Review of Literature and Introduction
1.1 Malaria
Malaria is estimated to be the 8th overall leading cause of mortality and the 3rd leading cause of mortality among communicable diseases globally. Unlike cerebrovascular diseases, ischemic heart diseases or cancers that primarily kill older people, malaria related mortality is mostly concentrated in young children.

![Figure 1.1 Stack bar representation of estimated global deaths caused by various factors. (The World Health Report 2008, WHO)](image)

The persistent interaction of malaria parasite with human host has led to profound changes in human genome. Several recessive alleles have been selected to high frequencies in populations residing in malaria endemic regions. For instance, sickle cell trait which is potentially fatal for homozygous individual has been selected because of its protective effects against severe malaria. There is also a distinct association between malaria and poverty. Malaria-endemic countries are not only poorer than non-malarious countries, but they also have lower rates of economic growth (Gallup and Sachs, 2001). Apart from these, malaria also played an important role in shaping human history. It may have been one of the major factors in protecting indigenous Africans from foreign invaders. America and Australia, which initially did not have malaria, were extensively colonized unlike Africa.

1.2 Current scenario of malaria
World Health Organization estimates about half of world’s population (3.3 billion) lives in areas with risk of malaria transmission. Out of these, about 1.2 billion live in areas with a high risk of malaria. The largest number of people living in areas with a high risk of malaria is in Africa, followed by the South-East Asia. It is estimated that
there were 247 million clinical malaria cases worldwide in 2006, of which >90% were due to *P. falciparum*. The vast majority of cases (86%) were reported from Africa, followed by the South-East Asia (9%) and Eastern Mediterranean regions (3%).

In Africa, 19 of the most populous countries accounted for >90% of malaria cases in 2006. Outside Africa, India is estimated to have approximately 30% of the malaria and 15% of *Plasmodium falciparum* cases. There were an estimated 881,000 deaths due to malaria worldwide in 2006, of which >90% were in the Africa. An estimated 85% of deaths due to malaria occur in children below 5 years.

South-East Asia has the highest rate of drug resistance in the world and multi-drug resistance has contributed to the re-emergence of malaria in many areas, especially along international borders. India is endemic for malaria except for the mountainous regions above 1800-meter sea level and well-drained coastal areas along the Western and Eastern Ghats. In the island territories, Lakshadweep is a malaria free island, however in Andaman and Nicobar Islands malaria has presence in the coastal regions. Orissa, Jharkhand, Bengal, Assam, Chhattisgarh and parts of Rajasthan, Gujarat and Uttar Pradesh are high-risk areas for malaria in India. Orissa state with only 4% population of India contributes about 22% of total malaria cases and 43% of *P. falciparum* cases. About half of national malaria related deaths occur in Orissa.

### 1.3 Plasmodium life cycle

Malaria is caused by a protozoan parasite belonging to the genus *Plasmodium*. There are four *Plasmodium* species that infect humans namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*. A fifth species *Plasmodium knowlesi*, previously known as a simian malaria parasite has recently been reported to infect humans as well (Singh et al., 2004). The complex life cycle of *Plasmodium* requires two hosts. *Plasmodium* completes asexual stage in vertebrate host whereas sexual stage is completed inside female Anopheles mosquitoes.
1.3.1 Asexual stage

Plasmodium infection in humans is initiated by the inoculation of sporozoites with the bite of infected female Anopheles mosquitoes. Sporozoites are injected into the human host through the saliva of infected mosquitoes during a blood meal. Usually 10-20 sporozoites are injected in a single infective bite. Once inside the human host the sporozoites reach the liver with blood circulation where they infect hepatocytes and start multiplying by schizogony. Depending upon the Plasmodium species, it may take 5-15 days for a single sporozoite to develop into 40,000-50,000 merozoites by liver schizogony. Merozoites from liver hepatocytes are released into liver sinusoids as membrane bound vesicles called merosomes (Sturm et al., 2006). Merosomes are vesicular structures that protect merozoites from phagocytic cells such as Kupffer cells in the liver. Merosomes are formed by budding of parasite filled vesicles after parasite induced death and detachment of infected hepatocytes. The host origin of meroosome membrane protects merosomes from phagocytosis.
Merozoites released from merosomes infect erythrocytes to initiate blood stage infection. Merozoites invade erythrocytes and ring shaped parasite can be observed inside infected erythrocyte (IE) within hours of infection. Subsequently, the ring matures into metabolically active trophozoite stage. Trophozoites develop into schizonts in which multiple merozoites form after repeated cell divisions. Merozoites are released upon rupture of mature schizonts. The length of this erythrocytic stage of the parasite life cycle depends on the parasite species: 48 hours for \textit{P. falciparum}, \textit{P. vivax}, and \textit{P. ovale} and 72 hours for \textit{P. malariae}. The released merozoites invade fresh erythrocytes and repeat the cycle, however a small proportion of blood stage parasites develop into male and female gametocytes. The gametocytes, if ingested by mosquito start sexual stage of parasite life cycle.

In \textit{P. vivax} and \textit{P. ovale} some of the sporozoites enter a dormant phase known as the hypnozoite. Hypnozoites are sporozoites that do not immediately undergo asexual replication. Reactivation and schizogony of hypnozoite at later time may result in relapses.

1.3.2 Sexual stage

Once inside mosquito midgut, the gametocytes undergo gametogenesis to form male and female gametes. During gametogenesis microgametocytes undergo three rounds of nuclear replication to form 8 microgametes by a process known as exflagellation. Low temperature, high pH (7.8-8.2) and mosquito-derived gametocyte activating factor xanthenuric acid acts as inducers of exflagellation (Hirai et al., 2001). The macrogametocytes develop into macrogametes without any division. Fertilization of male and female gametes results in formation of zygotes. Zygotes transform into ookinetes and cross the midgut epithelium 24–48 hours post infection. Ookinetes transform into non-motile oocysts on the outer wall of the midgut. Over the next 10 days a meiotic cell division followed by several rounds of mitosis produces thousands of haploid sporozoites within each oocyst. This process is called sporogony. Mature sporozoites are released into hemocoel after 14-16 days of infection. Sporozoites then migrate and invade salivary glands. When infected Anopheles mosquitoes bite human hosts for a blood meal, the sporozoites are released in blood stream to complete the cycle.
1.4 Clinical features of malaria

Clinical malaria is defined as fever or history of fever with asexual malaria parasitemia. Malaria presentation may range from asymptomatic infection in hyperimmune adults to life threatening cerebral malaria. Malaria presentation varies between the different epidemiological settings, depending considerably on intensity of malaria transmission, host factors and the Plasmodium species involved. In high endemic areas severe malaria is presented mostly in young children. Adults are generally protected from severe malaria with the exception of paucigravidae pregnant women. In areas of low endemicity and seasonal malaria transmission, both children and adults are susceptible to severe malaria. Several host genetic factors have been identified that affect clinical presentation of malaria. Sickle cell trait (Flint et al., 1998), α- thalassemia (Flint et al., 1986), Glucose 6-phosphate dehydrogenase deficiency (Kwiatkowski, 2005), absence of Duffy antigen on erythrocytes surface (Chitnis and Miller, 1994; Miller et al., 1976), blood group antigens, CR1 polymorphism (Cockburn et al., 2004) and polymorphism in CD36 (Aitman et al., 2000) etc. may affect the outcome of malaria infection. *P. falciparum* causes most of the severe malaria cases and accounts for almost all the malaria related death. *P. vivax, P. malariae* and *P. ovale* are generally benign. However, in recent reports *P. vivax* has been implicated in severe malaria (Price et al., 2007).

The characteristic malaria fever is presented by cycles of fever and chills followed by a sweating stage. Transient headaches, vomiting, delirium, anxiety and restlessness may accompany the febrile paroxysms. In *vivax* and *ovale* malaria, this typical pattern of fever recurs once every 48 hours and is referred to as tertian malaria. In *falciparum* infections, this pattern may not be seen often and the paroxysms tend to be more frequent (Sub-tertian). In *P. malariae* infection the relapses occur once every 72 hours and it is referred to as quartan malaria.

1.4.1 Non-severe malaria

The clinical symptoms of non-severe malaria are primarily due to schizont rupture and destruction of erythrocytes. Mild malaria patients often present symptoms similar to those of common viral infections; this may lead to a delay in the diagnosis. The majority of patients experience fever, chills, headaches, and diaphoresis. Other common symptoms include dizziness, malaise, myalgia, abdominal pain, nausea, vomiting, diarrhea and cough.
1.4.2 Severe malaria

Severe malaria is a condition of multi-system disorder presented as a range of clinical complications. Complications may involve the nervous, respiratory, renal, and/or hematopoietic systems. Metabolic acidosis and hypoglycemia are the most common metabolic complications. The major symptoms of severe malaria include cerebral malaria, severe anemia and/or bleeding, pulmonary edema, acute renal failure and pregnancy associated malaria in women. Any of these complications can develop rapidly and progress to death within hours or days. In many patients, several of these complications coexist or evolve in rapid succession within a few hours.

Cellular basis of severe malaria pathogenesis is still not completely understood. There are two theories explaining pathophysiology of severe malaria. Cytokine theory suggests that inflammatory mediators such as cytokines and nitric oxide released by host cells exposed to malaria toxins play a major role in the onset of the pathology (Clark et al., 1989). Whereas, the mechanical theory suggests that severe malaria is attributable to an excessive sequestration of *P. falciparum* IEs in the micro-vasculature of vital organs leading to obstruction of blood flow (Beare et al., 2009), hypoxia, tissue damage and, ultimately, organ failure. It has been demonstrated that sequestration itself can lead to increased secretion of proinflammatory cytokines (Tongren et al., 2000), and proinflammatory cytokines can up regulate the surface expression of certain receptors such as intercellular adhesion molecule 1 (ICAM1/CD54) involved in cytoadherence on microvascular endothelial cells (Wong and Dorovini-Zis, 1992). Both mechanisms may act separately or in conjunction leading to severe malaria.

Host factors associated with high risk of severe malaria include age less than 5 years, gender (female in pregnancy associated malaria), nonimmune status, coexisting medical conditions, no anti-malarial prophylaxis, delay in treatment, and severity of the illness at the time of admission.

1.4.2.1 Cerebral malaria

Cerebral malaria is defined as a clinical syndrome characterized by unrousable coma (Blantyre coma score <2 in children or Glasgow coma score <9 in adults) at least 1 h after termination of a seizure or correction of hypoglycemia, with detection of asexual
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forms of *P. falciparum* malaria parasites in peripheral blood smears and no other probable cause of encephalopathy.

In sub-Saharan Africa, cerebral malaria annually affects 575,000 children less than 5 years of age and approximately 110,000 die (19% case fatality rate). Children who survive cerebral malaria may experience developmental and behavioral abnormalities. Every year, 9,000–19,000 children less than 5 years of age in Africa experience neurological complications lasting up to 6 months (Murphy and Breman, 2001).

The incidence of cerebral malaria depends upon the intensity of malaria transmission, with the highest rates in areas of low-to-moderate malaria transmission and the lowest rates in areas of intense malaria transmission. In endemic areas cerebral malaria and subsequent neurological impairments are most common in children. Children < 18 months old are less susceptible to cerebral malaria as compared to the older children in endemic areas (Snow et al., 1997).

The pathogenesis of cerebral malaria is still incompletely understood. It appears to be a multi-factorial condition. Sequestration of IEs in the cerebral blood vessels seem to be an essential component of the pathogenesis (MacPherson et al., 1985). The adhesion of IEs to ICAM1 has been implicated in the pathogenesis of cerebral malaria (Turner et al., 1994). Post-mortem studies have revealed increased number of IEs present in cerebral blood vessels in the patients who died of cerebral malaria as compared to those who died of other severe malaria complications. ICAM1 expression is up regulated in the cerebral vascular endothelium in patients who died of cerebral malaria (Turner et al., 1994). An ICAM1 polymorphism (ICAM1Kilifi), which is common in Africa that changes protein binding affinity to IEs, was associated with susceptibility to cerebral malaria in Kenyan children (Fernandez-Reyes et al., 1997) but not in The Gambia (Bellamy et al., 1998).

1.4.2.2 Severe malarial anemia

Plasmodium infections in humans frequently lead to anemia. *P. falciparum* causes the most severe form of anemia, which is a risk factor for malaria related mortality. Severe anemia may lead to profound hypoxia and congestive cardiac failure. Severe malarial anemia is defined as Hemoglobin <5 g/l or hematocrit (HCT) < 15 % in presence of malaria parasitemia of any density. Malaria is the most common cause of severe anemia in children and pregnant women admitted to hospital in malaria.
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Prevalence of anemia (HCT <33%) in malaria endemic areas varies between 31-90% (Bradley-Moore et al., 1985; Premji et al., 1995) in children and 60-80% in pregnant women (Matteelli et al., 1994; Menendez et al., 1994). Severe anemia is more common in infants and young children (<3 years) as compared to adults living in malaria endemic areas.

The severity of malarial anemia does not always correlate with parasitemia, suggesting that destruction of IEs may not be the only cause of malarial anemia and other factors may be involved. The pathophysiology of malaria related anemia involves increased destruction as well as reduced production of erythrocytes. Malnutrition particularly iron deficiency and certain anti-malarial drugs may worsen anemia.

Increased erythrocyte destruction: IEs are destroyed upon completion of erythrocyte cycle to release the merozoites. Activated macrophages also destroy IEs and uninfected erythrocytes (UEs) with rigid membranes. Destruction of UEs is thought to be a major underlying mechanism of persistent anemia after clearance of parasitemia. Spleen extracts parasite from IEs without destroying erythrocytes, however, such erythrocytes may have decreased life span (Angus et al., 1997). High doses of anti malarial drugs such as quinine may induce the destruction of erythrocytes (Van den Ende et al., 1998). Autoimmune haemolysis may also be a contributory factor in malaria related anemia.

Decreased erythrocyte production: During malaria infection the growth of early erythrocyte precursors is delayed. Serum erythropoietin (EPO) levels are raised in anemia patients that induce erythrocyte production. In studies on Thai and Sudanese adults with malaria, the levels of EPO were not as high as expected for the intensity of anemia (Burgmann et al., 1996), suggesting that malaria may reduce the production or release of EPO. In malaria infection the levels of Th1 cytokines (TNF-α and IFN-γ) and nitric oxide are high, which may lead to bone marrow depression. High levels of Th2 cytokines (IL10, IL12) may protect against development of severe malarial anemia (Othoro et al., 1999). The immune system is depressed during malaria and secondary infections are common. For example about 90% children in the developing world are infected by Parvovirus B19 by the age of 2 years. B19 has a tropism for erythroid progenitor cells and is toxic to them. Such opportunistic
infections might also contribute to malaria related anemia especially in patients with sickle cell disease.

Iron deficiency and malnutrition also contribute to malarial anemia. Plasmodium infection can cause reduced iron absorption from gut and loss of iron by haemoglobinuria. Pyrimethamine inhibits Plasmodium dihydrofolate-reductase (DHFR) more potently than the mammalian DHFR. High doses of pyrimethamine may lead to anemia (Fleming et al., 1974).

1.4.2.3 Pregnancy associated malaria

Clinical immunity to malaria is acquired after repeated infections of the parasite in children living in malaria endemic areas. Adults in endemic areas are generally protected against symptomatic malaria (Bejon et al., 2008). An important exception to this rule is pregnant women. In endemic areas malaria is more prevalent and severe in pregnant women compared to non-pregnant women (Brabin, 1983). Sequestration of IEs in the intervillous spaces of the placenta is the underlying mechanism for onset of pregnancy associated malaria (PAM). Impact of PAM on mother and fetus contributes to premature delivery, intrauterine growth retardation, stillbirth, maternal anemia, and increased neonatal and maternal mortality (Menendez, 1995). In endemic areas, PAM is distributed in a parity dependent manner being more common and severe among paucigravidae women as compared to multigravidae women. This indicates that protective immunity to PAM is acquired as a function of parity (Brabin, 1983). In sub-Saharan Africa alone approximately 25 million pregnant women are at risk of \textit{P. falciparum} infections every year, and one in four women have evidence of placental infection at the time of delivery. Low birth weight associated with malaria in pregnancy is estimated to result in 100,000 infant deaths in Africa each year. \textit{P. vivax} infections can also lead to severe malaria in pregnant women albeit of milder nature. In a study on pregnant women in Thailand, \textit{P vivax} malaria was found to be more common in primigravidae than in multigravidae and was associated with mild anemia and an increased risk of low birth weight. However, unlike \textit{P. falciparum} malaria, \textit{P vivax} malaria in pregnant women was not associated with miscarriage, stillbirth, or preterm delivery in this study (Nosten et al., 1999).

The proteoglycan chondroitin sulphate A (CSA) was identified as the major host receptor implicated in PAM. Parasites isolated from placenta preferentially bound CSA, whereas parasites from non-pregnant hosts rarely bound CSA (Fried and
Duffy, 1996). Initially, VAR1CSA was identified as the parasite ligand binding to CSA. However the major parasite ligand involved in PAM was later identified as VAR2CSA. Var2CSA is a member of the \textit{P. falciparum} Erythrocyte Membrane Protein 1 (PfEMP1) family, and it is the leading vaccine candidate for PAM. The estimated molecular weight of VAR2CSA is 350 kDa. It has six Duffy binding-like domains (DBLs), out of which four can bind to CSA namely, DBL-2x, DBL-3x, DBL-5e and DBL-6e. The \textit{var2csa} gene is found in all parasite isolates but is transcriptionally up regulated only in placental isolates and laboratory parasites selected to bind CSA (Salanti et al., 2003). Experiments with \textit{var2csa} knock out parasites suggest that it is the major parasite ligand involved in massive adhesion in the placenta (Viebig et al., 2007).

1.4.2.4 Severe malaria (Other)

1.4.2.4.1 Prostration
Prostration is defined as inability to sit unaided in children old enough to be able to sit, or inability to breastfeed in children too young to be able to sit. Prostration may be the most prevalent sign among severe malaria cases in endemic areas. For example more than half of the children (55%) admitted to hospital with severe malaria in rural Mozambique suffer from prostration (Bassat et al., 2008). There is a difference of opinion about pathogenesis of prostration; some consider it a sign of central nervous system (CNS) dysfunction while others suggest that it is a state of generalized weakness involving severe systemic illness. Frequent occurrence of seizures in malaria infected children manifesting prostration suggest neurological involvement (Idro et al., 2007). However, prostration is presented in other illnesses not involving CNS, suggesting systemic involvement. The mechanisms, by which malaria leads to prostration remains poorly understood.

1.4.2.4.2 Acidosis and respiratory distress
Blood lactate levels >5 mmol/L are suggestive of acidosis. Acidosis may lead to respiratory distress, which is defined as the presence of any of the following signs: alar flaring, chest recession (intercostal or subcostal), the use of accessory muscles of respiration, or abnormally deep (acidotic) breathing. Multiple factors are involved in the pathogenesis of acidosis, which is a result of high lactate levels beyond bodies
buffering capacity. Anorebic respiration in poorly perfused tissues may lead to acidosis. Possible mechanisms include an increase in tissue oxygen demand in severely ill patients, reduction in peripheral blood flow in hypovolemic patients and obstruction in blood flow leading to decreased tissue oxygen supply due to sequestration. Acidosis deteriorates the complications in severe malaria patients. Acidosis in combination with coma is a risk factor for malaria related death as compared to coma and acidosis as isolated findings (English et al., 1997).

1.4.2.4.3 Hypoglycemia
Hypoglycemia is a condition in which blood glucose levels are lower than value of normal (70-110 mg/dl). Glucose levels of 40 mg/dl and below constitute severe hypoglycemia, a life-threatening emergency. Hypoglycemia is common in malaria; as malaria parasitized erythrocytes utilize glucose 75 times faster than uninfected cells. In addition, treatment with quinine and quinidine stimulate insulin secretion (hyper-insulinamia), reducing blood glucose.

1.4.2.4.4 Multiple seizures
Multiple seizures are defined as two or more generalized convulsions within a 24 hours period. Pathogenesis of multiple seizures involves CNS. In malaria endemic areas falciparum infections are a major cause of multiple seizures. In a study on Kenyan children admitted to hospital over 80% of the seizures were associated with infections, with 58% cases due to falciparum infections (Idro et al., 2008).

1.5 Cytoadherence of \textit{P. falciparum}
\textit{P. falciparum} exhibits unique adhesive phenotypes which are collectively referred to as cytoadherence. Cytoadherence leads to sequestration of IEs in host blood vasculature and is thought to be a major virulence factor in \textit{P. falciparum} malaria. Several lines of evidence support cytoadherence as a main process leading to severe malaria. First, among all human malaria parasites only \textit{P. falciparum}, which is responsible for almost all the malaria related deaths is known to sequester. Second, autopsy studies have shown sequestered IEs containing trophozoites and schizonts in capillaries and post-capillaries venules of the brain and other organs of individuals
who had died of cerebral malaria (MacPherson et al., 1985; Nakazawa et al., 1995; Taylor et al., 2004). Third, estimation of the sequestered parasite biomass based on plasma histidine-rich protein-2 shows that disease severity increases with increased parasite sequestration (Dondorp et al., 2005). Finally, organ-specific accumulation of 

*P. falciparum* suggests that IEs can exhibit preferential binding in the body (Montgomery et al., 2007). Cytoadherence can lead to obstruction in blood flow (Beare et al., 2009), endothelial cell activation (Turner et al., 1994) and release of proinflammatory cytokines (Tongren et al., 2000). Local disturbance in physiology in certain organs such as the brain may lead to severe malaria. However, recent reports that platelets (McMorran et al., 2009) and macrophages (Patel et al., 2004) can kill *P. falciparum* IEs, suggests that at least some of cytoadherence phenotypes may be a result of host innate immune responses.

There are three major known adhesion phenotypes of *P. falciparum* IEs, namely adhesion to host endothelium, platelet-mediated clumping and rosetting. Cytoadherence, leading to sequestration of IEs in host organs, offers the parasite the possibility of avoiding passage through the spleen and its non-specific clearance mechanisms. Although this has not been formally proven, the rapid loss of sequestration in splenectomized animals strongly supports this hypothesis (David et al., 1983). For the host, sequestration may be a pathological event of varying degrees depending on the site and extent of sequestration. Blocking sequestration by means of vaccines or drugs may thus protect against severe malaria. However, the enormous receptor-ligand diversity involved and poorly understood pathogenic mechanisms have severely hindered any significant progress in this direction.
The parasite ligands involved in cytoadherence are almost exclusively PfEMP1s. Mature stage *P. falciparum* IEs have electron dense structures on their surface called knobs. Knobs are the sites where parasite ligands for cytoadherence are localized. In static adhesion assays irrespective of presence or absence of knobs, parasite lines can adhere to endothelial cells. However, under physiological shear stress conditions only parasite lines expressing knobs can adhere to host receptors, suggesting that knobs may be necessary for *in-vivo* sequestration (Crabb et al., 1997). Ring stage *P. falciparum* IEs do not show cytoadherence. However, febrile temperature may induce early PfEMP1 trafficking to IE membrane resulting in cytoadherence of ring stage IEs (Udomsangpetch et al., 2002).

1.5.1 Adhesion to host microvascular endothelial cells

In peripheral blood smears of *P. falciparum* infected patients only ring stage IEs can be detected, because trophozoite and schizont sequester in host capillary and post-capillary venules. The microvasculature in different host organs is heterogeneous and expression of several known cytoadherence receptors is modulated by local environmental factors such as proinflammatory cytokines. Accordingly the extent of adhesion of IEs may differ in different host organs. Adhesion to host blood microvascular endothelial cells in placenta and brain is implicated in pathogenesis of
pregnancy associated malaria and cerebral malaria respectively. *P. falciparum* uses a variety of endothelial cell surface receptor for adhesion such as thrombospondin (TSP) (Roberts et al., 1985), CD36 (Ockenhouse et al., 1989), ICAM1 (Berendt et al., 1989), platelet/endothelial cell adhesion molecule (PECAM/CD31) (Treutiger et al., 1997), vascular cell adhesion molecule-1 (VCAM-1) (Ockenhouse et al., 1992), endothelial leukocyte adhesion molecule-1 (ELAM-1) (Ockenhouse et al., 1992), normal immunoglobulin (IgG) (Flick et al., 2001), CSA (Fried and Duffy, 1996; Rogerson et al., 1995) and hyaluronic acid (HA) (Beeson et al., 2000). Disregarding the levels of adhesion, CD36 (Chaiyaroj et al., 1996) and TSP (Sherwood et al., 1987) are used by almost all the *P. falciparum* field isolates whereas CSA is used only by the placental isolates as adhesion receptors. Under flow conditions adhesion of *P. falciparum* IEs to host vascular endothelium is a multi-step process involving margination, rolling, and static arrest, which may require multiple receptor–ligand interactions (Cooke et al., 1994), (McCormick et al., 1997), (Ho and White, 1999).

Host genetic factors such as sickle cell trait also affect cytoadherence. The surface expression of major adhesion ligands PfEMP1 is least in mature stage *P. falciparum* IEs from homozygous recessive sickle trait erythrocytes (SS) followed by heterozygous (AS) and maximum in non-sickle erythrocytes (AA). Accordingly, SS and AS erythrocytes have reduced levels of adhesion to endothelial cells and rosetting relative to AA erythrocytes infected with *P. falciparum* (Cholera et al., 2008; Fairhurst et al., 2005).

### 1.5.2 Platelet-mediated clumping

*P. falciparum* IEs were observed to form aggregates which were earlier attributed to adhesion of IEs to other IEs. This aggregation was termed as auto-agglutination (Roberts et al., 2000). It was later realized that platelets were responsible for the phenomenon of auto-agglutination and this phenomenon is now referred to as platelet-mediated agglutination or clumping (Pain et al., 2001). The two receptors identified for platelet-mediated clumping are CD36 and P-selectin (Wassmer et al., 2008). Evidence for association of platelet-mediated clumping with severe malaria (Pain et al., 2001), severe anemia (Wassmer et al., 2008), cerebral malaria (Wassmer et al., 2008), and high parasitemia (Chotivanich et al., 2004) have been reported. However, in other studies no association between platelet-mediated clumping and severity has been observed (Rowe et al., 2009).
Platelets are increasingly recognized as a component of the innate immune system. Platelet secreted peptides are reported to kill bacteria (Taylor et al., 2006) and fungi (Yeaman et al., 1993). Recently, platelets were reported to kill *P. falciparum* (McMorran et al., 2009). In the light of such emerging evidence, platelet-mediated clumping appears to be a host mechanism to kill malaria parasites. At low parasitemia platelets can effectively protect *P. chabaudi* infected mice from severe malaria, however as the parasitemia increases platelets becomes ineffective in controlling parasitemia. At this point platelet-mediated clumping may become detrimental to the host, leading to severe malaria.

1.5.3 Rosetting

Another cytoadherence phenotype is the ability of *P. falciparum* IEs to adhere to UEs to form rosettes (David et al., 1988). Unlike sequestration, rosetting is a property of only some strains, varying quite dramatically in the extent to which they form rosettes (Wahlgren et al., 1992). Host receptors for rosetting include complement receptor 1(CR1) (Rowe et al., 2009), heparan-sulphate-like molecules (Chen et al., 1998) and blood group antigens A and B (Carlson and Wahlgren, 1992). Rosettes are observed in the *falciparum* patients with O blood group, however these rosettes are smaller and more easily disrupted than rosettes formed in group A, B, or AB erythrocytes (Carlson and Wahlgren, 1992; Taylor et al., 2006). Rosetting is reported to be associated with severe malaria (Rowe et al., 2009), cerebral malaria (Carlson et al., 1990; Treutiger et al., 1992) and severe anemia (Newbold et al., 1997). However in other studies no association between rosetting have been reported (al-Yaman et al., 1995; Traore et al., 2000). Polymorphism in CR1 (Cockburn et al., 2004) and presence of O blood group are known to protect against severe malaria probably by reducing rosetting (Rowe et al., 2009).

1.6 Antigenic variation and variant surface antigens of *P. falciparum*

For a parasite to successfully establish and propagate in the host, the parasite must avoid constant surveillances by the host immune system. Malaria parasite uses two mechanisms to change its antigenic properties, namely, antigenic diversity and antigenic variation. Antigenic diversity is defined as the expression of antigenically different alleles of a gene in different parasite strains, whereas antigenic variation is the process by which a clonal parasite strain can switch its antigenic phenotype. The
fact that acquired immunity to \textit{P. falciparum} has a distinct strain-specific component is suggestive of antigenic diversity. Studies on infections with \textit{P. falciparum} for therapeutic purpose of neurosyphilis showed that heterologous strains result in higher parasitemia upon re-infection as compared to homologous strains (Jeffery, 1966). Evidence for antigenic variation was first suggested by immuno fluorescence studies, which showed that parasites isolated during secondary and recrudescent peaks in monkeys expressed erythrocyte associated surface antigens that were different from parasites isolated during the primary infection (Hommel et al., 1983). All malaria parasites exhibit antigenic variation; however, this phenomenon is best studied in \textit{P. falciparum} largely because of its involvement in cytoadherence.

There are atleast 4 families of proteins involved in antigenic variation of \textit{P. falciparum} namely, PfEMP1, SURFIN, RIFIN and STEVOR.

\subsection{1.6.1 PfEMP1}

The first identified variant antigen on the surface of malaria-infected erythrocytes was schizont infected cell clumping antigens (SICA) in \textit{P. knowlesi} (Howard et al., 1983). The first variant antigen family identified in \textit{P. falciparum} was PfEMP1. Members of PfEMP1 family are large protein molecules with multiple adhesive domains. The ability of \textit{P. falciparum} strain specific sera to block specific cytoadherence phenotypes (David et al., 1983) and pull down PfEMP1 with specific molecular weights (David et al., 1988) suggested that cytoadherence of \textit{P. falciparum} may be mediated by these variant surface antigens (Biggs et al., 1991). \textit{Var} genes that encode PfEMP1 (Smith et al., 2000) were the first identified gene family responsible for antigenic variation and are widely accepted as the major player in antigenic variation in \textit{P. falciparum}. About 60 \textit{var} genes exist in the \textit{p. falciparum} genome. In a clonal parasite population, expression of \textit{var} genes switches at a rate of about 2\% per erythrocyte cycle. Switching of \textit{var} gene expression attributes new antigenic and cytoadherence phenotypes to the IEs (Roberts et al., 1992) and provides a mechanism for the parasite to avoid host immune response.

![Figure 1.4 Representative domain architecture of PfEMP1.](image)
1.6.1.1 Domains of PfEMP1

Members of PfEMP1 family are large protein molecules with sizes ranging between 200-400 kD. PfEMP1 are encoded by var genes that are made up of two exons. The first exon encodes the extracellular region and transmembrane domain. The intracellular region that contains acidic terminal segment (ATS) is encoded by the second exon. Extra cellular domain of PfEMP1 contains several domains namely N-terminal sequence (NTS), Duffy-binding like domains (DBLs), C2 domain and cysteine rich inter domain regions (CIDRs). NTS is the most N terminal domain, which is present in all PfEMP1. Length of NTS varies between 75-107 amino acids, with a conserved central alpha helical region. NTS is a non adhesive domain and may have role in PfEMP1 transport and localization on erythrocyte surface.

Duffy-binding like (DBL) domains are present in multiple numbers in PfEMP1. DBL domains are so named because they share sequence homology with the receptor binding domain of P. vivax Duffy-binding protein (PvDBP) and P. knowlesi Duffy-binding protein (PkDBP) that are expressed by merozoites and bind the Duffy antigen to mediate invasion of erythrocyte. Apart from PfEMP1, DBL are also present in erythrocyte binding antigens (EBA) such as P. falciparum EBA-175. EBA-DBLs are characterized by 12 invariant cysteine residues, conserved hydrophobic amino acid sequence and absence of length variation among homologous EBA-DBLs. PfEMP1-DBL domains on the contrary exhibit large length variations and only first 10 of the invariant cysteines are conserved among the PfEMP1 family members. PfEMP1-DBL domains are classified in 5 sequence classes that are named as DBL-α, β, γ, δ and ε. All DBL sequence classes contain 10 homology blocks, within which are present conserved sequence class specific residues. Ten hyper-variable blocks are present flanking the homology blocks, which have few conserved residues and account for much of the sequence variation found in DBL domains.

There are three CIDR sequence classes, namely, CIDR α, β and γ. On the basis of function CIDR sequences can be divided into three regions, M1, M2 and M3. CIDR M1 and M2 regions are more conserved then M3. Region M1 contains 6 invariant cysteine residues. Region M2 of MC var I has been shown to be the minimal binding region for CD36. All CIDR sequence classes contain a cysteine rich motif (CX_{7-12}CX_{3-5}CX_5CX_{1-2}CX_3WX_{7-9}W) in M2 region. Region M3 is the most divergent
among the three regions and generally contains stretches of charged amino acid residues.

The C2 domain varies in length between 140-217 amino acids. There are conserved motifs in C2 domain, which are hallmark of C2 domains such as YX_5 YXHXE, CX_2QX_2FC, and ACXC. DBLβ-C2 domains from the JDP81cvar PfEMP1 have been reported to bind ICAM1 (Chattopadhyay et al., 2004).

1.6.1.2 PfEMP1 domain arrangement and nomenclature
Domains within PfEMP1 are not arranged in random sequence and certain domain pairs are present at high frequency. NTS is always the N terminal most domain in PfEMP1, and is always followed by DBLα domain. The combination of DBLα-CIDRα combination is observed in high frequency, however, DBLα – CIDRγ domains are also present, example include R29 PfEMP1. The NTS, DBLα and CIDR domains composes the head of PfEMP1. The PfEMP1 head structure may be followed either by DBLβ, DBLγ or DBLδ domains. C2 domain, when present is always found in combination and downstream to DBLβ domains. The CIDRβ or γ domains always follow DBLδ domains.

From the N-terminal, the DBL and CIDR domains are numbered 1, 2, 3, …n. For example, the first DBLα will be named DBL1α, and the first CIDRα following it will be named as CIDR1α.

1.6.1.3 Var gene organization
In P. falciparum 3D7 two third of the 61 var genes are located in the sub-telomeric regions of 14 chromosomes while rest are located in the central chromosomal regions. Typically, there may be one, two or three var genes in each chromosomal region, followed by a group of rif, stevor or other multi-gene families. Many var genes on sub-telomeric regions contain two var genes arranged in tail-to-tail fashion relative to each other with one or more rif genes in between them. Var genes located in the central regions of a chromosome may exist as single or in tandem arrays arranged head to tail. These tandem arrays may contain 3-7 var genes in a cluster. Moreover, the 5’ non-coding upstream promoter region of var genes is found to be specific for var gene locations and direction of transcription. Based on homology, the upstream promoter sequence (Ups) is divided into four groups UpsA, UpsB, UpsC and UpsE. UpsA and UpsE containing var genes are sub-telomeric and transcribed towards the
telomere. UpsB containing \textit{var} genes are transcribed away from telomeres, and are either sub telomeric or central where they are present in tandem arrays with other UpsB and UpsC containing \textit{var} genes. UpsC\textit{var} genes are centrally located.

The functional difference in different Ups sequences is not known. However, UpsA and UpsB var genes have been reported to be associated with rosetting as well as severe malaria.

1.6.2 \textbf{SURFIN proteins}

SURFINS are high molecular weight proteins encoded by a small family of surface-associated interspersed genes (\textit{surf} genes). In 3D7 there are 10 \textit{surf} gene including 3 predicted pseudo-genes, which have sub-telomeric location.

![Figure 1.5 Domain architecture of SURFIN protein. (Winter et al., 2005)](image)

SURFIN proteins have an N terminal cysteine rich domain (CRD) followed by a region of high variability, a putative transmembrane domain and 1-4 tryptophan rich domains towards the C terminus. A 3D7 SURFIN, SURFIN\textsubscript{4.2} has been localized on the surface of mature stage IEs as well as near the apical end of merozoites. Anti-SURFIN\textsubscript{4.2} antibodies inhibits erythrocyte invasion by \textit{p. falciparum} merozoites, suggesting that SURFIN\textsubscript{4.2} may be involved in invasion process (Winter et al., 2005).

1.6.3 \textbf{RIFIN proteins}
RIFIN proteins are encoded by repetitive interspersed family (rif) genes. rif gene family is the largest multi-gene family of *P. falciparum* with 150-200 genes per haploid genome located in sub-telomeric regions of chromosomes. rif are small (approximately 1000 base pairs, bp), two exon genes. RIFIN proteins have conserved domain architecture beginning with a putative signal peptide at N-terminus, a conserved domain (C1), a variable region and a conserved C terminus domain (C2). Two predicted transmembrane domains flank the variable region, which is predicted to have extracellular location. Based on sequence similarity, RIFIN proteins are subdivided in two groups; A-type and B-type RIFIN. A-type RIFIN proteins are generally larger in size and numbers in 3D7 as compared to B-type RIFIN proteins. In A-type RIFIN proteins there is an extra 25 amino acid stretch present in conserved domain C1 that makes them larger than B-type RIFIN proteins. Another distinctive feature between the two RIFIN protein subtypes is the number of conserved cysteine residues. In A-type RIFIN there are 10 conserved cysteines whereas in B-type there are only 6-conserved cysteines. RIFIN proteins are expressed throughout human stage parasite life cycle; their function apart from antigenic variation remains largely unknown.

### 1.6.4 Sub-Telomeric Variable Open Reading Frame (STEVOR) family

*stevor* genes are located in sub-telomeric chromosomal regions, along with the *var* and *rif* genes. There are 28 putative *stevor* genes in 3D7 genome. *stevor* genes have similar two exon structure as *rif* genes, moreover STEVOR protein architecture is also quite similar to RIFINS.
STEVOR proteins have an N-terminal signal sequence followed by conserved region and a variable region. Like RIFIN, two transmembrane domains flank the STEVOR variable region. STEVOR proteins are localized on the surface of IEs and mediate antigenic variation.
1.7 Introduction

One of the important virulence mechanisms associated with *P. falciparum* infection is the unique ability of *P. falciparum* trophozoites and schizonts to sequester in the vasculature of diverse host organs. Sequestration of *P. falciparum* IEs in the microvasculature of the brain is associated with severe pathological outcome of cerebral malaria. *P. falciparum* IEs can also adhere to platelets to form platelet-mediated clumps, a cytoadherence phenomenon that is associated with severe disease [8-10].

The endothelial receptors used by *P. falciparum* for adhesion include TSP (Roberts et al., 1985), CD36 (Ockenhouse et al., 1989), ICAM1 (Berendt et al., 1989), PECAM (Treutiger et al., 1997), VCAM-1 (Ockenhouse et al., 1992), ELAM-1 (Ockenhouse et al., 1992), IgG (Flick et al., 2001), CSA (Fried and Duffy, 1996; Rogerson et al., 1995) and HA (Beeson et al., 2000). Expression of ICAM1 is upregulated on cerebrovascular endothelium and *P. falciparum* IEs co-localize with ICAM1 in cerebral vessels of patients who die of cerebral malaria suggesting that adhesion to ICAM1 plays a key role in cerebral sequestration. Adhesion of *P. falciparum* IEs to host vascular endothelium under flow conditions involves three distinct events, namely, margination, rolling and static arrest/tethering, which may require multiple receptor-ligand interactions. Adhesion to endothelial cells under flow requires binding of *P. falciparum* IEs to ICAM1 as well as CD36. Expression of ICAM1 on brain endothelium is upregulated during blood stage *P. falciparum* infection. However, the expression of CD36 on brain endothelial cells is minimal. Platelets, which have been shown to accumulate in brain microvasculature of patients who die of cerebral malaria, express CD36 on their surface and may act as bridges for adhesion of *P. falciparum* IEs with brain vascular endothelium. Alternatively, other as yet unidentified host receptors may play a role in adhesion of *P. falciparum* IEs to cerebral capillaries and for platelet-mediated clumping.

Adhesion of IEs to vascular endothelium is mediated by interaction of the PfEMP1 family of variant surface antigens with host receptors. Variant surface antigens are important targets of naturally acquired immunity in the adults living in endemic areas. Antibodies directed against PfEMP1s may block parasite adhesion to host receptors and thus protect from severe malaria. Adhesive domains of specific PfEMP1s such as VAR2CSA, which are involved in adhesion to CSA in placenta (Salanti et al., 2003), are considered as promising vaccine candidates against
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pregnancy associated malaria.
All mature stage *P. falciparum* IEs sequester completely, both in immune individuals with asymptomatic parasitemia and in nonimmune patients with life-threatening illness. Therefore, not all but specific cytoadherence phenotypes may be involved in the pathophysiological mechanisms leading to severe malaria. Correlations between adhesion to ICAM1 and severity have been reported by some (Newbold et al., 1997; Traore et al., 2000) but not by others (Rogerson et al., 1999). The pathophysiological significance of rosette and platelet-mediated clumping is also unclear. Some studies have shown correlation between rosetting and cerebral malaria (Carlson et al., 1990; Treutiger et al., 1992), severity of disease other than cerebral symptoms (Rowe et al., 1995) and severe anemia only (Newbold et al., 1997), but these results have not been replicated in other studies (al-Yaman et al., 1995; Traore et al., 2000). Similarly, platelet-mediated clumping has been associated with severe malaria (Pain et al., 2001), severe anemia (Wassmer et al., 2008) and cerebral malaria (Chotivanich et al., 2004; Wassmer et al., 2008), but other studies have not been able to show this association (Arman et al., 2007). Adhesion to CD36 has been implicated in uncomplicated malaria in few studies (Newbold et al., 1997; Rogerson et al., 1999; Traore et al., 2000). Moreover, recent reports suggest that some of cytoadherence phenotypes may even have a protective role in the context of the host innate immune responses (McMorran et al., 2009; Patel et al., 2004). Understanding role of specific cytoadherence phenotypes in malaria pathogenesis is important for developing novel anti-malaria interventions.

1.8 Objectives

Here we have studied use of gC1qR as a cytoadherence receptor by *P. falciparum*. Our specific objectives were as follows:

1. To test if *P. falciparum* uses gC1qR as a receptor for adhesion to human brain microvascular endothelial cells (HBMEC).
2. To test if *P. falciparum* uses gC1qR as a receptor for platelet-mediated clumping.
3. To identify potential ligand of *P. falciparum* that mediates its adhesion to gC1qR.
4. To investigate if gC1qR plays a role in the pathogenesis of severe malaria.