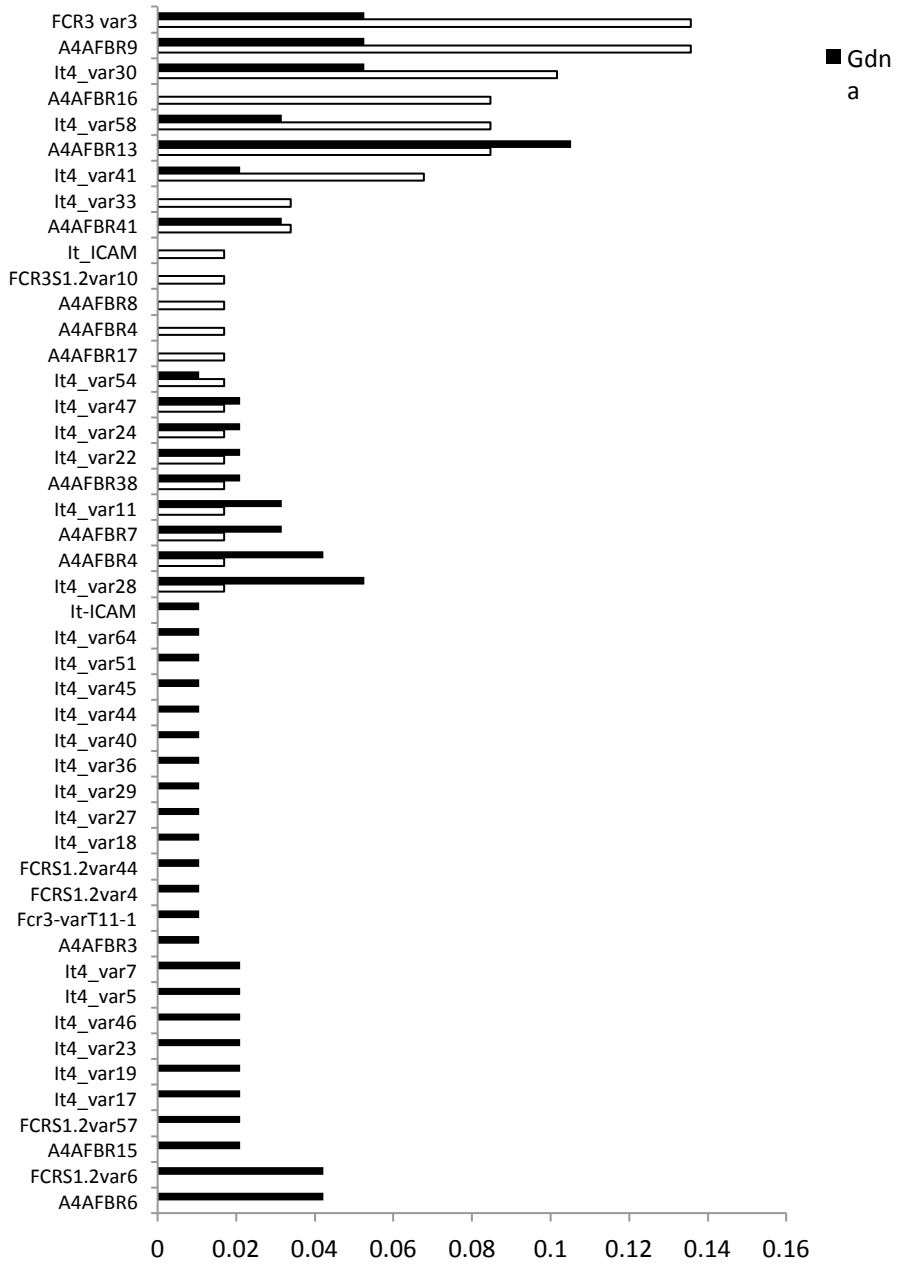


Figure 4.8 Primer bias n the AFBR primer set

DBL tags were amplified from cDNA or gDNA from clone 21 from the IT/C10 clone panel. There was an over representation of some clones in the cDNA. A total of 48 different sequences were amplified from gDNA.

The sequences that were obtained from the amplification and sequencing of the DBL region were translated using the online translating tool http://www.ebi.ac.uk/Tools/st/emboss_transeq/. The resulting sequences were searched through archived sequences of the *Plasmodium* genome project at http://blast.ncbi.nlm.nih.gov/ to identify homologous sequences from parasite line IT4. The majority of the clumping clones expressed IT4*var*30 as the main variant present. Clumping IT/C10 clones 37, 12, 39, 40, 7 and 2 expressed higher levels of IT*var*30 compared to non-clumping clones. Clumping frequency was positively correlated with expression of IT4*var*30 r=0.64, P=0.05 (Figure 4.9A). Only four clones were positive for IT4*var*58. Expression of IT4*var*58 appeared had a positive correlation with clumping frequency but was not statistically significant (r=0.14, p=0.6) (Figure 4.9B). IT*var*34, present in both clumpers and non-clumpers, was not associated with the clumping phenotype (r=0.06, p=0.80) (Figure 4.9C). A4AFBR13 was expressed by both clumpers and non-clumpers but appeared to have a negative correlation with clumping frequency, which was not statistically significant (r=0.29, p=0.24) (Figure 4.9D). IT-ICAM and FCR-*var*2 were minor variants in in the IT/C10 clones that were not significantly associated with clumping frequency (Figure 4.9E and 4.9F).

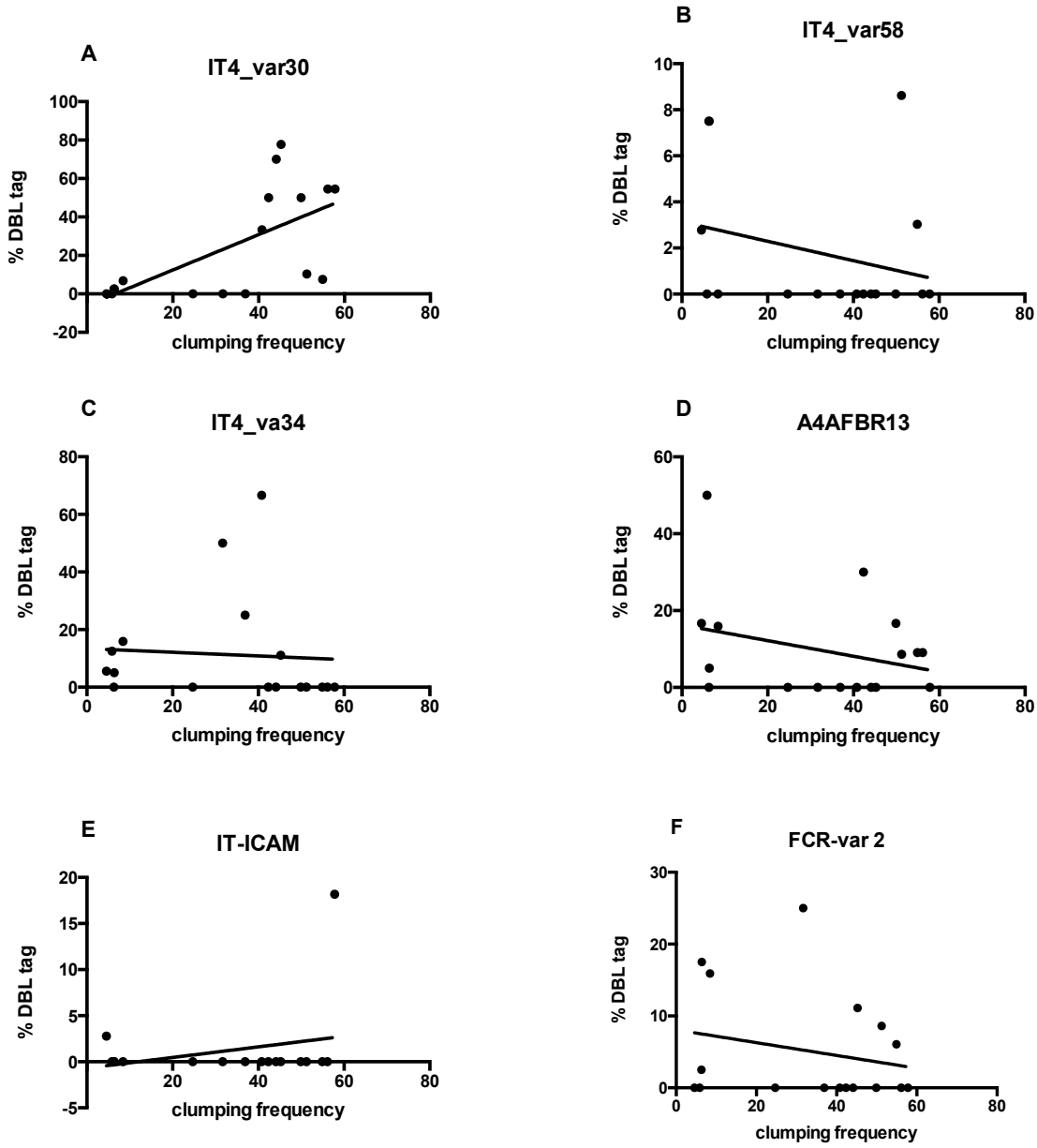


Figure 4.9 Common DBL-1 tags expressed by IT/C10

DBL tags expressed by IT/C10 clones (A) IT*var*30 is common among clumping clones (B) IT*var*58 is only expressed by non-clumpers (C) IT*var*34 is expressed in both clumpers and non-clumpers (D) A4AFBR13 common among non-clumpers (E) IT-ICAM is only expressed by non-clumpers (F) FCR3_*var*2 is expressed among non-clumpers.

4.3.4 IT/C10 clones are positive for the KAHRP transcript

The adhesion phenotype of malaria-infected erythrocytes is partially determined by the presence of knobs on the IE surface as evidenced by variation in binding phenotypes of isogenic parasite clones with different knob expression profiles (Horrocks, Pinches *et al*. 2005). To determine the knob phenotype of the IT/C10 panel of clones, ring-stage RNA from the clones was reverse-transcribed to make cDNA. RNA from knobless parasite line T996 was included as a negative control. All tested parasite clones in the panel were positive for KAHRP transcript by reverse-transcriptase PCR (Figure 4.10).

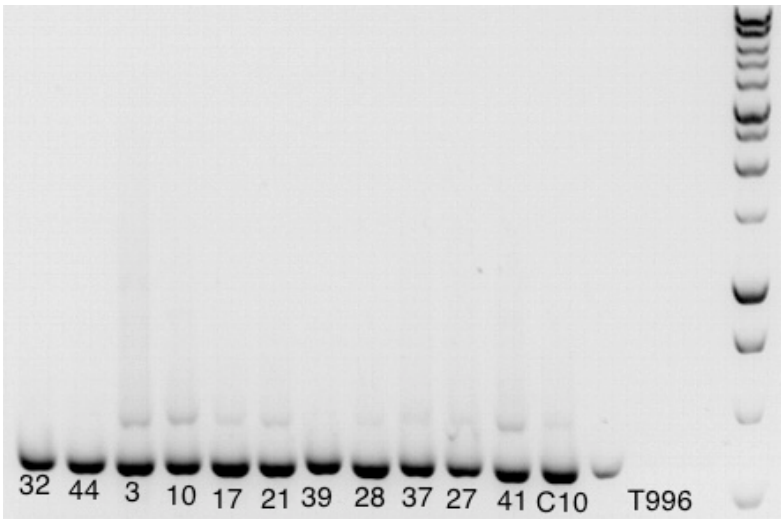


Figure 4.10 IT/C10 clones are positive for the KAHRP transcript

The RIII region of the *KAHRP* gene was amplified by PCR from cDNA of selected IT/C10 clones control and analysed in a 1% agarose gel. All IT/C10 clones are positive for the KAHRP transcript.

4.4 Discussion

Platelet-mediated clumping is an adhesion phenotype of a subset of *Plasmodium falciparum*-infected erythrocytes that has been associated with severe malaria in children and in adults (Pain, Ferguson *et al*. 2001, Chotivanich, Sritabal *et al*. 2004, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011). Here I have described the clumping phenotype in considerable detail for laboratory parasite line IT/C10. A clone tree of isogenic parasites from the parasite line IT/C10 was developed, screened for clumping phenotypes in the presence of platelets, characterized for binding phenotypes to immobilized receptors and finally *var* gene transcription was determined by sequencing DBL tags. The results have shown that the platelet-mediated clumping phenotype is associated with a ligand whose characteristics closely resemble those of PfEMP-1. Further analysis showed that IT4_*var*30 is positively correlated with clumping frequency in IT/C10 parasites.

In order to identify the parasite ligand associated with platelet-mediated clumping, a panel of isogenic parasite clones was developed by limiting dilution from parasite line IT/C10. The clones were screened for the platelet-mediated clumping phenotype in the presence of platelets and it was found that 6/18 of the clones exhibited low clumping frequencies (<10%) as compared to the remaining 12 clones and the IT/C10 parent clone (>10%). The 12 clumping clones exhibited a distinct clumping phenotype in the presence of PRP in which IEs agglutinated to form clumps. In various field isolates and laboratory-adapted parasite lines, it has been shown that platelets use CD36 and P-Selectin to interact with an unidentified parasite ligand to bring about clumping (Pain, Ferguson *et al*. 2001, Wassmer, Taylor *et al*. 2008). PECAM-1 is a platelet receptor implicated in platelet-platelet

interactions and a known receptor for parasite ligands on the IE surface (Treutiger, Heddini *et al*. 1997). It was hypothesized that PECAM-1 on platelets binds to the parasite ligand for clumping. In binding assays to determine the relative importance of these receptors in adhesion I found binding to CD36 by all IT/C10 clones. None of the non-clumpers showed binding to PECAM-1 while four clumpers bound to P-Selectin. Interestingly there was a positive correlation between P-Selectin binding and CD36 binding. I did not find any appreciable binding to PECAM-1 and gC1qR in the IT/C10 clone tree. Neither were there any significant correlations between the clumping phenotype and individual binding phenotypes.

IT/A4 parasites are used in adhesion studies maintain the ability to adhere to receptors after numerous passages in culture. To determine the relationship between platelet-mediated clumping and adhesion to purified receptorsI did not find any significant correlation between among the IT/C10 clones. However all IT/C10 clones bound to CD36 as expected. There was wide variation in ability to bind to P-Selectin among the IT/C10 clones and virtually no binding to PECAM-1 and gC1qR by the IT/C10 clones as compared to controls of FCR selected parasites. It is possible that these receptors are involved in clumping but do not bind to the IEs on their own.

Malaria-infected IEs have protrusions on the surface that are sites of expression of PfEMP-1 known as knobs. The truncation of *kahrp* gene leads to development of knobless parasites. To determine if the differences of clumping phenotype among IT/C10 clones could be explained by presence or absence of knobs, I tested for the presence of the

KAHRP gene transcripts in selected IT/C10 clones. All IT/C10 clones as expected were positive for the KAHRP transcript.

Chapter 5

Host factors that influence platelet-mediated clumping

5.1 Introduction

Platelet-mediated clumping refers to the agglutination of mature-stage *P. falciparum*-infected erythrocytes (IEs) in close contact with platelets (Pain, Ferguson *et al*. 2001), a phenomenon reported to be a characteristic of both laboratory-adapted and field isolates *P. falciparum* parasites (Pain, Urban *et al*. 2001, Chotivanich, Sritabal *et al*. 2004, Arman, Raza *et al*. 2007, Biswas, Hafiz *et al*. 2007, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011). Platelet surface receptor CD36 is required for development of platelet-mediated clumping phenotype in most laboratory strains and field isolates (Pain, Ferguson *et al*. 2001). P-Selectin and gC1qR/HABP1 expressed on the platelet membrane have also been implicated as receptors mediating platelet-mediated clumping in some parasites (Biswas, Hafiz *et al*. 2007, Wassmer, Taylor *et al*. 2008). The platelet surface has a plethora of other receptors that could potentially interact with parasite-derived ligands on the IE surface leading to the development of platelet-mediated clumping. To describe further the interactions involved in clumping, I investigated the relative importance of three platelet receptors in the development of the platelet-mediated clumping phenomenon that interact with ligands expressed on laboratory parasite lines. I used monoclonal antibodies to test

the effect on platelet mediated clumping of selected parasite line by delineating platelet receptors CD36, P-Selectin and PECAM-1.

P-Selectin is implicated in the involvement of platelet-mediated clumping (Wassmer, Taylor *et al*. 2008). To further dissect the mechanisms by which P-Selectin contributes to platelet-mediated clumping, I conducted clumping assays with IT/C10 parasites in the presence of reagents targeting platelet P-Selectin and measured their effect on the clumping frequency. P-Selectin is a well-established marker for platelet activation. Here, I used the putative physiological ligand for P-Selectin known as P-Selectin Glycoprotein Ligand-1, the tetrasaccharide sialyl Lewis-a with sialic acid residues that interact with P-Selectin and finally fucoidin – a fucose sugar that intercts with the lectin ligand on P-Selectin (Ho, Schollaardt *et al*. 1998, Senczuk, Reeder *et al*. 2001).

Platelets circulate in a resting state and are activated by agonists in order to perform their haemostatic functions. Platelets are activated in the presence of *P. falciparum*-infected erythrocytes *in vitro* through a CD36-dependent mechanism (Polack, Peyron *et al*. 1990, Srivastava, Cockburn *et al*. 2008). Platelet activation is measured by using PAC-1 antibody that recognizes an epitope on the glycoprotein IIb/IIIa complex expressed on activated platelets near the platelet fibrinogen receptor. Platelet activation can also be determined by measuring upregulation of P-Selectin expression on the platelet surface. Indeed, early electron micrographs of platelet-mediated clumps of IEs clearly show platelets extending activation pseudopodia around the surface of knobbed IEs (Pain, Ferguson *et al*. 2001). To further understand the role of platelet activation in clumping, clumping assays were conducted in the presence of specific inhibitors of platelet activation

including salicyclic acid, prostacyclin, colchicine and CD39 (For review see (Michelson 2002)).

Sialic acid residues expressed on the erythrocyte surface interacted with platelet P-Selectin on platelet monolayers and on the surface of endothelial cells (Ho, Schollaardt *et al*. 1998). Furthermore, P-Selectin mediates adhesion of sickled erythrocytes on activated endothelium (Matsui, Borsig *et al*. 2001). I hypothesized that erythrocyte sialic acid residues may interact with platelet receptors in the development of the clumping phenotype. To test this hypothesis, IEs were treated with neuraminidase, an enzyme that cleaves off sialyl residues, before they were tested for the clumping phenotype.

Finally, I tested the importance of sulphatides in the development of the clumping phenotype. Sulphatides, glycosphingolipids expressed on both platelets and erythrocytes, are implicated in adhesion of erythocytes and in aggregation of platelets (Merten and Thiagarajan 2001).

This chapter therefore, attempts to explore some of the host factors, that may influence the development of the platelet-mediated clumping phenotype specifically platelet receptors, platelet activation, erythrocyte sialic acid and erythrocyte sulphatides.

5.2 Specific methods

5.2.1 Inhibition of clumping with anti-platelet monoclonal antibodies

Blood (10ml) was collected from a malaria naïve donor into sodium citrate anticoagulated tubes. Platelet Rich Plasma (PRP) was prepared by centrifugation as described in Chapter 2 (Section 2.2.1). Platelet concentration in PRP was adjusted to 150-200 x 10^9 platelets per L by diluting in PBS. Aliquots of the resulting platelet suspension were incubated in the presence of monoclonal antibodies directed against CD36 (Clone SMθ, Serotec), P-Selectin (Clone AK-6, Serotec) or PECAM-1 (Clones 9G11, R & D Systems) at 37^{O}C for 1 hour. Slides were prepared in triplicate and examined under a fluorescent microscope. Clumping was scored as the proportion of IEs in agglutinates of three or more IES out of 1000 IEs and compared with PRP.

5.2.2 Inhibition of clumping with P-Selectin antagonists

To further investigate the role of platelet P-Selectin in development of the platelet-mediated clumping phenotype, P-Selectin interactions were delineated with antagonists of P-Selectin. Clumping assays were conducted under standard conditions described in chapter 3. Here, in the first experiment PRP was incubated with the physiological, putative receptor for P-Selectin known as P-Selectin Glycoprotein Ligand-1 (PSGL-1) (R&D Systems) or PBS at 37^{O}C for 1 hour. In the second experiment, PRP was treated with different concentrations of tetrasaccharide sialyl Lewis-a (Sle-a) at 37^{O}C for 1 hour before clumping assays were conducted. In the third experiment, to determine if IEs interact with the lectin domain of P-Selectin on platelets, the effect of fucoidin, a homopolymer of fucose, was determined. PRP was incubated with fucoidin (Sigma) at 37^{o}C for 1 hour

before clumping assays were conducted. Clumping assays were conducted under standard conditions described in chapter 3.

5.2.3 Co-incubation of IEs and PRP

To investigate the changes that occur on the surface of the platelet membrane with regards to platelet activation when brought to close contact with *P. falciparum* parasites, washed trophozoite-stage IEs or uninfected erythrocytes were incubated for 30 minutes with platelet-rich plasma (PRP). FITC-PAC-1 antibody (BD PharMingen) or FITC-P-Selectin antibody was then added for 20 min prior to fixation with 1% formalin. The expression of these platelet receptors was determined by flow cytometry and compared with resting platelets.

5.2.4 Inhibition platelet-mediated clumping with platelet activation inhibitors

To determine the importance of platelet activation in the development of the platelet-mediated clumping, experiments were conducted in the presence of platelet-activation inhibitors. PRP was treated with salicyclic acid (Sigma), Colchicine (Sigma), CD39 (R&D Systems) or prostaglandin (Sigma) and incubated for 30 minutes at 37^{O}C. In all experiments control PRP was incubated with PBS for 30 minutes at 37^{O}C before clumping assays were conducted as described before. Clumping was scored as the proportion of IEs in agglutinates of three or more IEs out of 1000 IEs and compared with the control PRP.

5.2.5 Neuraminidase treatment of IEs

To determine the role erythrocyte sialic acid may play in the development of the clumping phenotype, IEs were treated with neuraminidase before the clumping assay. To further determine if the neuraminidase effect was specific to one stage of parasite development, parasite line IT/C10 ring-stage IEs were treated with neuraminidase and re-cultured to the mid-trophozoite stage whereas an aliquot from the same culture was treated with neuraminidase at the trophozoite stage. To treat IEs with neuraminidase, IEs were incubated in a neuraminidase-supplemented buffer (or wash buffer for control) (Calbiochem). The IEs were washed in incomplete culture medium then stained with ethidium bromide and tested for the clumping phenotype in PRP as described before.

5.2.6 Inhibition of clumping with anti-sulphatide and anti-sialyl antibodies

To determine if targets for anti-sulphatide residues on the erythrocyte surface contribute to the development of the platelet-mediated clumping phenotype, IT/C10 parasites were incubated in the presence of monoclonal antibodies against sulphatides, sialyl Lewi-a (Sle-a), sialyl Lewi-x (Sle-x) or sulphatide oligoendrocyte marker (O4) at 37ºC for 1 hour. The effect of blocking the receptors was determined by comparing the clumping frequencies with control PRP under similar experimental conditions.

5.3 Results

5.3.1 Platelet receptors for clumping

I sought to determine the importance of three platelet receptors CD36, P-Selectin and PECAM-1 in the development of the platelet-mediated clumping phenotype in IT/C10 parasite line and two selected parasite lines on PECAM-1 and P-Selectin respectively. The PECAM-1 selected parasite line was a gift from Prof Artur Scherf. PRP was incubated in the presence of monoclonal antibodies or reagents to delineate specific platelet receptors. Clumping assays were conducted comparing clumping frequency between the antibody-treated PRP and controls to show the effect of these monoclonal antibodies on the platelet-mediated clumping phenotype.

a) IT/C10 parent line with anti-CD36, anti-P-Selectin and anti-PECAM-1 monoclonal antibodies

The IT/C10 parasite line has been used in several previous studies for platelet-mediated clumping (Pain, Ferguson *et al*. 2001, Wassmer, Taylor *et al*. 2008). Antibodies directed to CD36 (Clone SMθ) inhibited clumping in this parasite by 88.09% (p=0.0001), anti-P-Selectin antibody (Clone AK-6) inhibited clumping by 33.7% (p=0.001) and antibodies directed against PECAM-1 inhibited clumping by only 7.6% (p=0.2) (Figure 5.1).

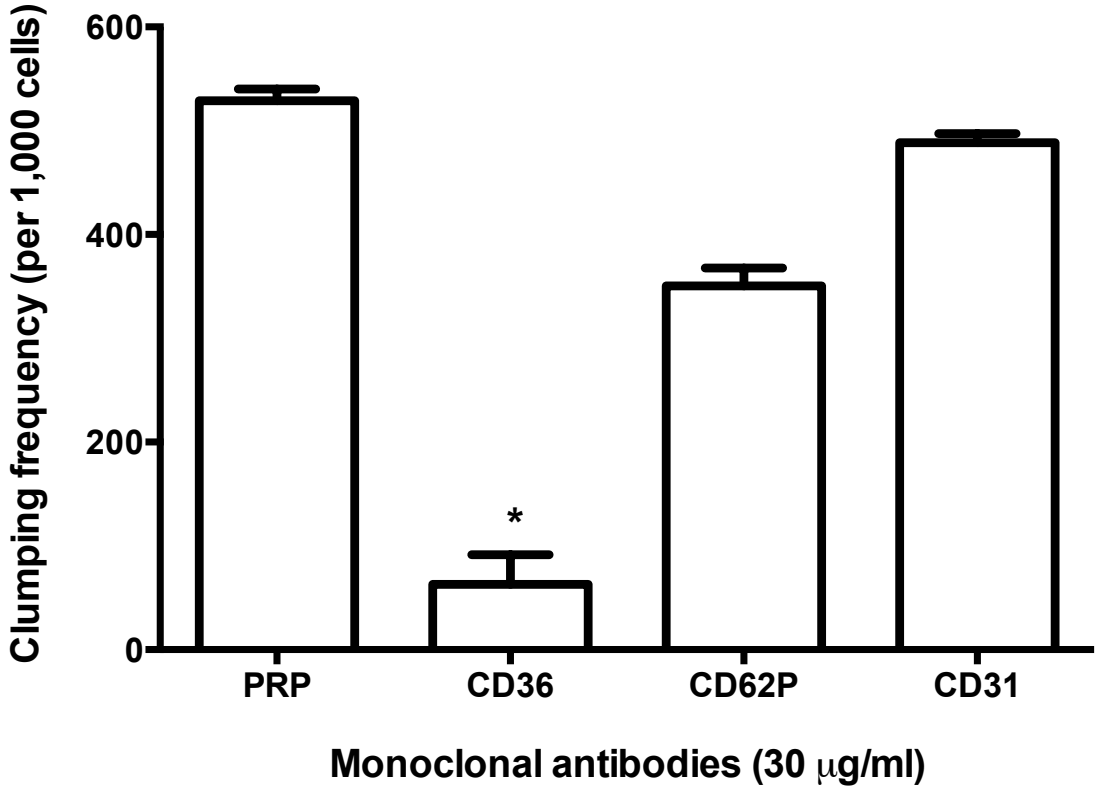


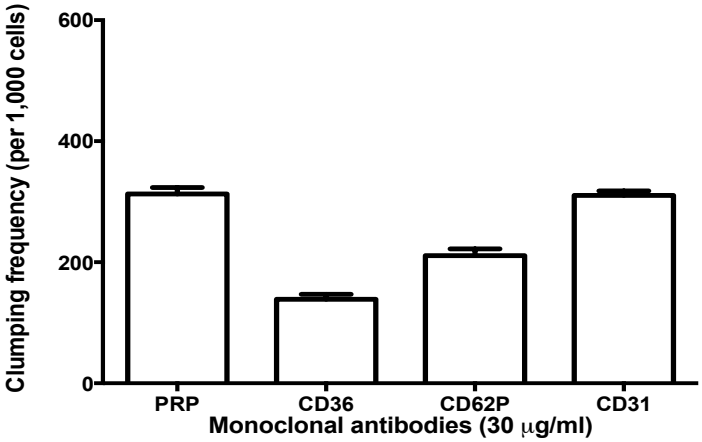
Figure 5.1 Clumping of IT/C10 in presence of anti-platelet monoclonal antibodies

PRP was pre-incubated with monoclonal antibodies (anti-CD36, anti-P-Selectin or anti-PECAM-1) to block corresponding platelet receptors before clumping assays were conducted and compared control PRP. Three experiments were conducted in triplicate. Error bars represent standard deviations of three experiments.

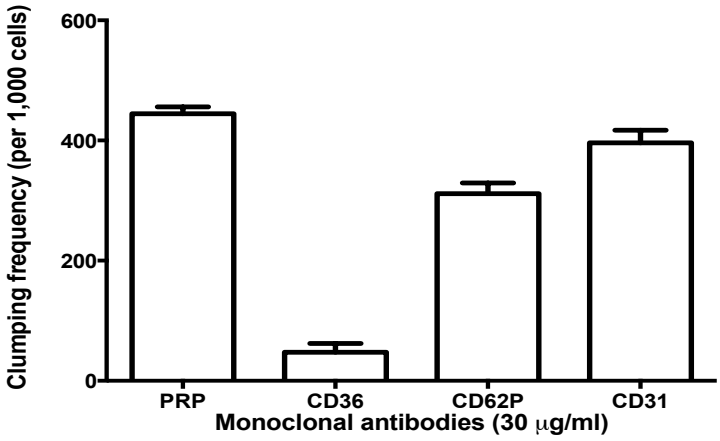
b) IT/C10 sub-clones with anti-CD36, anti-P-Selectin and anti-PECAM-1 monoclonal antibodies

Parasite clones derived from the IT/C10 parent clone by limiting dilution were also tested for their ability to clump in the presence of the monoclonal antibodies directed against CD36, P-Selectin and PECAM-1 respectively. Consistent with previous results, antibodies for CD36 (Clone SMθ) inhibited clumping in IT/C10 clone 4 by 56.7%, in clone 21 by 88.0% and in clone 39 by 91.3% showing that all three IT/C10 clones depended on platelet CD36 as a main receptor for the development of clumps (Figure 5.2 A-C). The monoclonal antibody directed against platelet P-Selectin (clone AK-6), inhibited clumping in clone 4 by 34.1%, clone 21 by 33.7% and clone 39 by 31.2%, all of which attained statistical significance ($p<0.05$). Finally, an antibody directed to platelet PECAM-1 (clone 9G11) inhibited clumping in clone 4 by 4.3%, in clone 21 by 7.6% and in clone 39 1.7%. None of the inhibitor effects of anti-PECAM-1 antibody reached statistical significance when compared with clumping with control PRP without antibody ($p>0.05$).

A


Clumping frequency (per 1,000 cells)
600
400
200
0
PRP
CD36
CD62P
CD31
Monoclonal antibodies (30 μg/ml)

B


Clumping frequency (per 1,000 cells)
600
400
200
0
PRP
CD36
CD62P
CD31
Monoclonal antibodies (30 μg/ml)

C

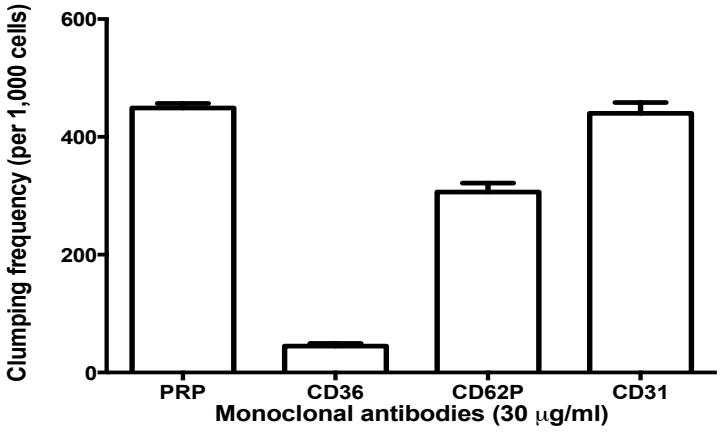

Clumping frequency (per 1,000 cells)
600
400
200
0
PRP
CD36
CD62P
CD31
Monoclonal antibodies (30 μg/ml)

Figure 5.2 Clumping of IT/C10 clones in presence of anti-platelet mAbs

PRP was pre-incubated with monoclonal antibodies (anti-CD36, anti-P-Selectin or anti-PECAM-1) to block corresponding platelet receptors before clumping assays were conducted and compared control PRP. A) In clone 4, anti-CD36 ($p=0.0002$) and anti-P-Selectin ($p=0.002$) antibodies inhibited platelet-mediated clumping significantly while anti-PECAM-1 antibodies did not ($p=0.08$) B) In clone 21, anti-CD36 ($p=0.0001$) and anti-P-Selectin ($p=0.001$) antibodies inhibited platelet-mediated clumping significantly while anti-PECAM-1 antibodies did not ($p=0.08$) C) In clone 39, anti-CD36 ($p=0.0001$) and anti-P-Selectin ($p=0.001$) antibodies inhibited platelet-mediated clumping significantly while anti-PECAM-1 antibodies did not ($p=0.67$).

c) Selected parasite lines (A4PSel and FCR-PECAM)

To further investigate the relative importance of the known platelet receptors for clumping namely CD36, P-Selectin and PECAM-1. I used parasites that were specifically selected to bind to recombinant purified P-Selectin and PECAM-1 namely A4PSEL and FCR-PECAM respectively. In the A4PSEL parasite line, platelet-mediated clumping was inhibited significantly by both the anti-CD36 monoclonal antibody (40.1%) and the anti-P-Selectin monoclonal antibody (49.3%) both of which attained statistical significance ($p=0.002$) and ($p=0.001$) respectively. On the other hand the anti-PECAM-1 antibody failed to inhibit clumping in this parasite line at all. In the FCR-PECAM parasite line, both the anti-CD36 monoclonal antibody and the anti-PECAM-1 monoclonal antibody significantly inhibited platelet-mediated clumping by 53.4% and 56.7% respectively. The anti-P-Selectin antibody failed to inhibit clumping of the FCR-PECAM parasite line.

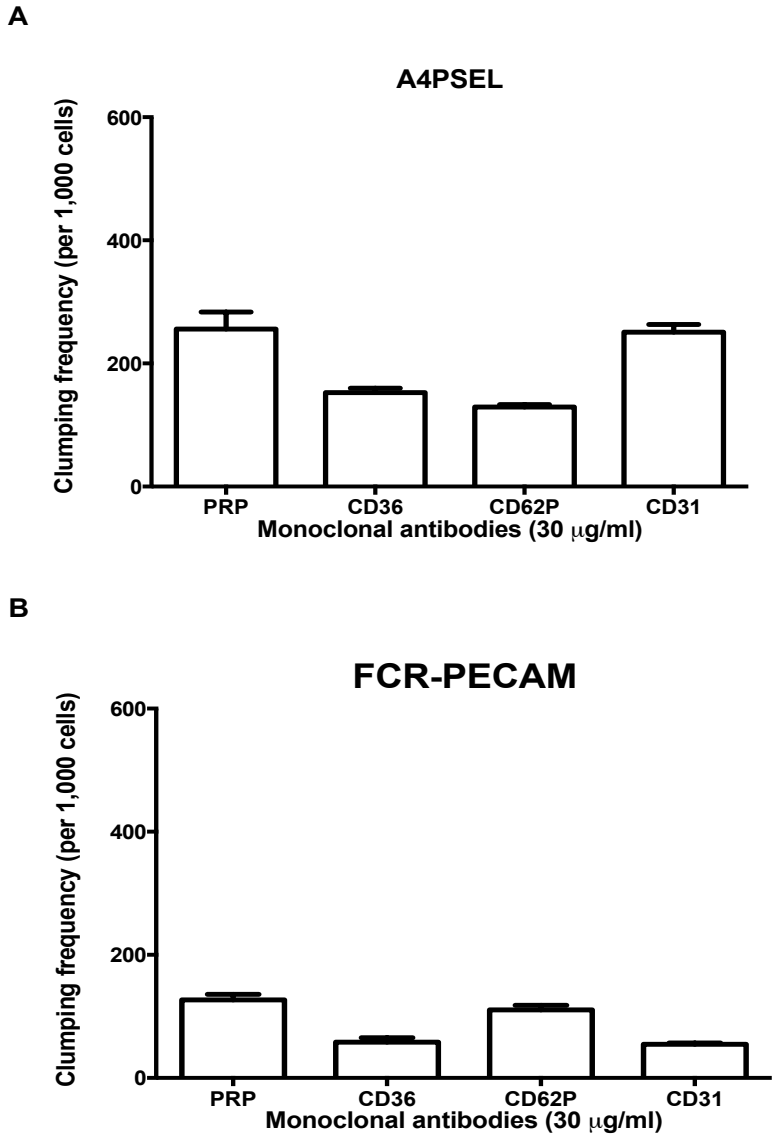


Figure 5.3 Clumping of A4PSEL and FCR-PECAM in presence of anti-platelet mAbs

PRP was pre-incubated with monoclonal antibodies (anti-CD36, anti-P-Selectin or anti-PECAM-1) before clumping assays were conducted and compared control PRP. A) In A4PSEL, anti-CD36 (p=0.002) and anti-P-Selectin (p=0.01) antibodies inhibited platelet-mediated clumping significantly while anti-PECAM-1 antibodies did not (p=0.87) B) In FCR-PECAM, anti-CD36 (p=0.004) and anti-PECAM-1 (p=0.001) antibodies inhibited platelet-mediated clumping significantly while anti-P-Selectin antibodies did not (p=0.24).

5.3.2 P-Selectin antagonists Sialyl Lewis, PSGL-1 and fucoidin

In some parasites P-Selectin appears to be important in mediating platelet-mediated clumping (Wassmer, Taylor *et al*. 2008). To further dissect the interactions that P-Selectin exploits in development of clumps, I used a P-Selectin-binding parasite to conduct experiments in the presence of antagonists known to interfere with P-Selectin interactions including PSGL-1, sialyl lewis-a and fucoidin.

a) PSGL-1 and sialyl lewis

To determine the interaction involved in P-Selectin clumping in the IT/C10 parasite line, PRP was pre-treated with Sialyl Lewis-a before clumping assays were conducted compared to control PRP. Sialyl lewis-a, a tetrasacccharide with sialic acid residues inhibited clumping significantly at 10μg/ml and 20μg/ml ($p<0.05$) (Figure 5.4A). In the presence of PSGL-1, the putative physiological receptor for P-Selectin in leucocyte rolling, the clumping phenotype was also inhibited significantly ($p=0.006$) (Figure 5.4B).

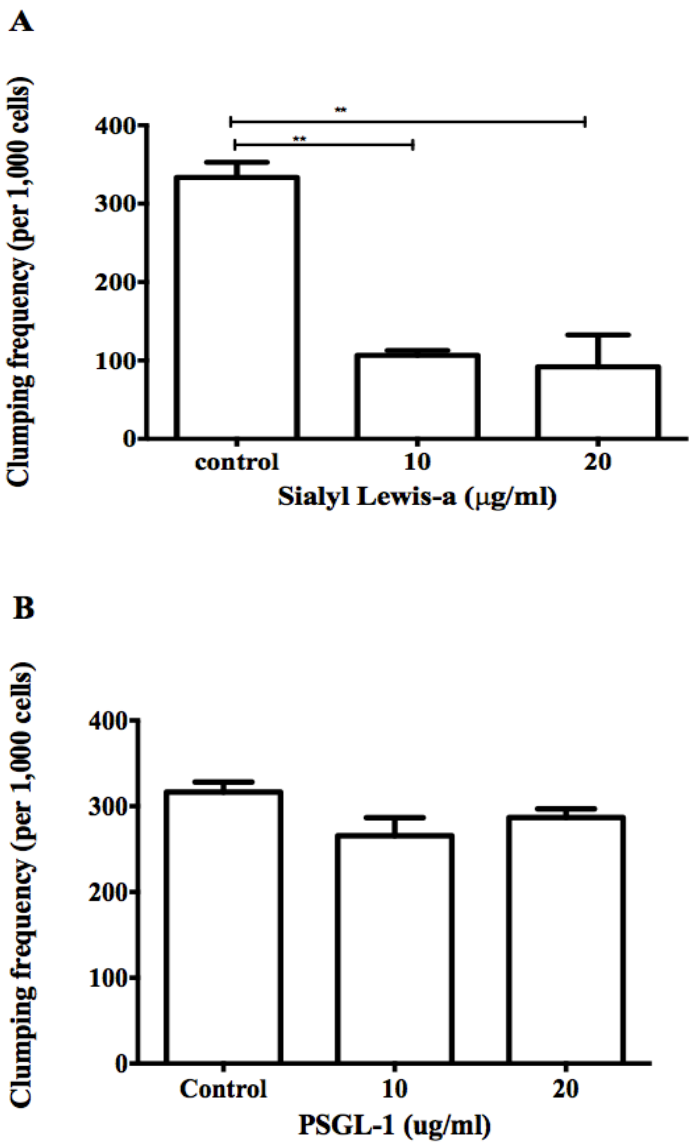


Figure 5.4 P-Selectin in clumping

PRP was pre-incubated with P-Selectin antagonists Sialyl Lewis A or PSGL-1 for 1 hour before clumping assays were conducted and compared to control PRP (A) Platelet-mediated clumping of the IT/C10 was significantly inhibited at 10 μg/ml (p=0.0004) and 20 μg/ml (p=0.005) (B) Platelet-mediated clumping was significantly inhibited by incubation with PSGL-1 10 μg/ml (p=0.006) and 20 μg/ml (p=0.08).

b) fucoidin

Fucoidin is a sulphated glycoconjugate that interacts with the lectin domain of P-Selectin. Clumping assays wer eperformed in the presence of fucoidin (Sigma), which is known to compete with PfEMP-1 to bind P-Selectin. In the A4PSEL parasite line, a P-Selectin-binding parasite line, the platelet-mediated clumping assay was inhibited by fucoidin at 100μg/ml (p=0.0001) and at 200 μg/ml (p=0.0001) (Figure 5.5).

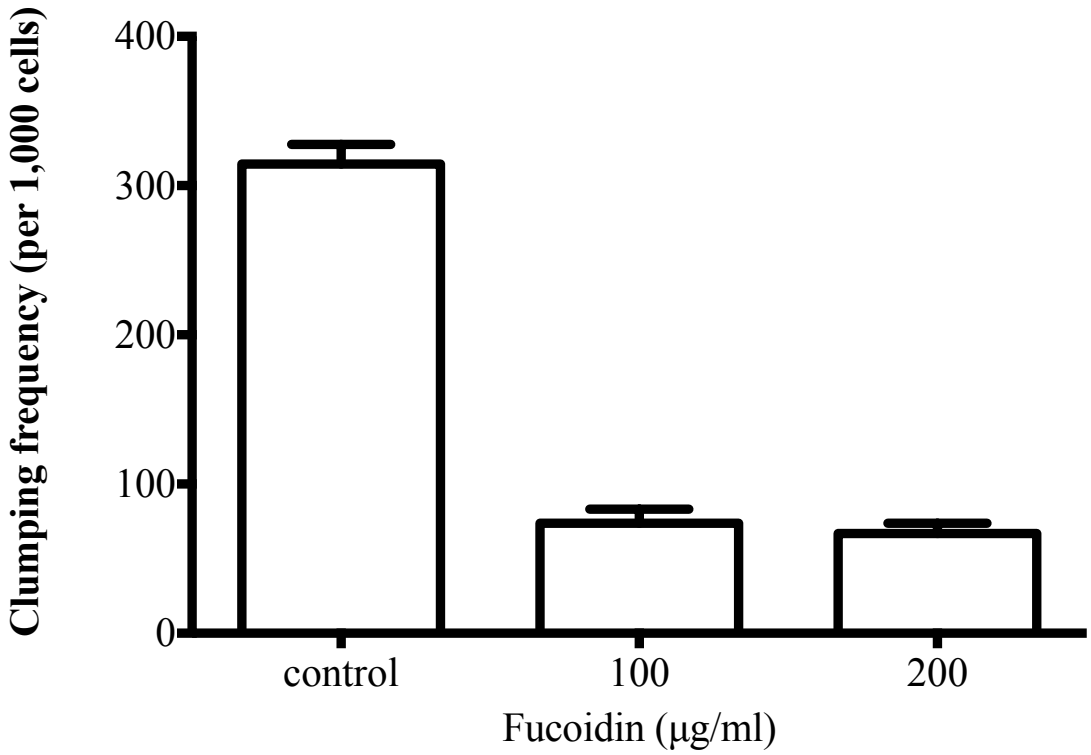


Figure 5.5 Fucoidin inhibits clumping

Platelet-mediated clumping assays for IT/C10 parasites were conducted in the presence of fucoidin and compared with control PRP. The platelet-mediated clumping phenotype was significantly inhibited by fucoidin at 100µg/ml (p=0.0001) and 200µg /ml (p=0.0001).

5.3.3 IT/C10 parasites activate platelets

To determine if IT/C10 parasites activated platelets during clumping, I co-incubated resting platelets with IT/C10 IEs or uninfected erythrocytes. The mixture was stained for markers of platelet activation (anti-P-Selectin or anti-PAC-1), before the suspension was then analysed by flow cytometry. The proportion of platelets positive for platelet activation markers P-Selectin ($p=0.002$) and PAC-1 ($p=0.002$) increased significantly after co-incubation with IT/C10 IEs as compared to platelets co-incubated with uninfected erythrocytes. Platelets were activated by IT/C10 clumping parasites but not by uninfected erythrocytes. Platelets that were co-incubated with uninfected erythrocytes expressed similar levels of PAC-1 and P-Selectin as control platelets suggesting the importance of platelet activation in the development of the platelet-mediated clumping phenotype.

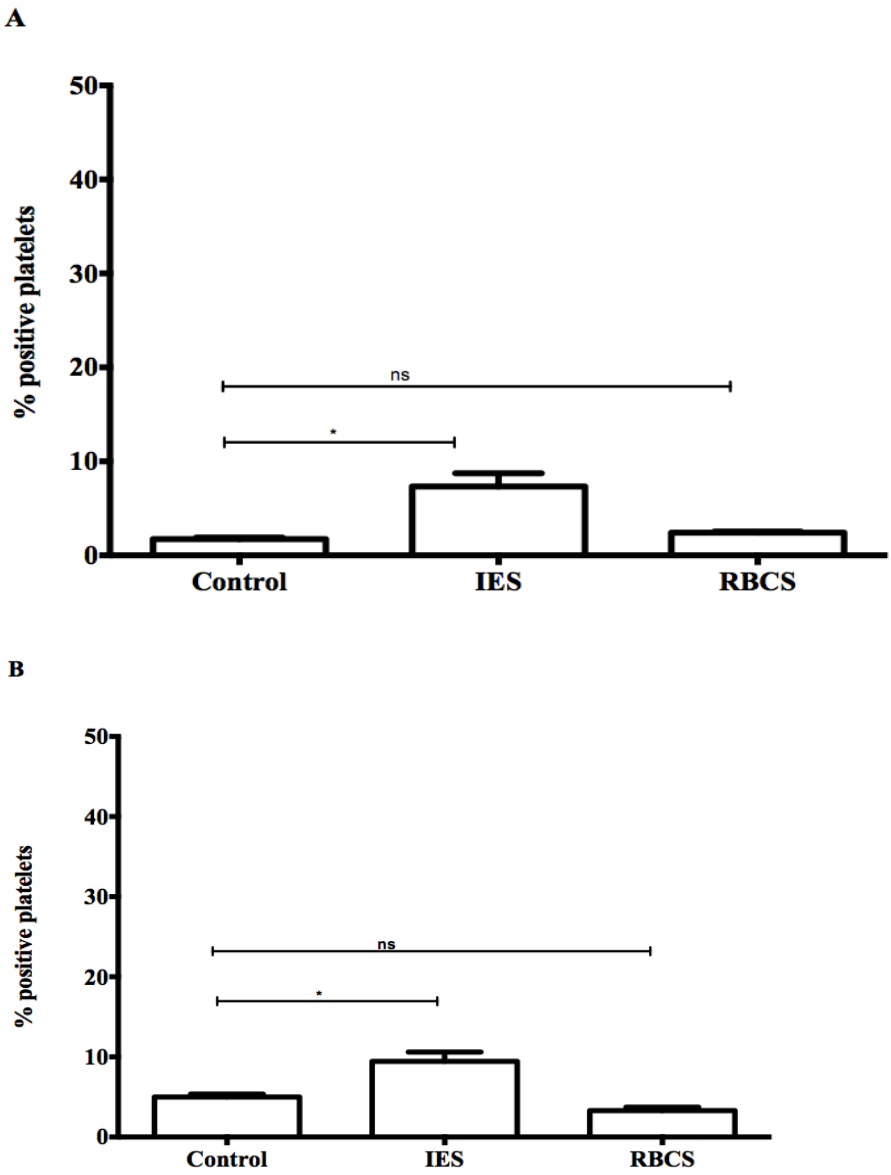


Figure 5.6 Platelet are activated by IT/C10

Platelets were co-incubated with IT/C10 IEs or uninfected erythrocytes for 30 minutes. The mixture of IEs and platelets was stained with FITC-conjugated anti-PAC-1 or anti-P-Selectin. Platelet suspensions were then analysed by flow cytometry to determine the proportion of positive platelets for the two markers. A) A greater proportion of platelets were positive for PAC-1 after incubation with IEs compared to when platelets were

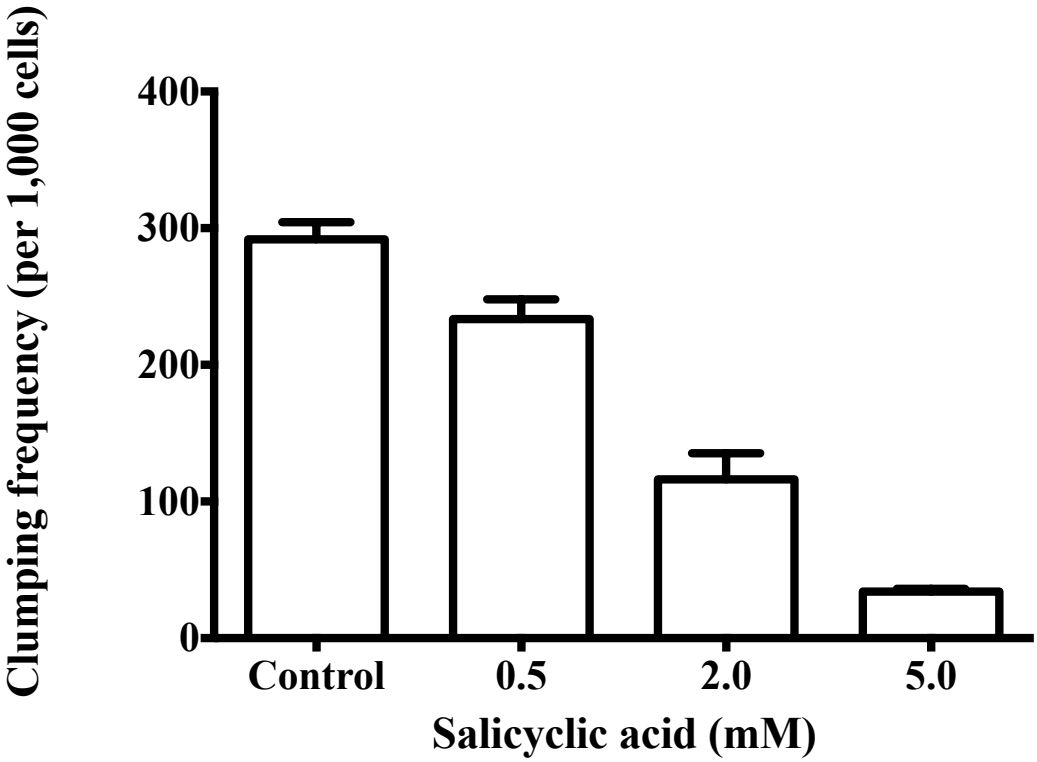
incubated with uninfected erythrocytes (p=0.002) (B). The proportion of platelets that were positive of P-Selectin after incubation with IEs was increased (p=0.002) compared to when the platelets were incubated with uninfected erythrocytes.

5.3.4 Platelet-activation inhibitors inhibit platelet-mediated clumping

Having shown that platelet-activation appears to be involved in development of platelet-mediated clumps, I sought to determine the effect of four inhibitors of platelet activation on the clumping phenotype. PRP was treated with the reagents; salicyclic acid (0.5-5.0mM), prostacyclin (50-200μM), colchicine (0.62-5.0mM), or CD39 (2.5-10μg/mL) before the pre-treated platelets were used to screen for the clumping phenotype in parasites of the IT/C10. Each of these reagents targets and inhibits a specific component of the platelet activation cascade.

a) Salicyclate

The salicyclate group is the active component of aspirin that inhibits platelet activation by inhibitng cyclooxygenase I. To determine if inhibition of platelet activation through cyclooxygenase I could inhibit the platelet-mediated clumping phenotype, PRP was treated with salicyclic acid dissolved in sodium hydroxide before clumping assays were performed and compared with clumping in control PRP. I found that salicyclic acid inhibited clumping in to a statistically significant degree ($p<0.05$) (Figure 5.7) As expected treatment of platelets with salicyclic acid inhibited platelet-mediated clumping significantly at all the concentrations tested ($p<0.05$).



Platelet-mediated clumping assays were conducted with PRP pre-treated with salicyclic acid at different concentrations. Platelet-mediated clumping was significantly inhibited by salicyclic acid at 0.5mM ($p=0.03$), 2 mM ($p=0.001$) and 5mM ($p=0.0001$).

b) Prostacyclin

Prostacyclin is a prostaglandin that inhibits platelet activation by activating adenylyl cyclase. To determine if inhbition of platelet activation through adenylyl cyclase would affect the clumping phenotype, clumping assays were conducted with PRP that was treated with prostacyclin. Prostacyclin treatment lead to a siginificant inhibition of clumping as compared to control PRP. Clumping was inhibited at the tested concentrations (20-200 μM) and this inhibition was statistically significant ($p<0.005$), showing that platelet activation is important for development of the clumping phenotype.

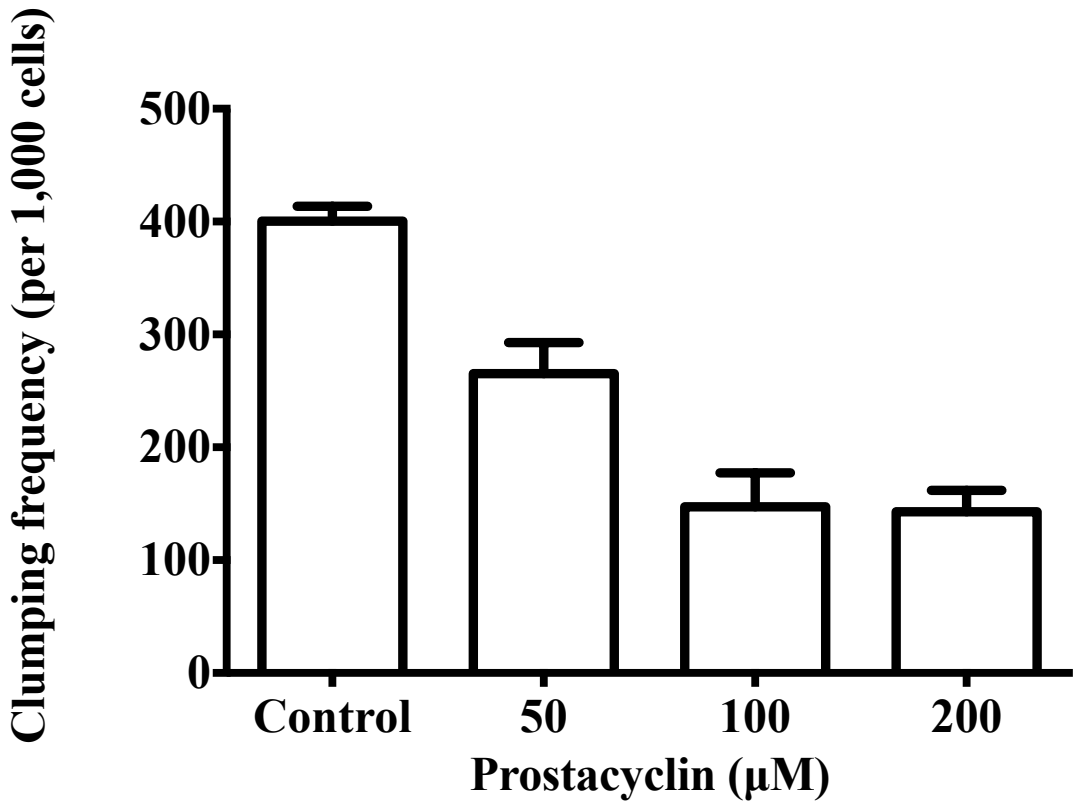


Figure 5.8 Effect of prostacyclin on platelet-mediated clumping in IT/C10

Platelet-mediated clumping assays were conducted with PRP pre-treated with prostacyclin at different concentrations. Platelet-mediated clumping was significantly inhibited by prostacyclin at 50μM ($p=0.01$), 100μM ($p=0.001$) and 200μM ($p=0.0004$).

c) Colchicine

Platelet activation is often accompanied by changes in the cytoskeletal components in the plasma membrane (Cerecedo, Stock *et al*. 2002). To determine if the platelet surface changes that occur during platelet activation are required in the development of the clumping phenotype, particularly in the plasma membrane microtubule network, PRP was treated with colchicine, a reagent that inhibits microtubule activity, before clumping assays were conducted and clumping compared with control PRP. Colchicine treatment of PRP at 5mM led to inhibition of the clumping phenotype in parasite line IT/C10.

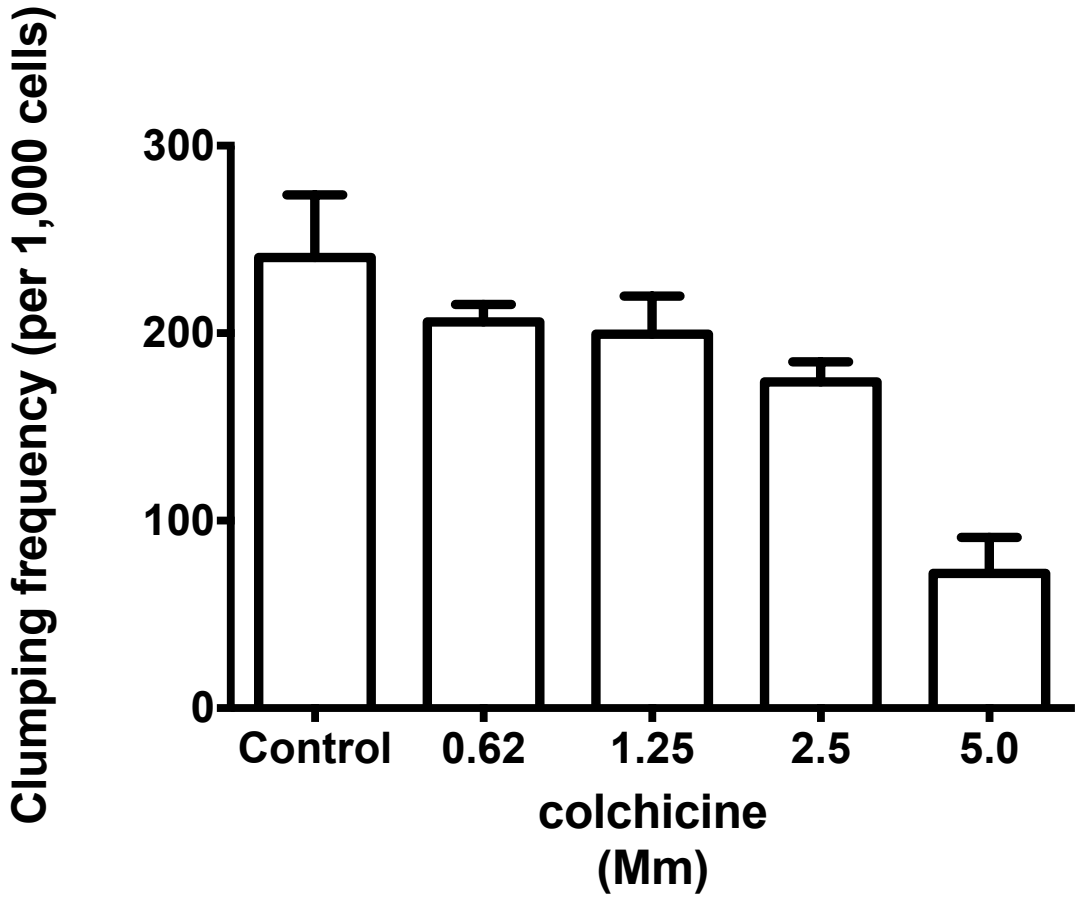


Figure 5.9 Effect of colchicine on platelet-mediated clumping in IT/C10

Platelet-mediated clumping assays were conducted with PRP pre-treated with colchicine at different concentrations. Platelet-mediated clumping was significantly inhibited by colchicine at 5mM ($p=0.01$).

d) CD39

CD39 also known as NTPDase hydrolyses ATP dinucleotides and may therefore inhibit platelet activation. I sought to determine if pre-treatment of PRP with CD39 would lead to a noticeable effect on the clumping phenotype of IT/C10 parasites. PRP was treated with CD39 before clumping assays were conducted and compared to control PRP. Platelet-mediated clumping was inhibited in IT/C10 clumping by treatment of PRP with CD39 and this inhibition was statistically significant ($p<0.05$) at the concentrations that were tested (Figure 5.10).

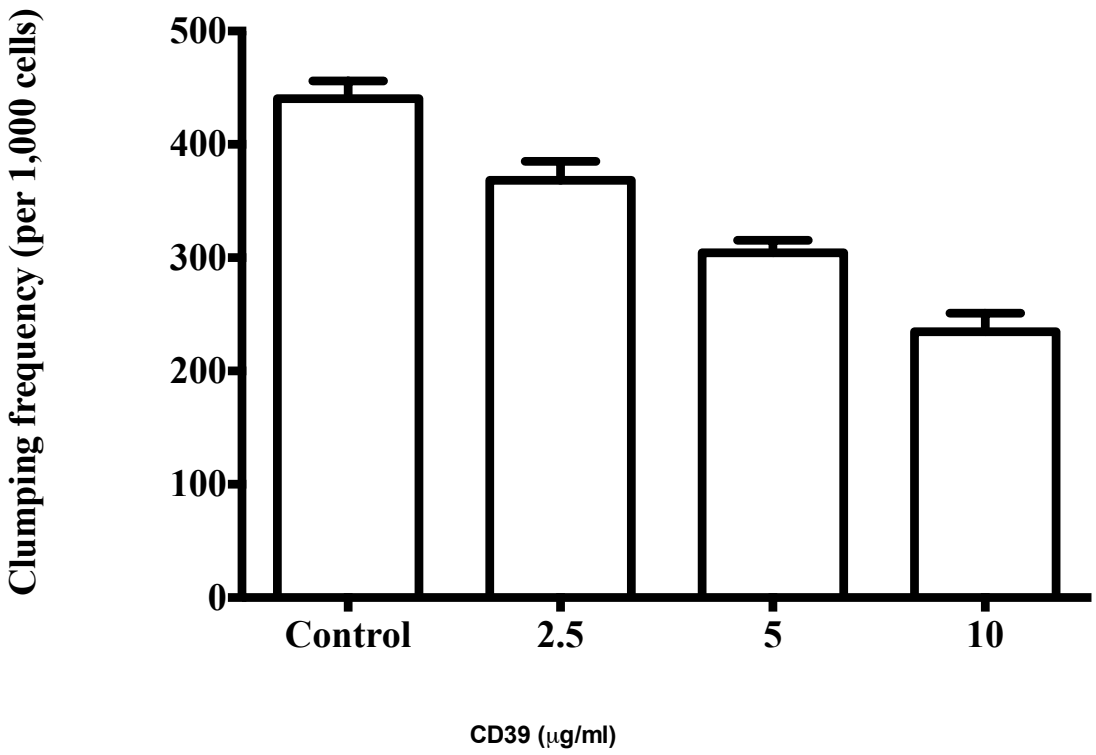


Figure 5.10 Effect of CD39 on platelet-mediated clumping in IT/C10

Platelet-mediated clumping assays were conducted with PRP pre-treated with recombinant CD39 at different concentrations. Platelet-mediated clumping was significantly inhibited by CD39 at 2.5μg/ml (p=0.03), 5μg/ml (p=0.002) and 10μg/ml (p=0.0008).

5.3.5 Erythrocyte sialic acid residues mediate platelet-mediated clumping

Since it is known that P-Selectin may contribute to development of the clumping phenotype, I sought to determine if sialylated residues on IEs is involved in the interaction with platelet P-Selectin and to determine the location and importance of such sialylated residues. An aliquot of an IT/C10 parasite culture at the ring stage was treated with neuraminidase and returned to culture and grown to the trophozoite stage. A second aliquot of the culture was harvested IEs at the trophozoite stage and treated with neuraminidase at 37ºC for one hour. The two neuraminidase-treated aliquots (ring-treated and trophozoite-treated) were tested for the effect of neuraminidase treatment on the clumping phenotype and compared with untreated controls. I found that neuraminidase-treated IEs exhibited lower clumping frequency than control IEs (Figure 5.11). This inhibition was maintained irrespective of the parasite stage at which the IEs were neuraminidase-treated; clumping was inhibited in ring treated IEs significantly ($p=0.007$) and in the trophozoite-treated IEs ($p=0.008$).

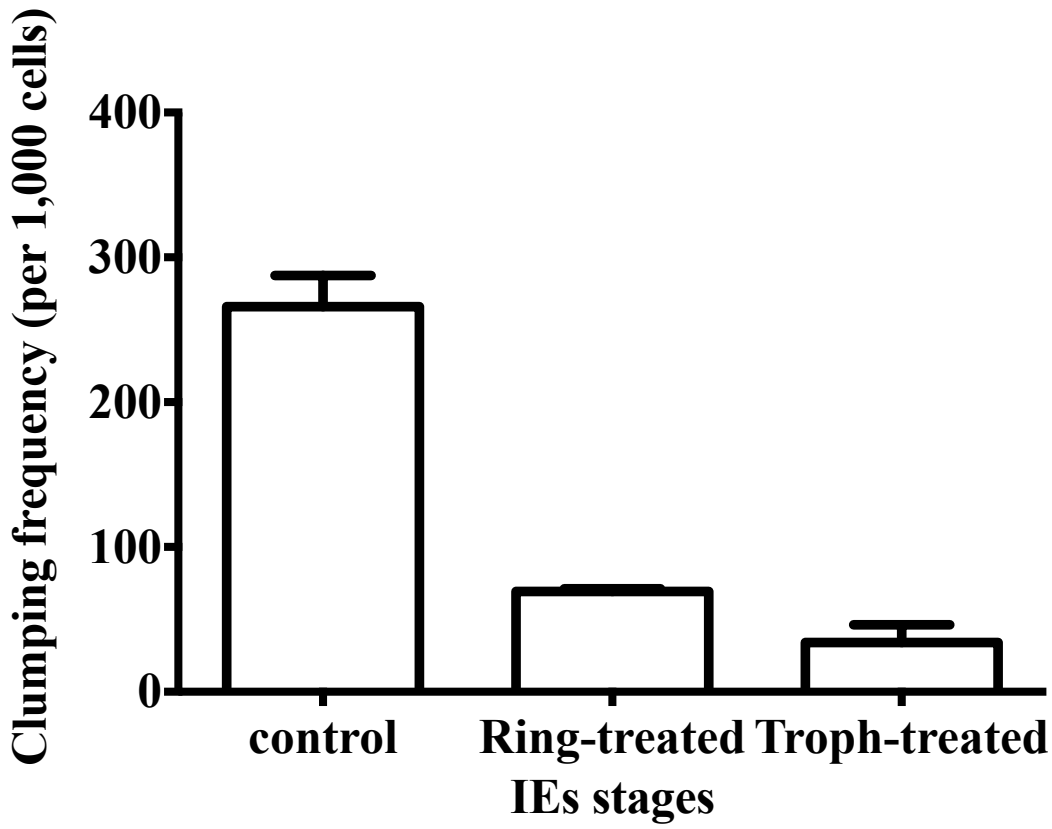


Figure 5.11 Clumping depends on erythrocyte sialylated residues

Clumping assays were conducted with neuraminidase-treated IEs (treated at ring or trophozoite stage) and compared to control IEs. The clumping phenotype was inhibited by neuraminidase treatment regardless of the stage at which the IEs were treated. Neuraminidase treatment of IEs inhibited the platelet-mediated clumping phenotype significantly both at the ring ($p=0.008$) and trophozoite ($p=0.007$) stages.

5.3.6 Anti-sulphatide and anti-sialyl Lewis-x antibodies

To determine if sulphatide residues on the erythrocyte surface contribute to the development of the platelet-mediated clumping phenotype, the sulphatide residues were blocked with monoclonal antibodies by incubating IEs with monoclonal antibodies for Sle-x, Sle-a or O4 for 1 hour at 37ºC. Platelet-mediated clumping assays were then conducted and clumping frequencies were compared with control PRP. In my hands, the platelet-mediated clumping phenotype was maintained after blocking the sulphatide residues Sle-a and Sle-x whereas blocking of O4 led to a small decrease in clumping frequency in the IT/C10 parasite that was statistically significant compared to clumping in control PRP (ANOVA, $p<0.05$).

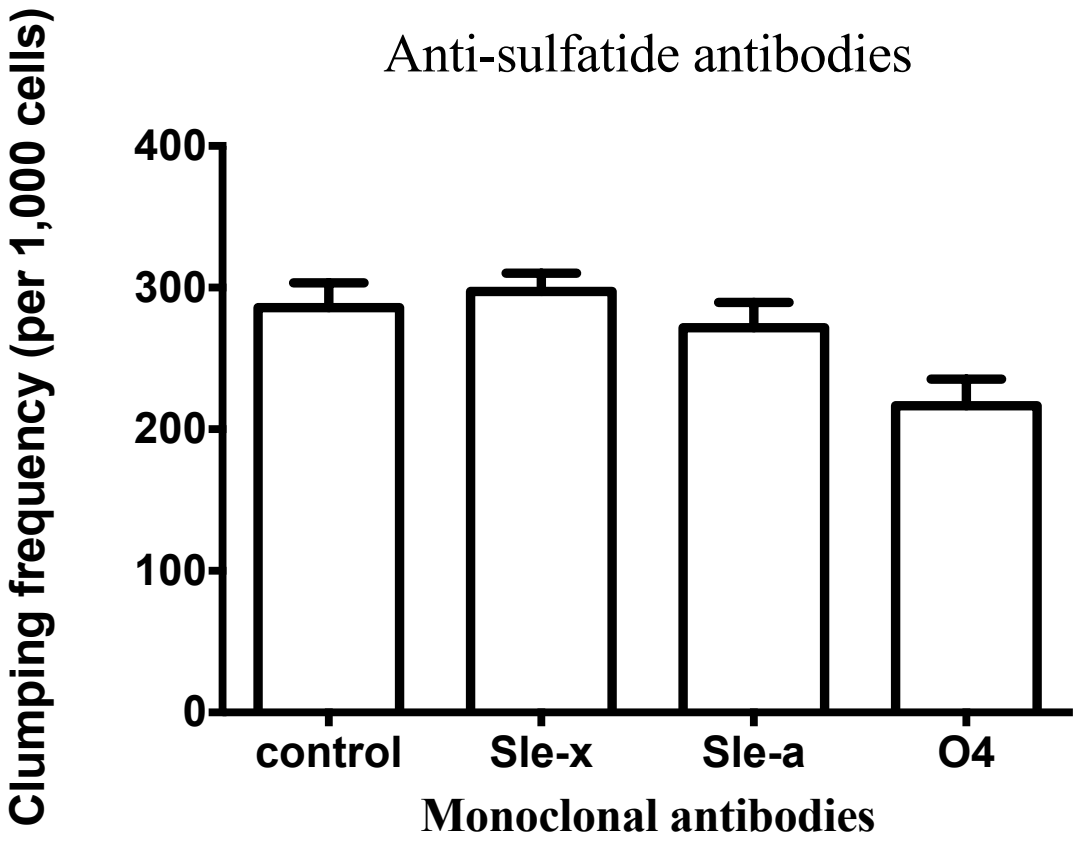


Figure 5.12 Anti-sulphatide antibodies

Platelet-mediated clumping assays for mature stage IT/C10 parasites were conducted in the presence monoclonal antibodies for sulphatide antigens on the erythrocyte surface and compared to control PRP. The monoclonal antibodies for Sle-a, Sle-x did not significantly inhibit the platelet-mediated clumping phenotype in IT/C10 parasites but anti-O4 monoclonal antibody significantly inhibited the clumping phenotype.

5.4 Discussion

Platelet-mediated clumping relies on specific platelet receptors and also on platelet activation. CD36 is suggested to be the most important host receptor in development of this phenotype so far described (Pain, Ferguson *et al*. 2001). Here, I have shown, consistent with previous studies that antibodies against platelet CD36 inhibit clumping significantly in IT/C10 clones and also in parasite lines selected for binding to PECAM-1 and P-Selectin (Figure 5.1 and Figure 5.2). I have also shown that platelet P-Selectin may contribute to clumping through a lectin domain and may interact with sialic acid residues on erythrocytes. I also have shown that platelet activation is an integral part of the platelet-mediated clumping phenotype.

Platelet-mediated clumping is one of the adhesion phenotypes of *P. falciparum*-infected erythrocyte. The platelet receptors reported for clumping are CD36 (Pain, Ferguson *et al*. 2001) and P-selectin (Wassmer, Taylor *et al*. 2008) and gC1qR (Biswas, Hafiz *et al*. 2007). In this study, my results agree with those showing that antibodies that block platelet surface CD36 inhibit clumping but entirely in the CD36 binding parasite lines. Anti-CD36 antibodies also significantly inhibit clumping in parasite lines developed by selecting on purified P-Selectin and PECAM-1 (Figure 5.3). In our hands a gC1qR-binding parasite line failed to clump. Although the PECAM-1 binding parasite showed very low levels of clumping, I were able to demonstrate that this clumping dependent on CD36 and PECAM-1. To our knowledge, this is the first time that a PECAM-1 parasite has been shown to exhibit the clumping phenotype.

P-Selectin expressed on endothelial cells is a receptor for IE rolling (Ho, Schollaardt *et al*. 1998, Senczuk, Reeder *et al*. 2001). Here, I showed that in platelet-mediated clumping the parasite ligand(s) uses an epitope that competes with fucoidin and sialyl lewis-a but not the epitope for PSGL-1 (Figure 5.4). Fucoidin, a sulphated tetrasaccharide, may interfere with lectin-like interactions whereas sialyl lewis has sialyl residues that interact with the lectin epitopes on P-Selectin. In this study I have shown the mechanisms by which P-Selectin may contribute to the development of the clumping ligand.

P-Selectin is a marker for platelet activation when it is upregulated on the surface following release from intracellular vesicles. Here I have also shown the role of platelet activation in platelet-mediated clumping by using four different inhibitors of platelet activation. Some of which may be explored as an anti-clumping drug once the mechanisms for clumping are fully described. Interestingly, excessive and chronic use of aspirin in malaria-endemic areas has been associated with salicyclate poisoning and metabolic acidosis, hence aspirin may not be recommended in malaria (English, Marsh *et al*. 1996).

Neuraminidase enzyme cleaves off the sialic acid residues on the IE surface leading to inhibition of the clumping phenotype. Interestingly no sialidases are known in *Plasmodium* genome (Gardner, Hall *et al*. 2002). Neuraminidase treatment of IEs inhibited rolling on purified P-selectin and activated platelet monolayers (Ho, Schollaardt *et al*. 1998). The known ligand for P-Selectin, PSGL-1 failed to reduce clumping suggesting different epitopes used in binding compared to binding and rolling. Treating platelets with

Sialyl Lewis-a (Sle-a), a tetrasaccharide that is has sialic acid residues that bind to the lectin domains of P-selectin also inhibited platelet–mediated clumping.

This chapter has reviewed how host factors contribute to the development of the clumping factor. Consistent with previous work, I showed that clumping is dependent on the platelet receptors CD36, P-Selectin. PECAM-1 appears to be a receptor for clumping in some parasite isolates. Platelet activation is an important part in the development of clumping. Cleavage of erythrocyte surface sialic acid residues also does inhibit the clumping phenotype.

Chapter 6

Study of the platelet-mediated clumping phenotype in clinical isolates

6.1 Introduction

The importance of platelets in the pathogenesis of malaria was discussed in Chapter 1 (Section 1.4). The ways in which platelets have been reported to be involved in pathogenesis of malaria include; decreased peripheral platelet counts, accumulation of platelets in various tissues or organs, inhibition of parasite growth and the platelet-mediated clumping of IEs (Reviewed in (Cox and McConkey 2010)). Platelet-mediated clumping refers to agglutination of *P. falciparum*-infected erythrocytes when in contact with platelets, a phenomenon that has been associated with severe malaria-causing parasites in field studies of both children from endemic areas and adults in areas with unstable malaria transmission (Pain, Ferguson *et al*. 2001, Chotivanich, Sritabal *et al*. 2004, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011). Only one study so far has not demonstrated an association between platelet-mediated clumping and severe disease but instead reported an association with parasitaemia (Arman, Raza *et al*. 2007).

The molecular interactions involved in platelet-mediated clumping are only partially understood. Platelet surface receptors CD36 (Pain, Ferguson *et al*. 2001), P-Selectin (Wassmer, Taylor *et al*. 2008) and gC1qR (Biswas, Hafiz *et al*. 2007) have been shown to mediate the platelet-mediated clumping phenotype. In the published literature thus far, the erythrocyte surface ligand that mediates clumping had not been identified. Virtually all

parasite isolates bind to stationary recombinant CD36 but intriguingly not all CD36-binding isolates exhibit the clumping phenotype in the presence of platelets (Newbold, Warn *et al*. 1997, Pain, Ferguson *et al*. 2001, Mayor, Hafiz *et al*. 2011). Partial blocking of known platelet receptors using monoclonal antibodies does not entirely abrogate the platelet-mediated clumping phenotype suggesting involvement of cryptic receptors or yet to be identified mechanisms. The erythrocyte membrane is modified by the intracellular parasite making it more rigid, adhesive and less deformable resulting from exported parasite-derived proteins that are inserted into the erythrocyte membrane (Reviewed in (Moxon, Grau *et al*. 2011)). It is plausible that the exported proteins and modified erythrocyte cytoskeletal proteins may influence the clumping and adhesive phenotype of the IEs. I have shown in chapter four that within the parasite line IT/C10 the *var* transcript for IT4var30 was over-represented among clumping parasite clones compared to non-clumping parasite clones.

In this chapter I studied the platelet-mediated clumping phenotypes of parasite isolates, obtained from children suffering from well-defined clinical malaria in Kilifi, Kenya. I determined the importance of two platelet receptors in platelet-mediated clumping, CD36 and P-Selectin, by inhibiting them using monoclonal antibodies and comparing clumping between antibody-treated platelets with untreated platelets as controls. Unlike previous clinical studies on platelet-mediated clumping, in which parasite isolates were frozen at the ring stage, the parasite isolates were grown to the trophozoite-stage before glycerolyte cryopreservation. I took advantage of parasite isolates frozen for a different bigger study in Kilifi within which this study is nested. This enabled me to analyse all the samples within a short period. It is known that *P.falciparum*-infected erythrocytes maintain their

antigenic phenotypes after glycerolyte-cryopreservation (Kinyanjui, Howard *et al*. 2004). My experiments comparing the ability of trophozoite-frozen parasites to clump with fresh trophozoites showed that clumping was not significantly altered by the freeze-thawing process (Chapter 3). Finally, the relationships between the clumping phenotype of the parasite isolate to their respective *var* gene expression profile were analysed.

Specific aims

a) To investigate the clumping phenotype of Kilifi isolates

b) To investigate the platelet-receptor used for clumping by Kilifi isolates

c) To conduct correlations between clumping phenotypes and *var* gene sub-types expressed by the Kilifi isolates

6.2 Methods

6.2.1 Clinical study

Children presenting with malaria at the Kilifi District Hospital in coastal Kenya were recruited into this study with consent from the parents or guardian. Ethical approval for the study was approved by National Ethical Review Committee of Kenya Medical Research Institute (SSC Protocol Number 1131). Parasitaemia was determined by microscopy by qualified technologists at Kilifi District Hospital. The children had a parasitaemia ≥ 1 parasite per 500 uninfected erythrocytes or 100 parasites per 100 leukocytes. The children were classified into two groups according to the clinical features they presented namely, severe and non-severe malaria. Children with severe malaria required hospital admission and had any of three major overlapping clinical syndromes previously shown to be indicative of life-threatening disease namely impaired consciousness (Blantyre coma score <5), severe malarial anaemia (haemoglobin concentration <5g/dL) and severe respiratory distress (abnormally deep breathing) (Marsh, Forster *et al*. 1995) . The rest of the children were grouped as having non-severe malaria and were treated with anti-malaria therapy in the out-patient department.

6.2.2 Sample processing

Two mls of blood was collected from the study subjects into polypropylene, anticoagulated tubes. The blood was centrifuged at 1800rpm for 5 minutes to remove plasma from the cell pellet. The cell pellet made up of peripheral blood mononuclear cells (PBMCs) and erythrocytes was re-suspended in 5mls of incomplete culture medium. To remove PBMCs, the cell pellet was re-suspended in an equal volume of incomplete culture medium and then gently layered onto Lymphoprep™ (Axis-Shield PoC AS, Norway) at a

1:1 ratio. The tube was centrifuged at 1800rpm for 20 minutes and slowed with the centrifuge brakes turned off. PBMCs and granulocytes were harvested for use in other studies not described here. The remaining erythrocyte pellet was washed twice in incomplete culture medium at 1800rpm for 5 minutes. An aliquot of 100µl of the ring-stage infected erythrocytes was stored at -80°C in 1ml of Trizol® (Invitrogen, UK) for RNA extraction. The remaining pellet of ring-infected erythrocytes was suspended in complete culture medium and cultured at 37°C in the presence of 92% nitrogen, 3% oxygen and 5% carbon dioxide until they matured into pigmented trophozoites as assessed by examination of Giemsa-stained blood films. The cells were cryopreserved by suspending in 1ml aliquots in "glycerolyte" solution, transferred into cryovials in which they were stored. The tubes were stored at -80°C overnight and then transferred to the liquid phase of nitrogen (-196°C) until required for assays.

6.2.3 Thawing of cryopreserved clinical isolates

The parasites were thawed from liquid nitrogen on the day the experiment was performed. The vial containing the frozen parasites was transferred from liquid nitrogen tank and placed on ice to thaw. The parasites in glycerolyte were transferred to a 50ml centrifuge tube and shaken at approximately 10rpm at room temperature and a series of NaCl solutions of decreasing osmotic strength were added to the thawed pellet. Briefly, the thawed cells were shaken gently as 200µl of 12% NaCl solution was first added dropwise very slowly, followed by 10ml of 1.8% NaCl solution and a final 10ml of 0.9% NaCl solution. The suspension was centrifuged at 1800rpm for 5 minutes and the supernatant aspirated using a pipette by vacuum. The parasite pellet was washed twice in incomplete

culture medium at 1800rpm for 5 minutes each time. An aliquot (1µl) was taken to prepare a thin smear for parasitaemia estimation.

6.2.4 Parasitaemia estimation and dilution

Slides were prepared, stained and examined as described in Section 2.2.4. For each sample, parasitaemia was estimated by calculating the percentage of 500 intact erythrocytes containing pigmented trophozoites on random fields on the slide. Parasitaemia was also determined by flow cytometry. An aliquot of the thawed IEs was suspended in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and ethidium bromide (10µg/ml) and incubated for 30 minutes at room temperature. The cells were then washed in a 96-well round bottomed plate by suspending in 200µl of 0.5% BSA/PBS without ethidium bromide (by centrifuging at 1000rpm for 3 minutes. The IEs were then re-suspended in 0.5% BSA/PBS and analysed by flow cytometer (Beckman Coulter, UK)

6.2.5 Platelet-mediated clumping assays

To screen parasite isolates for the platelet-mediated clumping phenotype, clumping assays were conducted as described in Chapter 2. Here also platelets were prepared from a blood group AB donor and stored at 4ºC and used within a week. In brief, PRP was prepared from sodium citrate-anticoagulated blood from a healthy blood group AB donor. The thawed IEs were stained with ethidium bromide. The pellet was suspended in PRP or PPP at 10% haematocrit to a final platelet concentration of 100 to 200 x 10^9 per L to make the clumping suspension. Platelet-mediated clumping was scored by counting the proportion of the IEs in agglutinates of three or more out of a total of at least 1000 IEs.

6.2.6 Inhibition of platelet-mediated clumping with monoclonal antibodies

To determine the importance of major platelet receptors CD36 and P-Selectin, to an aliquot of PRP (18µl) was added a solution containing anti-CD36 (clone SMθ, Serotec) or anti-P-Selectin (clone AK6, Serotec) monoclonal antibody to a final concentration of 20µg/ml. A similar volume of PBS solution was added to control PRP. Reactions were incubated at 37ºC for 1 hour. Platelet-mediated clumping assays were conducted in parallel on the three different preparations described above. The level of inhibition by the respective monoclonal antibodies was determined by comparing clumping in the absence of antibodies.

6.3 Results

6.3.1 Patient characteristics

Platelet-mediated clumping assays were conducted for 71 samples collected from children with *P. falciparum* malaria at Kilifi District Hospital. Thirty-four children presented with severe malaria while 37 children were classified as having non-severe malaria. The difference in age between the two groups of children was not statistically significant (Mann-Whitney U test, p=0.81). The children in the severe disease group had significantly higher mean admission parasitaemia than those in non-severe malaria group (Mann-Whitney U test, p=0.002) which is in agreement with previous studies (Pain, Ferguson *et al*. 2001). The children in the severe malaria group were also significantly more anaemic than the children in the non-severe malaria group as shown by the mean haemoglobin (Mann-Whitney U test p=0.01). The children with severe malaria presented with severe malaria syndromes as follows; 26/34 (76.4%) children had impaired consciousness, 20/34 (58.8%) had cerebral malaria and 15/34 (41.1%) children had respiratory distress.

Table 6.1 Clinical laboratory characteristics of Kenyan children presenting with malaria at Kilifi District Hospital in Kenya

	Severe (N=34)	**Non-Severe (N=37)**	**P-value**
Age (months)	38.00 (4.1-86.1)	46 (2.5-167.7)	0.81
Parasitaemia (parasites / μl)	446,418.4 (15,768-1,175,460)	209,936.9 (83,22-927,080)	0.002
Haemoglobin (g/dL)	7.86 (4-11.9)	9.09 (4-14.50)	0.01

P-value for Mann-Whitney U test

Arithmetic mean $\pm$ standard deviation (minimum – maximum)

6.3.2 Clumping phenotypes of Kilifi field isolates

To determine the clumping phenotype of the parasite isolates from Kilifi and to show that the clumping phenotype depends on the presence of platelets, clumping assays were conducted concurrently in either PPP or PRP. For the majority of the isolates the clumping frequency was significantly increased in the presence of platelets (PRP) compared to (PPP), although for few isolates the increase was very small or no increase was recorded at all (Figure 6.1A). These results agree with previous published studies that showed that platelet-mediated clumping is a common phenotype in field isolates (Pain, Ferguson *et al*. 2001, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011).

To determine the importance of platelet-mediated clumping in the development of severe disease, clumping frequency of isolates from children in the two disease groups was compared using the Mann-Whitney U test. The results showed that isolates from children with severe disease had lower mean clumping frequency (mean 146.6$\pm$174.3) compared to isolates from children with mild malaria (mean 176.0$\pm$168.0). However the difference in mean clumping frequency between isolates from the two disease groups was not statistically significant (Mann-Whitney U test, p=0.56) (Figure 6.1B).

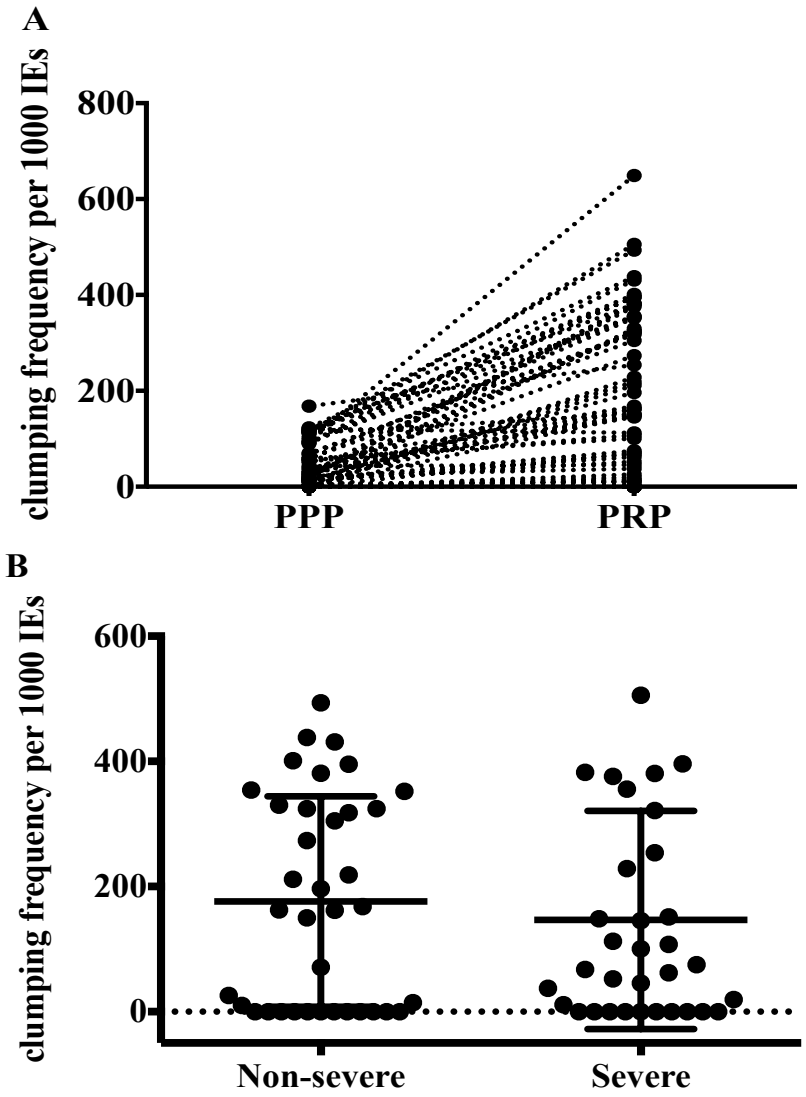


Figure 6.1 Platelet-mediated clumping phenotypes of Kilifi isolates

Parasite isolates from children were screened for platelet-mediated clumping phenotype. (A) The clumping phenotype was screened in PPP or PRP, clumping was significantly enhanced in PRP compared to PPP. (B) Clumping frequency in PRP was compared between isolates form children with severe disease and non-severe disease. Isolates from non-severe disease had a higher mean clumping frequency (176.0±168.0) than isolates from severe disease (146.6±174.3). The difference was not statistically significant (Mann-Whitney U test, p=0.56). The graph shows the mean ± standard deviations.

6.3.3 Platelet-mediated clumping is associated with assay parasitaemia

Previous published work showed that platelet-mediated clumping is independently associated with severe disease (Pain, Ferguson *et al*. 2001, Mayor, Hafiz *et al*. 2011). Here I investigated the correlation between assay parasitaemia and clumping frequency within our dataset by performing correlation tests between clumping frequency and assay parasitaemia. A statistically significant correlation between assay parasitaemia and clumping frequency in PRP was found (r=0.3, p=0.04) (Figure 6.2A). Next, I asked if this correlation held among the two disease groups (severe and non-severe malaria). Interestingly, the positive correlation between assay parasitaemia and clumping frequency was lost in the severe disease group (r=0.12, p=0.47) whereas the correlation was very strong in the non-severe disease group (r=0.56, p=0.003) (Figure 6.2B and C).

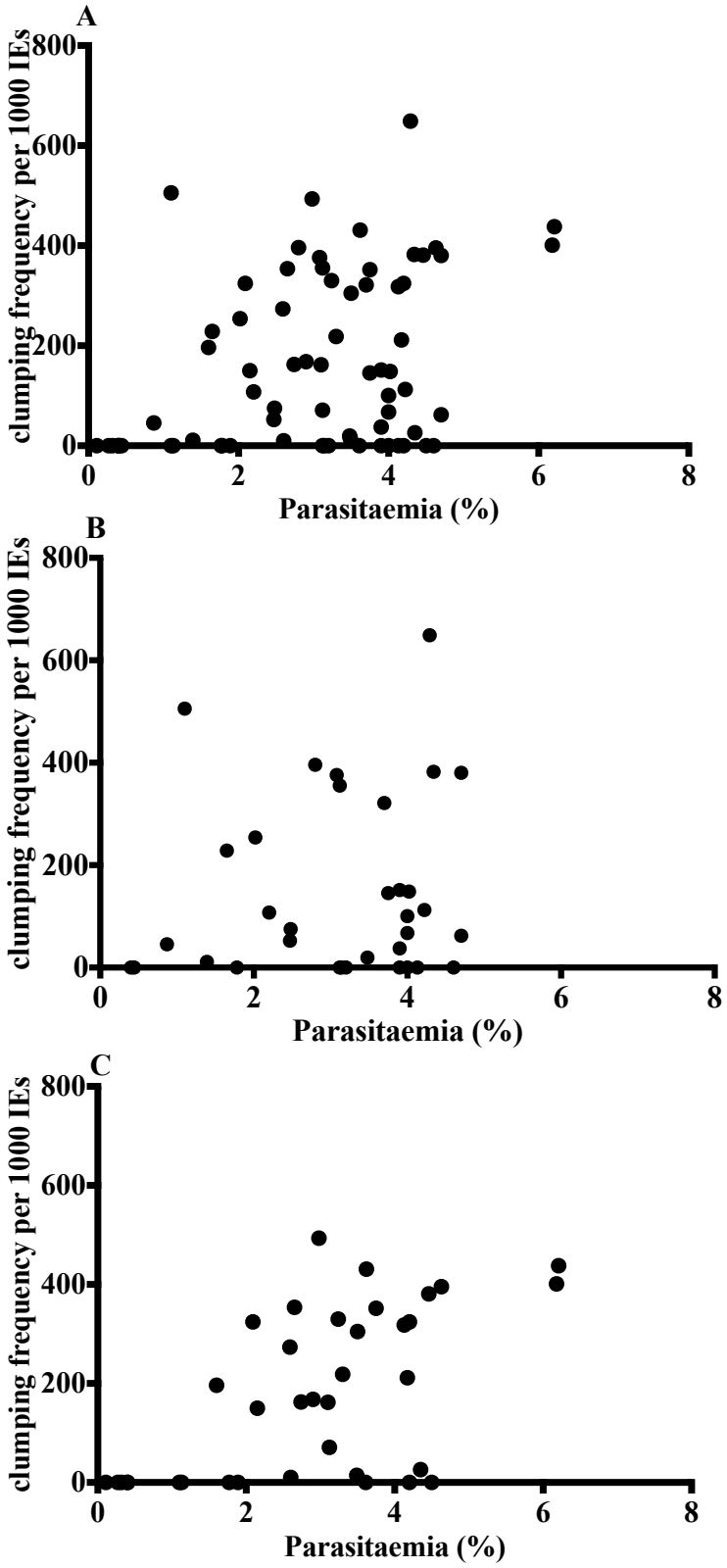


Figure 6.2 Relationship between clumping frequency and assay parasitaemia

Shown here are correlations between clumping frequency and assay parasitaemia. (A). Platelet-mediated clumping was positively correlated with assay parasitaemia for all cases (n=71) (r=0.3, p=0.04). (B). Platelet-mediated clumping is not correlated with assay parasitaemia for children with severe malaria (n=31) (r=0.12, P=0.47). (C). Platelet-mediated clumping was strongly associated with assay parasitaemia for children with non-severe disease malaria (n=37) (r=0.56, p=0.0003).

6.3.4 Platelet receptors for clumping

a) CD36

CD36 has been reported to be the main receptor for platelet-mediated clumping. To determine the importance of CD36 in clumping of field isolates from Kilifi, clumping assays were conducted in the presence of the antibody for CD36 and compared to controls. In agreement to previous published results platelet-mediated clumping was significantly inhibited by the monoclonal antibody against CD36 (Mann-Whitney U test U, p=0.002). 161.93±169.23 in PRP versus 50.17±69.39 in anti-CD36) (Figure 6.3A) (Pain, Ferguson *et al*. 2001). Since the antibody did not completely inhibit clumping, I derived a new variable to represent the platelet-mediated clumping sub-phenotype mediated by CD36, herein referred to as CD36-dependent clumping by subtracting the residual clumping frequency in the presence of anti-CD36 antibody from total clumping. I then compared the difference in CD36-dependent clumping between the two disease groups. I did not find a significant difference between the two disease groups although clumping in non-severe disease was slightly higher (Mann-Whiney U test, p=0.63) (119.5±124.6 for non-severe and 103.4±125.5 for severe disease) (Figure 6.3B).

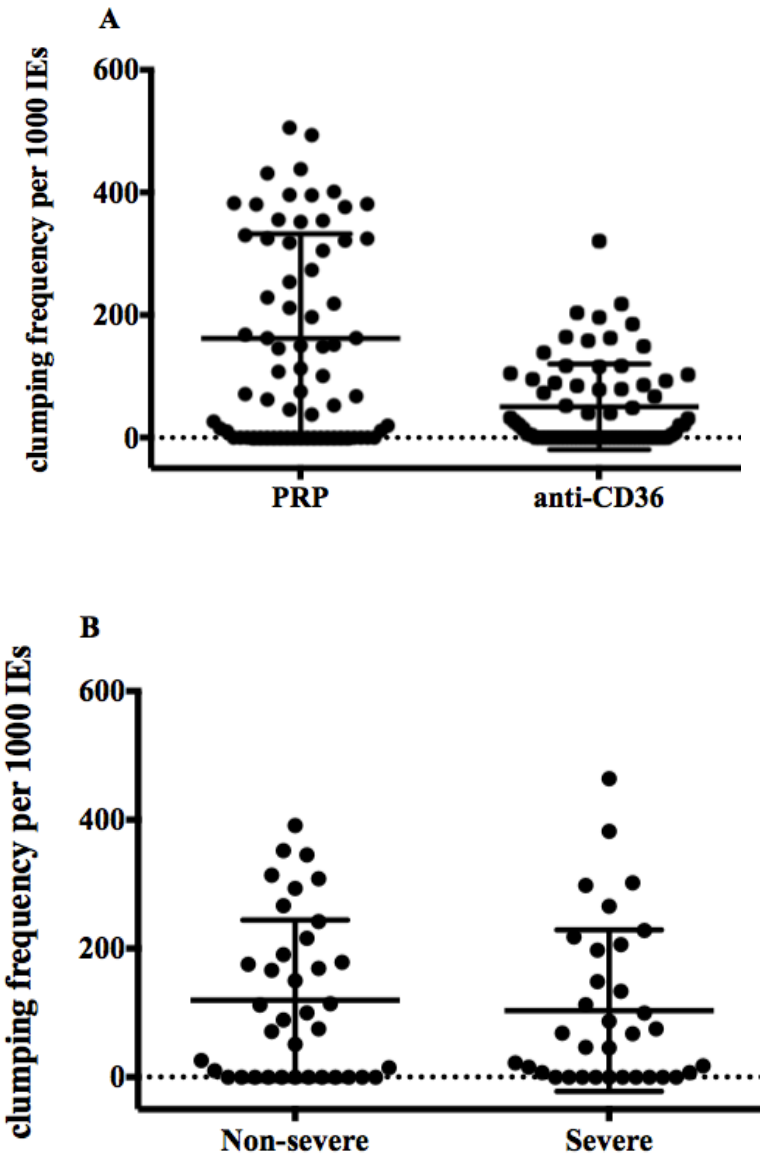


Figure 6.3 CD36 receptor in platelet-mediated clumping

Platelet-mediated clumping assays were conducted in the presence of anti-CD36 monoclonal antibody and compared to controls. (A) Platelet mediated clumping was significantly inhibited by incubation with anti-CD36 antibody (Mann-Whitney U test, p=0.002). (B) CD36-dependent clumping was not significantly different in the two disease subgroups compared by Mann-Whitney U test (p=0.63). The graph shows means and

interquartile ranges.

b) P-Selectin

P-Selectin is one of the reported platelet receptors for platelet-mediated clumping. Clumping assays were conducted in the presence of monoclonal antibody for P-selectin and compared to clumping in PRP. I found that platelet-mediated clumping of the isolates in Kilifi was not significantly inhibited by the monoclonal antibody against P-Selectin (Clone AK-6) as compared by Mann-Whitney U test (p=0.38) (159.2$\pm$169.9 IN PRP versus 131.9$\pm$150.5 in the presence of anti-P-Selectin antibody) (Figure 6.3A). To further investigate the importance of P-Selectin in clumping of field isolates, I derived a new variable to represent P-Selectin-dependent clumping by subtracting the residual clumping from the controls and compared this between the two disease groups as follows

P-Selectin-dependent clumping = Clumping in PRP – Clumping in anti-P-Selectin

A Mann-Whitney U test was then used to compare P-Selectin-dependent clumping in the two disease groups: severe and non-severe malaria. The difference in means between the two groups was not statistically significant (23.27$\pm$52.95 in non-severe versus 31.24$\pm$60.28 in severe disease) (p=0.45) (Figure 6.3B).

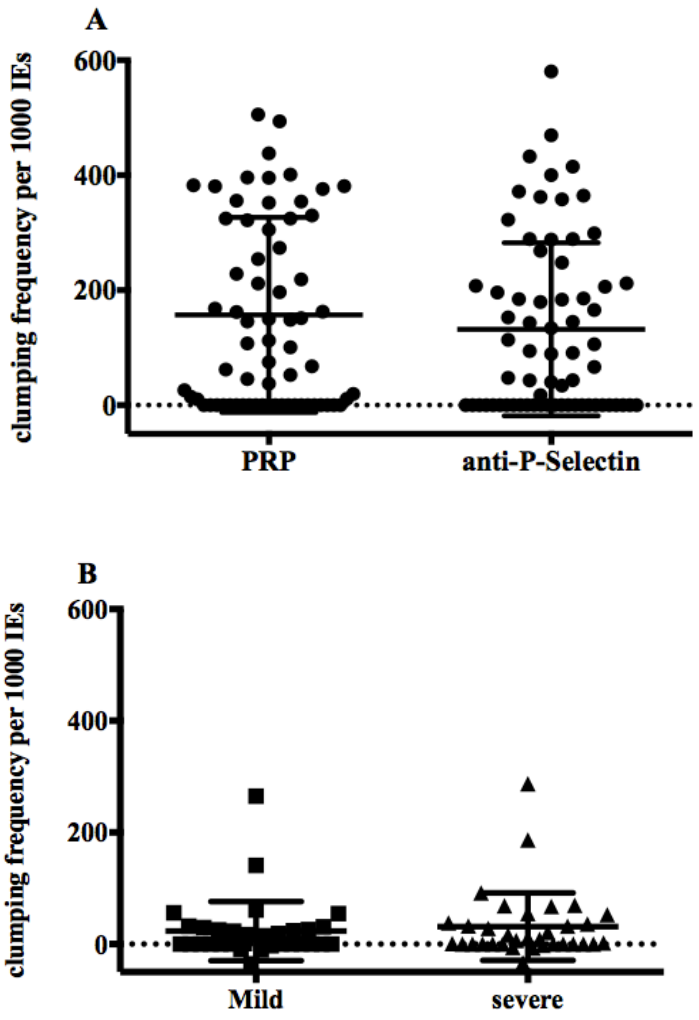


Figure 6.4 P-Selectin receptor in platelet-mediated clumping

Platelet-mediated clumping assays were conducted in the presence of anti-P-Selectin monoclonal antibody and compared to controls. (A) Platelet mediated clumping was not significantly inhibited by incubation with anti-P-Selectin antibody (Mann-Whitney U test, p=0.38) (B) P-Selectin-dependent clumping was not significantly different in the two disease subgroups compared by Mann-Whitney U test (p=0.45).

6.3.5 Associations of clumping with var gene sub-groups

This study is nested within a bigger published study from which a region of the *var* gene that transcribes PfEMP-1 was sequenced for the isolates used in this study (Warimwe, Keane *et al*. 2009). Two new variables to represent clumping were derived namely CD36-dependent clumping and P-Selectin-dependent clumping from the antibody inhibition experiments discussed above. To determine if the two platelet-mediated clumping sub-phenotypes were associated with any of the *var* gene subtypes I analysed the correlations between total clumping, CD36-dependent clumping and P-Selectin-dependent clumping with *var* gene expression respectively. Bull and colleagues classified *var* genes into biologically meaningful subsets based on small blocks of semi-conserved sequence. DBL sequences were divided into six sequence groups: sequence groups CP1-CP3 contain two cysteine residues, and sequence groups CP4 and CP5 are sequences that contain four cysteine residues and sequence group CP6 includes sequences with one, three, five, or six cysteines (Bull, Berriman *et al*. 2005).

a) Total clumping

Total clumping refers to the clumping frequency attained in PRP. Correlation were conducted to determine the relationship between the clumping frequency in PRP and the *var* gene subtypes expressed using Spearman's rank correlation. The statistical correlation between the proportions of IEs expressing CP1 subtype was statistically insignificant (r=0.03, p=0.7) (Figure 6.5A). There was no correlation between CP2 expression and clumping frequency (r=0.08, p=0.48) (Figure 6.5B). There was also no correlation between total clumping CP3 expression (r=-0.01, p=0.9) (Figure 6.5C) and CP4 expression (r=0.05, p=0.67) (Figure 6.5D). I also failed to find a correlation between

clumping frequency and CP5 expression (r=0.07, p=0.52) (Figure 6.5E). Finally also no correlation was found between clumping frequency and CP6 expression (r=0.002, p=0.74) (Figure 6.5F).

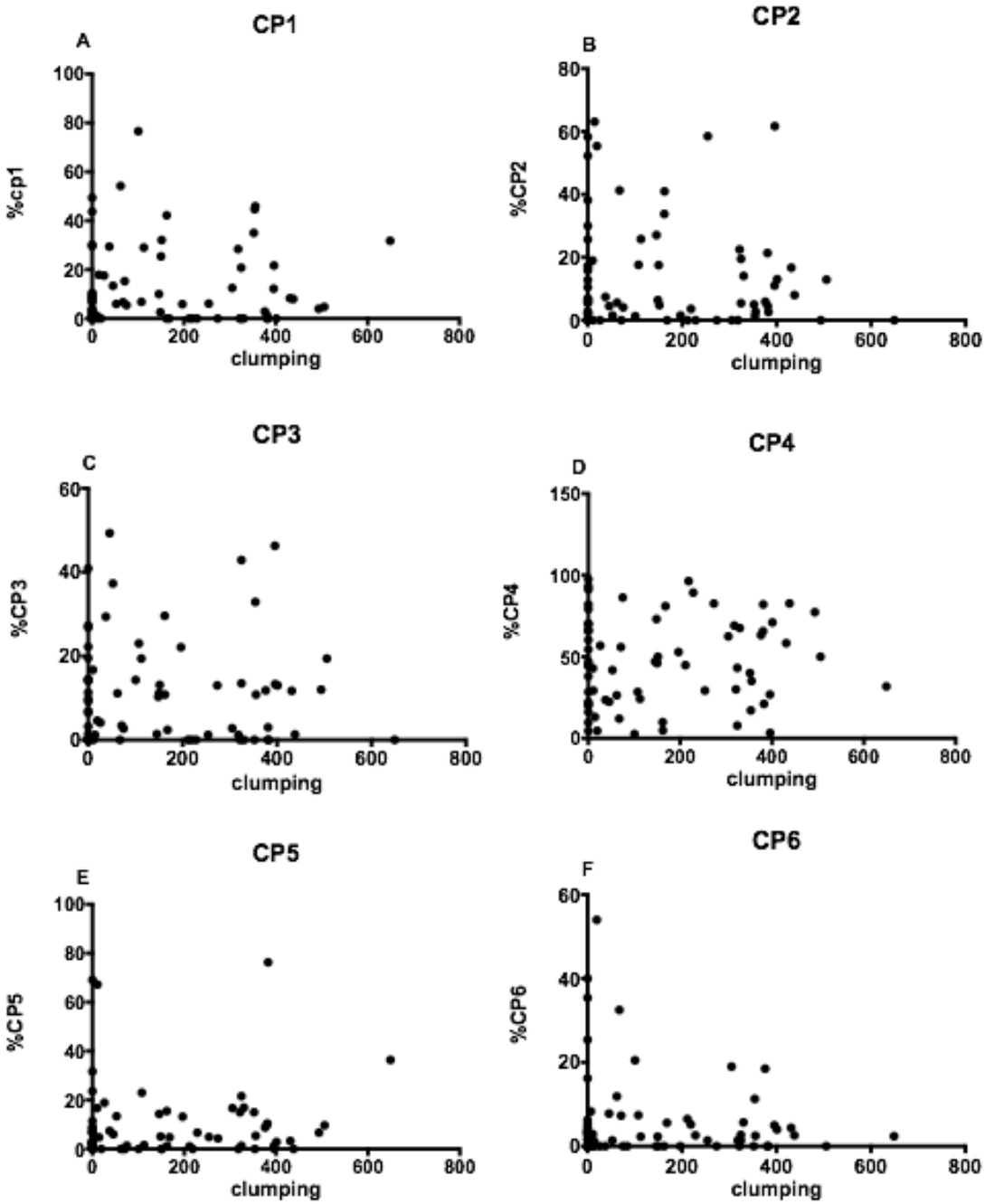


Figure 6.5 Correlations showing the relationship between total platelet-mediated clumping and *var* gene subtypes of PfEMP1

The figures show the relationship between platelet-mediated clumping of infected erythrocytes per 1000 IEs and the percentage expression of *var* gene subtypes

b) CD36-dependent clumping

To further analyse the relationship between platelet-mediated clumping and the clumping sub-phenotypes, correlations were investigated between CD36-dependent clumping and the expression of the PfEMP-1 sub-types. Given that most of clumping is mediated by CD36 a relationship was expected.

However, a significant correlation between CD36-dependent platelet-mediated clumping and *var* gene subtypes was not found with CP1 expression (r=0.03, p=0.74) (Figure 6.6A), similarly there was no correlation with CP2 expression (-0.08, p=0.48) (Figure 6.6B), Neither was there a correlation is the main receptor for platelet-mediated clumping with CP3 expression (r=-0.02, p=0.86) (Figure 6.6C), nor CP4 expression (r=0.03, p=0.77) (Figure 6.6D), Likewise, there was no correlation between clumping and CP5 expression (r=0.07, p=0.53) (Figure 6.6E), and CP6 expression (r=-0.19, p=0.10) (Figure 6.6F).

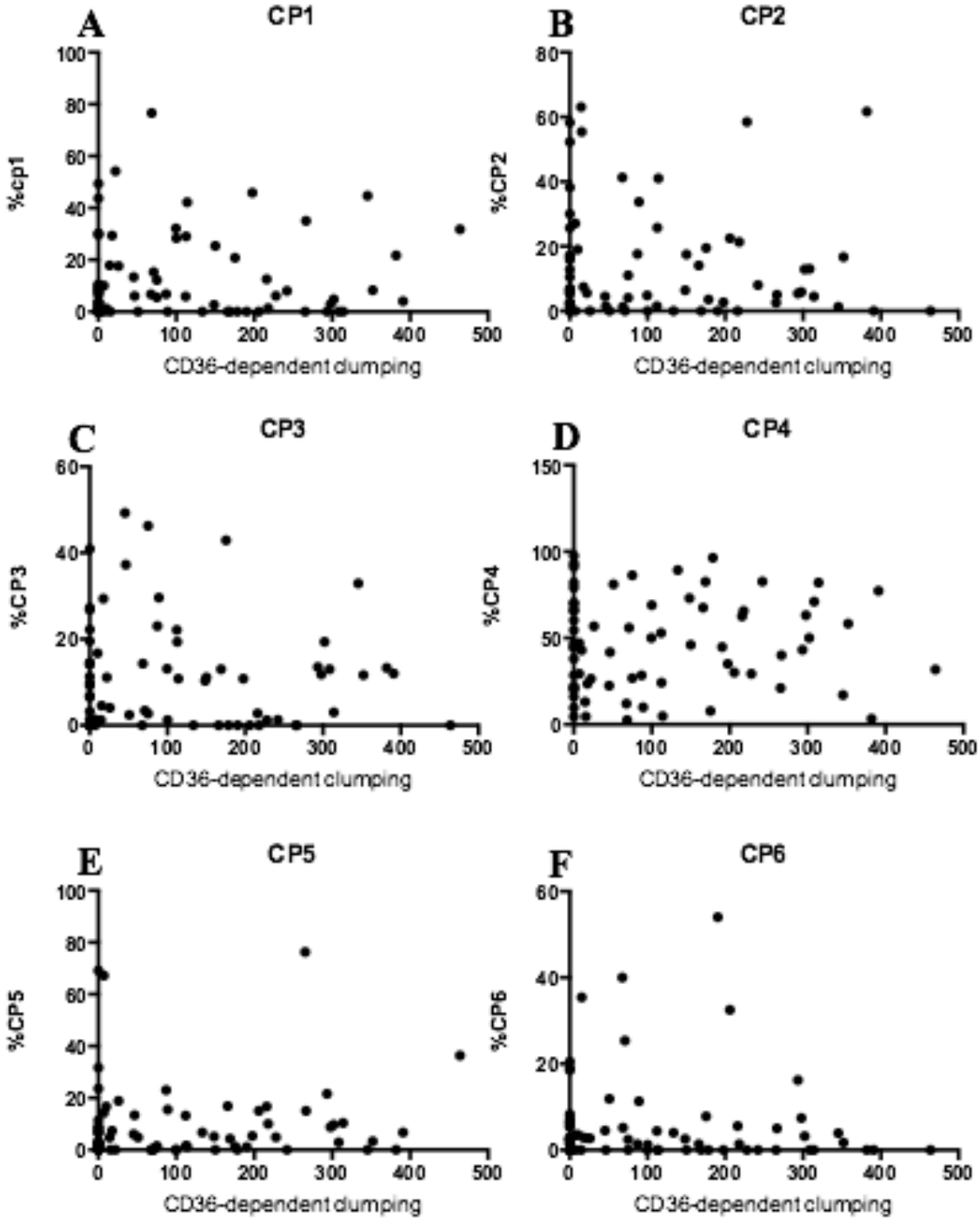


Figure 6.6 Correlations showing the relationship between CD36-dependent clumping platelet-mediated clumping and *var* gene subtypes of PfEMP1

The figures show the relationship between CD36-dependent clumping of infected erythrocytes per 1000 IEs and the percentage expression of *var* gene subtypes

c) P-Selectin-dependent clumping

A further analysis of the relationship between platelet-mediated clumping was conducted by investigating possible correlations between P-Selectin-dependent platelet-mediated clumping and *var* gene subtype expression. Here I found that there was no correlation between P-Selectin dependent clumping and CP1 expression (r=0.17, p=0.14) (Figure 6.7A), CP2 expression (r=-0.11, p=0.36) (Figure 6.7B), CP3 expression (r=-0.19, p=0.12) (Figure 6.7C) and CP6 (r=-0.05, p=0.66) (Figure 6.7F). However, I found a statistically significant correlation between P-Selectin-dependent clumping and CP5 expression (r=0.26, p=0.02). This was not thought to be biologically significant because the relationship is highly dependent on one isolate whose clumping was highly dependent on P-Selectin.

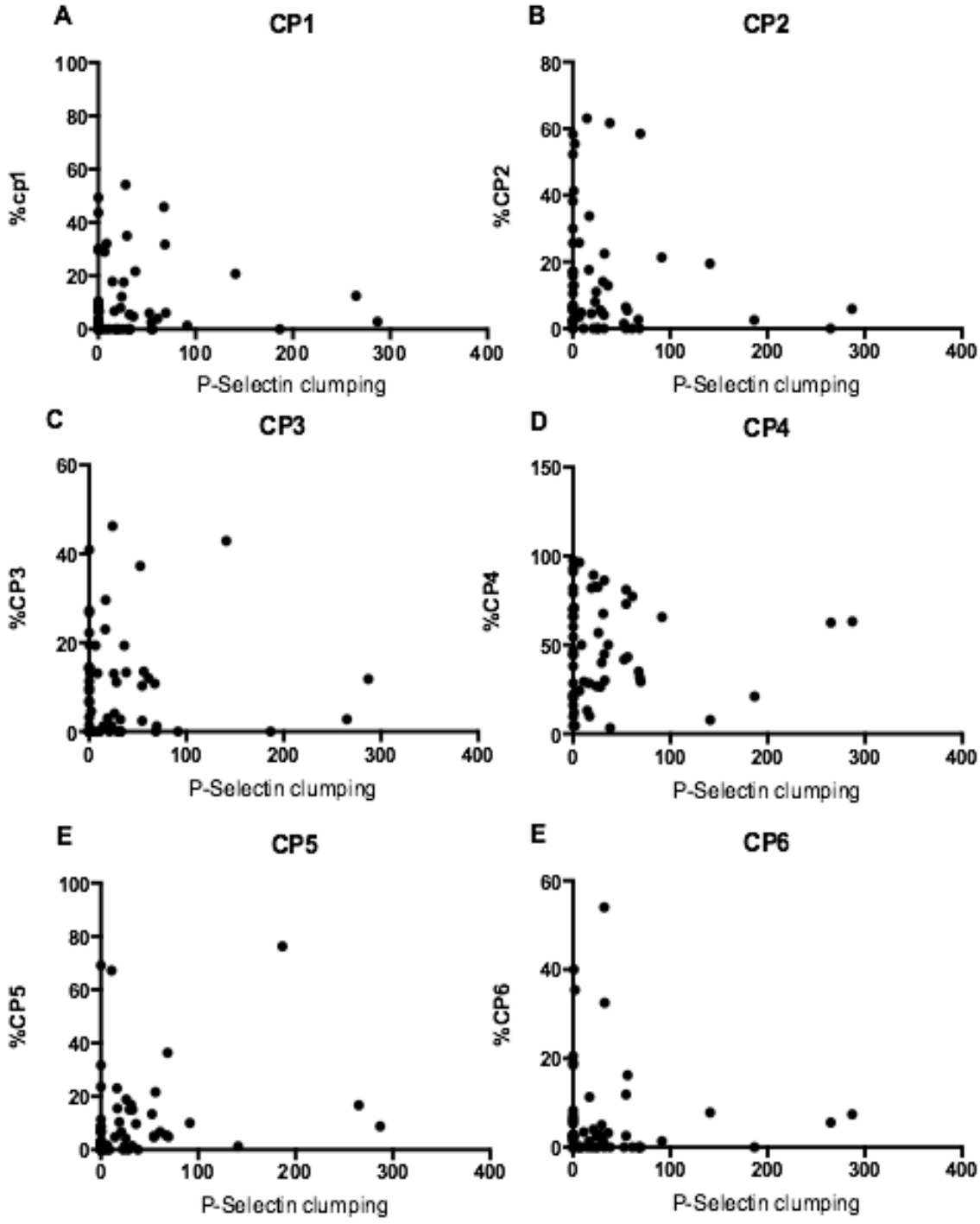


Figure 6.7 Correlations showing the relationship between P-Selectin-dependent clumping and *var* gene subtypes of PfEMP1

The figures show the relationship between P-Selecti-dependent clumping of infected erythrocytes per 1000 IEs and the percentage expression of var gene subtypes

6.4 Discussion

Platelet-mediated clumping was associated with severe disease and parasitaemia in previous field studies on the clumping phenotype (Pain, Ferguson *et al*. 2001, Chotivanich, Sritabal *et al*. 2004, Wassmer, Taylor *et al*. 2008, Mayor, Hafiz *et al*. 2011). Only one published study thus far has reported an association between platelet-mediated clumping frequency and parasitaemia but not severe disease (Arman, Raza *et al*. 2007). Here, the platelet-mediated clumping phenotype of isolates from children presenting with clinical malaria in Kilifi, Kenya was investigated. A positive association was found between platelet-mediated clumping and assay parasitaemia (r=0.3, p=0.04) (Figure 6.2), whereas the association between platelet-mediated clumping and disease severity was not statistically significant (Mann-Whitney U test, p=0.56). CD36 was previously reported to be the main platelet receptor for clumping (Pain, Ferguson *et al*. 2001). Consistent with previous findings, the main receptor for platelet–mediated clumping for Kilifi isolates is CD36 (Mann-Whitney U test, p=0.002) (Figure 6.3). Finally I investigated the relationship between platelet-mediated clumping and distinct PfEMP-1 sub-types and report a positive association between P-Selectin-dependent clumping with CP5 subtype (Figure 6.7).

Seventy-one children presenting with clinical malaria at the Kilifi District hospital were recruited in this study; 37 children with non-severe malaria and 31 children with severe malaria making it one of the smallest studies on clumping compared to the published clinical studies on platelet-mediated clumping which recruited between 49 to 182 participants (see Table 1.1). The clinical characteristics of children showed that the children with severe malaria had a significantly higher mean parasitaemia at time of admission compared to children with non-severe malaria (Mann-Whitney U test, p=0.002)

(Table 6.1) consistent with the expected higher parasitaemia in children with severe malaria compared to non-severe malaria.

Consistent with the findings of Arman and co-workers I found a positive association between clumping frequency and assay parasitaemia (r=0.3, p=0.04) (Figure 6.2A). This association was statistically significant in the non-severe malaria group (r=0.56, p=0.003) but was lost in the severe malaria group (r=0.12, p=0.47) (Figure 6.2B and C). These authors suggested that the reported association between platelet-mediated clumping with severe disease could be explained by parasitaemia (Arman, Raza *et al*. 2007). To tease apart the effect of parasitaemia the Arman study in Mali enrolled children with uncomplicated malaria and high parasitaemia and using multivariate analysis showed that clumping was significantly associated with parasitaemia but not disease category. The previous studies that reported an association between clumping and severe disease used controls of mild malaria with a significantly lower burden of parasitaemia (Pain, Ferguson *et al*. 2001, Wassmer, Taylor *et al*. 2008). However, a study performed in Mozambique in which both methods were used i.e. standardising parasitaemia and using admission parasitaemia reported a positive association between clumping frequency and severe disease in using both methods (Mayor, Hafiz *et al*. 2011).

In agreement with previous findings I found, by inhibition with monoclonal antibodies, that CD36 was by far the most important receptor for the clumping phenotype in the Kilifi field isolates (Figure 6.3A) (Pain, Ferguson *et al*. 2001, Mayor, Hafiz *et al*. 2011, Arman, Adams *et al*. 2013). Overall, inhibition of platelet-mediated clumping with P-Selectin antibodies was not statistically significant (Mann-Whitney U test, p=0.38), although in a

few of the isolates clumping was dependent on P-Selectin to a higher extent. Unlike previous studies, I derived new clumping variables by subtracting the residual clumping frequency in the presence of monoclonal antibodies from the clumping frequency in untreated controls. Comparing the new variables of CD36-dependent clumping and P-Selectin-dependent clumping between severe and non-severe malaria groups did not show any statistically significant differences (Figure 6.3B and Figure 6.4B). There were no differences in the mono-specific clumping frequency between the severe and non-severe disease groups.

The parasite ligand for platelet-mediated clumping is thought to be PfEMP-1. However, there are contradictions in what is understood about platelet-mediated clumping and CD36. CD36-binding PfEMP-1 variants are associated with non-severe disease whereas platelet-mediated clumping which has also been associated with severe disease relies on CD36. To determine the importance of PfEMP-1 in platelet-mediated clumping in PRP and for the two clumping sub-phenotypes, DBL-1 tag sequences of *var* gene sequences obtained in a previous studies (Warimwe, Keane *et al*. 2009) were analysed in relation with platelet-mediated clumping. The *var* gene expression of the isolates was analysed to find if there was any relationship between the transcribed *var* genes and the clumping phenotypes of the isolates. These data showed no correlation between total platelet-mediated clumping in PRP with any of the PfEMP-1 sub-types (Figure 6.4). This was the same for CD36-dependent clumping (Figure 6.4). This was expected since CD36 accounts for a major proportion of the clumping phenotype. A statistically significant positive correlation was found between P-Selectin dependent clumping and CP5 expression.

Chapter 7

Conclusion

In this study the first part is a brief summary of experiments conducted to standardize the method for the clumping assay. Consistent with previous findings, I showed that experimental conditions influence the reported clumping phenotype of a given parasite sample (Arman and Rowe 2008, Arman, Adams *et al*. 2013). Once this was done I investigated the role of PfEMP-1 in the clumping phenotype of laboratory-adapted parasite line IT/C10 and found that the *var* gene IT4*var*30 was overrepresented among platelet-mediating clumping sub-clones. Next I investigated the specific components of host platelets and erythrocytes involved in platelet-mediated clumping by using specific reagents to inhibit these host factors. Here, consistent with previous studies, I found that clumping appears to relie on platelet receptors CD36, P-Selectin and the process of platelet activation (Pain, Ferguson *et al*. 2001, Wassmer, Taylor *et al*. 2008). Finally, I used the knowledge developed in this study to investigate the clumping phenotype of clinical parasite isolates from children presenting with clinical malaria in Kilifi, Kenya. Here in agreement with Arman and co-workers I found an association between the clumping phenotype and assay parasitaemia but not disease severity (Arman, Raza *et al*. 2007).

Platelet-mediated clumping is a specific adhesion phenotype that is characteristic of a subset of malaria parasites. Previous studies show that the clumping phenotype is sensitive to assay conditions (Arman and Rowe 2008, Arman, Adams *et al*. 2013), using parasite line IT/C10 I determined the optimal experimental conditions required for a reproducible

and accurate assay to be used in screening parasites for the platelet-mediated clumping phenotype. The platelet-mediated clumping assay attempts to measure a property of parasites that has not been showed to occur *in vivo* in clinical malaria, although platelet accumulation in malaria has been reported in the brain of children that died from cerebral malaria (Grau, Mackenzie *et al*. 2003). It has been shown that CD36-binding IEs may bind to activated endothelia through vWf-decorated platelets as a bridge on surfaces that may otherwise lack CD36 (Bridges, Bunn *et al*. 2009). If the clumps of IEs observed *in vitro* in platelet-mediated clumping assays could form in vivo, it is plausible that they could obstruct of blood flow in post-capillary venules (Wassmer, Taylor *et al*. 2008).

P. falciparum interaction with platelets might also lead to platelet activation and release of inflammatory mediators (Srivastava, Cockburn *et al*. 2008, McMorran, Marshall *et al*. 2009, McMorran, Wieczorski *et al*. 2012). Accumulation of platelets has been reported in the brains of children dying from cerebral malaria (Grau, Mackenzie *et al*. 2003); however, the precise role of platelets in malaria pathology remains unclear. Platelets can also have antiparasite effects in vivo, and are able to bind to and kill IEs (McMorran, Marshall *et al*. 2009, McMorran, Wieczorski *et al*. 2012)

The IT/C10 parasite line was tested for clumping in different conditions where I varied haematocrit, parasitaemia, platelet concentration, plasma concentration, platelet buffers, anticoagulants used to prepare platelets and duration of incubation. My results were consistent with those of Arman and colleagues (Arman and Rowe 2008, Arman, Adams *et al*. 2013). I found that platelets support the platelet-mediated clumping effect of IEs in a variety of conditions, including after prolonged cold storage, in the absence of plasma or in the presence of anticoagulants sodium citrate or EDTA. On the other hand, heparin

anticoagulant disrupts the clumps and clumping appears to be calcium dependent consistent with previous findings (Chotivanich, Sritabal *et al*. 2004). In chapter 3 I described optimal assay conditions for screening a given parasite culture for the platelet-mediated clumping phenotype. I recommend conducting the clumping assay at a high haematocrit of 10%, which ensures that the clumping phenotype is detected, but also to control the parasitaemia to ensure that the size of clumps formed are limited to sizes that can be easily qualified under the microscope.

The assessment of *P. falciparum* clumping is affected by the precise conditions used to set up the clumping assay *in vitro,* with parasitaemia and haematocrit having a profound effect on the outcome of the assay. For field isolate studies, it is crucial that the effect of these parameters on clumping is taken into account during experimental design, otherwise the higher parasitaemia usually seen in parasite isolates from severe malaria patients compared to uncomplicated malaria controls could bias results. Possible solutions to the confounding effect of parasitaemia on studies of clumping and malaria severity include adjustment of all isolates to a standardized parasitaemia, or matching of cases and controls. For laboratory experiments on clumping, it is important that the limitations of the assay (eg. the difficulty in assessing the true maximum clumping frequency of a parasite because of the formation of giant clumps that cannot be counted accurately) are taken into account. However, the assay conditions alone cannot explain the variable association of clumping with severe malaria in different studies, which requires further investigation using carefully designed experiments and standardized techniques.

To my knowledge, there is no published study that has attempted to identify the parasite ligands used by IEs to interact with platelet receptors in platelet-mediated clumping. This is an important research question because platelet-mediated clumping has been associated with severe disease, making the clumping ligand a potential marker for severe disease or a candidate for development of adjunctive therapies to supplement existing malaria drug regimens. Some characteristics of the platelet-mediated clumping ligand are similar to PfEMP-1, the adhesion for other IE adhesion phenotypes including resetting (Rowe, Moulds *et al*. 1997), CD36 (Robinson, Welch *et al*. 2003), P-Selectin (Senczuk, Reeder *et al*. 2001), PECAM-1 (Treutiger, Heddini *et al*. 1997). For instance, I found that the platelet-mediated clumping ligand is very sensitive to trypsin treatment and is expressed at the early trophozoite stage of parasite development consistent with PfEMP-1. Furthermore, two of the known clumping ligand receptors, CD36 and P-Selectin are known receptors of PfEMP-1 (Senczuk, Reeder *et al*. 2001, Robinson, Welch *et al*. 2003, Joergensen, Bengtsson *et al*. 2010). Platelets also express PECAM-1, another receptor for PfEMP-1 (Treutiger, Heddini *et al*. 1997). Taken together, this led me to hypothesize that platelet-mediated clumping ligand is a PfEMP-1. To test this hypothesis I derived a number of parasite line IT/C10 sub-clones. Each one varied in its clumping and adhesive profiles to CD36, P-Selectin, PECAM-1 and gC1qR.

There are perceived contradictions on what is known about platelet-mediated clumping. On one hand all parasites that exist as wild isolates bind to CD36 while on the other hand not all CD36-binding parasites exhibit the clumping phenotype in the presence of platelets despite CD36 having been identified as the main platelet receptor for clumping. It has been proposed that this be explained by one of two scenarios. In the first scenario, the

clumping ligand and the adhesion ligand for CD36 exploit different distinct epitopes on CD36, while in the second scenario, the clumping ligand and the adhesion ligand may bind to different platelet receptors (Pain, Ferguson *et al*. 2001). In the IT/C10 parasite clones I found a single *var* gene that was over-represented among the clumping clones, IT4*var*30.

IEs have protrusions on the surface that are sites of expression of PfEMP-1 known as knobs. The truncation of *kahrp* gene, that encodes KAHRP, leads to development of knobless IEs. Knobless IEs are known to have reduced ability to bind to purified receptors compared to knobbed IEs (Horrocks, Pinches *et al*. 2005). To determine if the differences of clumping phenotype among IT/C10 clones could be explained by presence or absence of knobs, I tested for the presence of the KAHRP gene transcripts in selected IT/C10 clones. All IT/C10 clones as expected were positive for the KAHRP transcript therefore platelet-mediated clumping does not appear to be caused by knobs. Recent published data has shown that clumping parasites can be selected from knobless parasite line DD2 (Arman, Adams *et al*. 2013). It is possible however that these parasites are knot completely knobless but have a lower expression of the knobby phenotype.

The platelet-mediated clumping phenotype relies on CD36 on the platelet surface (Pain, Ferguson *et al*. 2001). Other platelet receptors reported to contribute to the clumping phenotype include P-Selectin (Wassmer, Taylor *et al*. 2008) and gC1qR (Biswas, Hafiz *et al*. 2007). Consistent with other studies, I showed that antibodies against platelet CD36 inhibit clumping significantly in IT/C10 clones, and also in parasite lines selected for binding to PECAM-1 and P-Selectin. Platelet P-Selectin may contribute to clumping by

interactions through a lectin domain of P-Selectin. In this study, my results agree with published results showing that antibodies that block platelet surface CD36 inhibit clumping almost entirely in the CD36-binding parasites and also significantly in clumping parasites developed by selecting on purified P-Selectin and PECAM-1 (Mayor, Bir *et al*. 2005). In my hands a gC1qR-binding parasite line failed to clump. Although the PECAM-1 binding parasites showed very low levels of clumping, I was able to demonstrate their clumping was dependent on CD36 and PECAM-1. To my knowledge, this is the first time that a PECAM-1-binding parasite has been shown to mediate the clumping phenotype.

P-Selectin expressed on endothelial cells is a receptor for IE rolling (Ho, Schollaardt *et al*. 1998, Senczuk, Reeder *et al*. 2001). From my data, it appears that the platelet-mediated clumping ligand(s) uses an epitope that competes with fucoidin and Sialyl Lewis-a but PSGL-1. P-Selectin is also a marker for platelet activation. I showed the role of platelet activation in platelet-mediated clumping by using four different inhibitors of platelet activation.

Neuraminidase enzyme cleaves off the sialic acid residues on the IE surface leading to inhibition of the clumping phenotype. Interestingly no sialidases are known in *Plasmodium* genome (Gardner, Hall *et al*. 2002). Neuraminidase-treatment of IEs reduced on purified P-selectin and activated platelet monolayers (Ho, Schollaardt *et al*. 1998). The known ligands for P-Selectin PSGL-1 and Sialyl Lewis A inhibited platelet-mediated clumping. Sialyl Lewis-a is a tetrasaccharide with sialic acid residues that bind to the lectin domains of P-selectin.

P-Selectin has been reported to be involved in clumping but the mechanisms by which it is involved are unclear (Wassmer, Taylor *et al*. 2008). P-Selectin is also a marker for platelet activation. It has been reported that platelets are activated in the presence of malaria IEs (Srivastava, Cockburn *et al*. 2008). In this study I have shown that the clumping phenotype can be inhibited blocking platelet activation. Taken together it can be inferred that in the presence of clumping IEs platelet activation takes place leading to upregulation of P-Selectin on the platelet surface and therefore contributing to the clumping phenotype by interacting with the clumping ligands on the IEs.

This study enrolled 71 cases making it one of the smallest studies to investigate the clumping phenotype so far, therefore these results are interpreted with caution because a study of this size may lack sufficient power to show differences among the groups. The clinical characteristics showed that the admission parasitaemia of the severe malaria group was significantly higher than that of the non-severe group consistent with the expected higher parasitaemia in children with severe malaria compared to non-severe malaria.

Consistent with the findings of Arman and co-workers a positive association was found between clumping frequency and assay parasitaemia. It has been proposed before that the reported association of platelet-mediated clumping with severe disease could be explained by parasitaemia (Arman, Raza *et al*. 2007). To show the true relationship between clumping frequency and disease severity, other researchers have suggested standardization of assay parasitaemia (Arman, Raza *et al*. 2007). This was proved to be unnecessary in a study performed in Mozambique in which both methods (unstandardized and

standardized) were used and the association between clumping frequency and disease severity was maintained (Mayor, Hafiz *et al*. 2011). Introduction of heterologous erythrocytes to the clumping assay may also affect the expression of the true clumping phenotype of the parasite isolates if erythrocyte ligands are involved as well.

In agreement with previous findings I found that CD36 was an important receptor for the clumping phenotype in the Kilifi field isolates by inhibition with monoclonal antibodies (Pain, Ferguson *et al*. 2001, Mayor, Hafiz *et al*. 2011, Arman, Adams *et al*. 2013). Unlike previous studies, I derived new clumping variables by subtracting the residual clumping frequency in the presence of monoclonal antibodies from the frequency in untreated controls. Comparing the new variables of CD36-dependent clumping and P-Selectin-dependent clumping between severe and non-severe malaria groups did not show any statistically significant differences. There were no differences in the mono-specific clumping frequency between the severe and non-severe disease groups

The parasite ligand for platelet-mediated clumping is thought to be PfEMP-1. However, this presents a paradox since CD36-binding PfEMP-1 variants are associated with non-severe disease whereas platelet-mediated clumping which has also been associated with severe disease relies on CD36. To determine the importance of PfEMP-1 in platelet-mediated clumping in PRP and for the two clumping sub-phenotypes, DBL-1 tag sequences of *var* gene sequences obtained in a previous studies (Warimwe, Keane *et al*. 2009) were analysed in relation with platelet-mediated clumping. The *var* gene expression of the isolates was analysed to find if there was any relationship between the transcribed *var* genes and the clumping phenotypes of the isolates. These data showed no correlation

between total platelet-mediated clumping in PRP with any of the PfEMP-1 sub-types. This was the same for CD36-dependent clumping. This was expected since CD36 accounts for a major proportion of the clumping phenotype. A statistically significant positive correlation was found between P-Selectin dependent clumping and CP5 expression.

Thus far there is no study that has formally shown that platelet-mediated clumping occurs *in vivo* in malaria patients. However circumstantial evidence points to a possibility of clumping occurring *in vivo*. Firstly, platelet deposits have been reported in post-morterm of children that died from malaria in Malawi along with malaria infected erythrocytes, hemozoin and leucocytes (Grau, Mackenzie *et al*. 2003). Secondly, the likelihood of the clumping phenotype occurring *in vivo* is shown by the size of clumps observed *in vitro*. If the size of clumps observed *in vitro* would occur *in vivo* it is plausible that they would obstruct post-capillary venules. Thirdly, some parasite isolates have been shown to have multiple adhesion phenotypes therefore it is possible that the adhesion phenotypes would occur concurrently including adhesion to endothelial cells on vascular endothelia, rosetting uninfected erythrocytes and platelet-mediated clumping. Another compelling question to malaria researchers is the contribution of clumping to development of cerebral malaria. It is plausible that platelet-mediated clumping contributes to development of cerebral malaria although current evidence cannot entirely explain the mechanisms behind it. Two clinical studies so far found that all parasite isolates from cerebral malaria patients were positive of the clumping phenotype (Chotivanich, Sritabal *et al*. 2004, Mayor, Hafiz *et al*. 2011). If cerebral malaria can be partly explained by obstruction of post-capillary venules in the brain it can be conjectured that clumping could contribute to platelet-mediated clumping. Furthermore platelets are CD36-rich have been found to bridge CD36-binding

parasites to brain endothelia that lacks CD36 therefore since platelet-mediated clumping brings parasites together it is plausible that if clumping occur in brain endothelia it would contribute to cerebral malaria (Bridges, Bunn *et al*. 2009).

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