1. Introduction
Mainly as a result of the survival advantage due to the protection against malaria conferred by the heterozygous state, inherited red cell disorders are the most common monogenic diseases affecting over a billion people around the world. Malaria is the most serious and widespread parasitic disease of humans. Each year, up to 500 million people are infected with malaria parasites and a million (predominantly infants and young children) die as a consequence of the infection (1). Four species of the malaria parasite *Plasmodium* infect humans, but almost all deaths are the result of *P. falciparum* infection. The clinical symptoms of malaria manifest when parasites invade and multiply inside red cells. A large number of interactions occur between various malarial and red cell proteins during all phases of the parasite life cycle (Figure 1), starting at the initial stages of invasion and continuing through 48 hours of intra-erythrocytic development and eventual rupture of the infected red cell at the end of the parasite life cycle. Intracellular development of the parasite is accompanied by a number of striking structural, biochemical and functional changes in red cells, a subset of which are strongly associated with parasite induced modifications to the red cell membrane. The induced cellular changes are responsible for the clinical symptoms and pathologies associated with malaria including cerebral malaria and malarial anaemia. Alteration of membrane protein organisation and the adhesive and mechanical properties of red cells is of particular

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**Figure 1: Red cell life cycle of the malarial parasite, *P. falciparum***

The various parasite proteins (RESA, PfEMP1, PfEMP3, MESA and KAHRP) expressed at different stages of parasite development that interact with red cell membrane are indicated.
importance since these traits are directly linked to increased destruction of red cells and to the sequestration of parasitised red cells in the microvasculature of infected individuals and the subsequent development of severe and frequently fatal clinical syndromes of severe anaemia and cerebral malaria. A disease of this severity operating over the course of thousands of years of human evolution has selected for a number of red cell genotypes that offer protection against severe forms of the disease including sickle cell disease, thalassaemias, hereditary ovalocytosis and G6PD deficiency. It is important to note that none of these inherited red cell disorders completely prevent parasite infection; they only reduce the severity of the clinical manifestations thus reducing the incidence of mortality. We will briefly describe our current understanding of the mechanistic basis for membrane and cellular changes induced in red cells by the parasite and their contribution to various clinical manifestations and the potential mechanisms responsible for the protective effect of various red cell disorders against severe forms of malaria.

2. The red blood cell surface and malaria parasite invasion

Parasite invasion of red cells occurs when the extracellular form of the parasite, the merozoite (Figure 2A), released from infected red cell attaches to the surface of an uninfected red cell. In a relatively short period of 30 seconds the invasion process is complete (2). The initial contact between the merozoite and the red cell is random but for successful invasion the merozoite actively re-orients using actin filaments and myosin-based motors, to bring its apical end in contact with the red cell membrane (3). A junctional zone at the site of apposition is formed and a dense undercoating of the red cell membrane is observed in this region. Specialised organelles in the apical end of the polar merozoite, called rhoptries and micronemes, discharge a mixture of proteins including proteases, phospholipases and lipids. These discharged components initiate structural changes in the red cell membrane including membrane invagination, phosphorylation of membrane skeletal components and stripping of the underlying red cell membrane skeleton from the region of merozoite invasion. Entry of the merozoite into this prepared region follows, again by virtue of actin-myosin motors in the parasite. Material of host and parasite origin is recruited into the parasitophorous vacuole membrane that surrounds the developing parasite and it is here that the parasite resides for the remainder of its life time within the red blood cell (4).

The various parasite ligands that are involved in invasion are proteins that contain one or more Duffy-binding ligand domains, regions of sequence characterised by the presence of 12 conserved cysteine residues within tryptophan and tyrosine-rich sequences (5). A large number of such parasite proteins have been identified in the
merozoite of *P. falciparum*, including EBA175, EBL-1 and EBA-140, for which cognate receptors on red cells (glycophorin A, glycophorin B and glycophorin C, respectively) have been identified. The other major group of parasite ligands are a family of proteins designated as the reticulocyte binding proteins. As their name suggests, these proteins are responsible for the preferential invasion of merozoites.
into reticulocytes. Finally, the red cell anion transporter protein, band 3, has recently been implicated in an initial interaction between the merozoite and the red cell. There are undoubtedly further complexities to be unraveled in ligands and receptors involved in the invasion process and it is known that field isolates may manifest even greater variability than that found in the comparatively small number of laboratory isolates in which these pathways have been defined. Apart from the involvement of individual red cell surface molecules in the invasion process, it is now becoming clear that the overall arrangement of surface molecules is also important. Specific membrane anchored proteins frequently associate with cholesterol-rich sub-domains within the red cell membrane called lipid “rafts”. The presence of raft-associated proteins of both red cell and parasite origin in the parasitophorous vacuolar membrane suggest that they play a role in the transport of macromolecules into malaria-infected cells. Rafts have been shown to play a critical role in parasite invasion. Experiments with red cells depleted of lipid rafts demonstrate that they become resistant to invasion with malaria parasites and recent evidence suggests that this is related to G-protein-coupled receptor signalling mechanisms involving the red blood cell β2-adrenergic receptor and the Gαs G-protein sub-unit (6).

An important role for state of red cell hydration in efficiency of parasite invasion has also been documented (7). Red cell dehydration resulting in cell haemoglobin concentration > 37 g/dL leads to decreased efficiency of invasion and at a cell haemoglobin concentration > 41 g/dL the red cells are resistant to invasion. The mechanistic basis for the effects of state of cell dehydration on parasite invasion has not been defined.

In terms of natural selection of red cell variants that confer protection against malaria by decreasing invasion efficiency, there exist a number of examples with mutations in gene encoding red cell membrane proteins that are receptors for merozoite surface proteins, as well as increased cell dehydration seen in haemoglobinopathies. For example, there is high incidence up to 30% of Gerbich variant of glycophorin C in endemic areas of Papua New Guinea, high incidence of Band 3 variant (10 to 30%) with deletion of 9 amino acids in the cytoplasmic domain in Melanesia and other areas of Far East Asia, and a large number of glycophorin A and glycophorin B variants in endemic areas of Brazil. Red cell dehydration is a feature of red cells in sickle cell disease, HbSC and HbCC diseases which are highly prevalent in Africa. In these red cell disorders, 5 to 30 % of the red cells have cell haemoglobin concentration > 37 g/dL and the presence of such a large fraction of dehydrated red cells will prevent development of high degree of parasitaemia and hence decrease the severity of disease. The protective mechanisms of RBC variants against malaria are summarised in Table 1.
3. Alterations in the cytoplasm and membrane skeleton of red blood cells following malaria parasite invasion

Once inside the red blood cell, the malaria parasite resides within a vacuole created partly of material of host origin and partly of material secreted from parasite organelles called the rhoptries. The parasite starts to increase in size and over the duration of development digests 70% of haemoglobin obtained from the red cell cytoplasm, depositing the undigested haem residue in a polymerised pigment material called haemozoin. A number of parasite proteases have been suggested to digest haemoglobin including the cysteine-protease falcipains and the aspartic-protease plasmepsins (8). Members of these protease families also appear to have a role in rupture of the red cell to allow merozoite release at the end of the parasite life cycle. Maturation of the malaria parasite causes striking structural and morphological changes in the infected red cell including loss of normal discoid shape, perturbations in the mechanical and adhesive properties of the cell and alterations in the state of phosphorylation of red cell membrane skeletal proteins (9). The red cell becomes more spherical and its surface becomes punctuated by up to 10,000 distinct electron-dense elevations called knobs that are associated with altered cellular adhesive properties of infected red cells.

Bio-informatic analysis and experimental validation suggest that over 400 proteins produced by maturing parasites are exported into the red cell cytoplasm and a number of these exported proteins interact with membrane skeleton (Figure 3) (10). Parasite proteins that associate with the red cell membrane skeleton become insoluble in the non-ionic detergent Triton X-100, and this is often used as an

Table 1: Protective mechanisms of RBC variants against malaria

<table>
<thead>
<tr>
<th>Red cell phenotype</th>
<th>Potential mechanism(s) of protection against severe forms of malaria</th>
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<tbody>
<tr>
<td>Glycophorin A variants or deficiency</td>
<td>Reduced invasion efficiency due to altered red cell receptor</td>
</tr>
<tr>
<td>Glycophorin B variants or deficiency</td>
<td>Reduced invasion efficiency due to altered red cell receptor</td>
</tr>
<tr>
<td>Glycophorin C variants or deficiency</td>
<td>Reduced invasion efficiency due to altered red cell receptor</td>
</tr>
<tr>
<td>Hereditary ovalocytosis due to mutant band 3</td>
<td>Reduced invasion efficiency due to altered red cell receptor and as consequence of increased membrane rigidity (?)</td>
</tr>
<tr>
<td>Sickle cell disease and Hb CC disease</td>
<td>Reduced invasion efficiency due to red cell dehydration and decreased ability of infected red cells to adhere to endothelial cells due to reduced expression of adhesive ligands on infected red cells</td>
</tr>
<tr>
<td>Alpha thalassaemia</td>
<td>Decreased anaemia due to increased numbers of circulating red cells with reduced haemoglobin content</td>
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operational definition of skeletal association. The function of a subset of these proteins is beginning to emerge; however, most remain without an identified function, although they are clearly associated with major structural and functional changes that occur in infected red cells. The best studied of parasite proteins that associate with the red cell membrane are RESA, MESA, PfEMP-3, KAHRP and PfEMP1 (Figure 3) (9). While RESA, PfEMP-3 and MESA are distributed evenly around the membrane of infected red cell, KAHRP and PfEMP1 tend to cluster together in higher density beneath membrane knobs. In early maturing parasites, a number of these proteins, and others such as PfSBP1 and MAHRP appear to be associated with discrete membrane bound structures, known as Maurers’ clefts (Figure 2B), which are scattered throughout the cytoplasm of the infected red cell. Recent studies have documented that PfSBP1 associated with Maurers’ clefts plays a critical role in assembly of the various parasite proteins at the red cell membrane (11).

At least some of these exported proteins are critically important for the normal growth and pathogenicity of malaria parasites. For example, targeted deletion of the kahrp gene results in a failure to form knobs on the surface of the infected red cell and abrogates their ability to adhere to vascular endothelial cells under conditions of flow. Similarly, disruption of the Pfemp3 gene can affect the trafficking of PfEMP1 to the red cell surface with a consequential decrease in their ability to cytoadhere. Further, although the precise function of MESA remains to be defined, its failure to bind to protein 4.1 in the membrane skeleton results in intracellular parasite death.

Figure 3: The membrane skeleton of a malaria-infected red blood cell

Various parasite proteins exported into the red cell cytoplasm interact with proteins of the red cell membrane skeleton and alter its structure and function.
Significant progress is being made in identifying the binding domains in both parasite proteins and red cell proteins that mediate protein-protein interactions and the functional sequelae of these interactions (Figures 1 and 3) (12-15). MESA binds to the 30kDA membrane binding domain of protein 4.1R, which also binds to red cell proteins glycophorin C, band 3 and p55. MESA binding interferes with the binding of p55 and it is likely that some of the effects of malaria on red cell morphology and membrane mechanical properties are mediated by the interruption of host protein interactions. RESA, expressed at the ring stage of parasite development, binds to repeat 16 of beta-spectrin, thereby stabilising the spectrin dimer-dimer interaction and increasing membrane mechanical stability. This stabilisation is likely to be important in enabling the parasite to continue to develop without loss of the structural integrity of the red cell. KAHRP binds to repeat 4 of alpha-spectrin and also to the cytoplasmic tail of PfEMP1, the parasite ligand expressed on the surface of the infected red cell that mediates all of the adhesive interaction of infected red cells. KAHRP and PFEMP1 are part of electron dense knob structures and play a key role in modulating the avidity of the adhesive interactions. PfEMP3, expressed at the late stages of parasite development binds to C-terminus of alpha-spectrin, thereby destabilising the spectrin-actin-protein 4.1R junctional complex and decreasing membrane mechanical stability. This destabilisation is likely to be important in enabling the release of merozoites from infected red cells.

At the conclusion of the asexual cycle, the red cell is ruptured to release merozoites for a fresh round of red cell invasion. While the details of the cell rupture are still being elucidated, at least two distinct stages have been defined. In the first, an internal membrane that surrounds the parasite, the parasitophorous vacuole membrane, is disrupted and merozoites enter the red cell cytoplasm. At the second stage red cell membrane is disrupted facilitating the release of merozoites. The first stage is prevented by addition of E64, an inhibitor of cysteine proteases, whereas the second stage is prevented by addition of leupeptin, inhibitor of trypsin-like serine proteases and serine proteases. There are a number of candidate parasite proteases that could be the targets of these inhibitors including SERA, ABRA and the falcipains. Development of inhibitors of haemoglobin degradation and of the red cell membrane disruption could be a valuable new therapeutic option for treatment of malaria.

4. Adhesive interactions of infected red blood cells

Red cells infected by mature forms of P. falciparum become adhesive for a number of different cells including vascular endothelial cells, platelets and other infected or non-infected red cells 9, 16. Adherence to endothelial cells and platelets is referred
to as cytoadherence, adherence to uninfected red cells is called rosetting (Figure 2C) and adherence to other infected RBCs is termed autoagglutination (Figure 2D).

From the parasites’ standpoint, imparting an adhesive phenotype on the red blood cells in which they reside is the key to both its survival and its pathogenicity, preventing destruction of infected red cells in the spleen and allowing the microaerophilic parasites to sequester and mature in a relatively hypoxic environment within the deep microvasculature of a variety of organs. For the infected human, however, the consequences of sequestration are often extremely detrimental, causing obstruction of blood flow particularly in small diameter vessels of the microcirculation. All of these interactions are mediated by parasite ligands expressed on the surface of the infected red cell, \textit{P. falciparum} erythrocyte membrane protein 1 (PfEMP1) family of proteins. PfEMP1s are encoded by various members of the \textit{var} multi-gene family. More than 60 \textit{var} genes have been identified within the \textit{P. falciparum} genome, but within one infected red cell, only one of the complement of \textit{var} genes appear to be transcribed. Immune responses against one PfEMP1 may not be effective against an antigenically-distinct form and the capacity to change which gene is transcribed results in a form of antigenic variation which favours survival of the parasite.

### 4.1 Cytoadherence

Cytoadherence has been studied in a number of \textit{in vitro} and \textit{ex vivo} systems and infected red blood cells have been demonstrated to be capable of adhering to a number of different receptors that are expressed on the surface of vascular endothelial cells or on syncytiotrophoblasts that line the placenta. The repertoires of receptors to which infected red cells can bind are diverse and include members of the immunoglobulin super family, integrins and glycosaminoglycans. These include CD36, ICAM1, VCAM1, E-selectin, PECAM1, chondroitin sulphate A and hyaluronic acid. The development of cerebral malaria, possibly the worst and most lethal complication of falciparum malaria infection is related to sequestration of infected red cells in the vasculature of the brain. The increased adhesiveness of infected red cells is of considerable interest, both because of pivotal role in the frequent development of severe clinical syndromes such as cerebral malaria, but also as a general example of pathological and inappropriate cell-cell adhesive interactions. It is now clear that the ability of PfEMP1 to mediate adhesion also depends on its interaction with other exported parasite proteins that are located inside infected red cells and that interact specifically with the red cell membrane skeleton. For example, if the cytoplasmic domain of surface-expressed PfEMP1 does not bind to the histidine-rich parasite-produced protein KAHRP, which clusters PfEMP1 at knobs on the red cell surface, then the infected red cells are incapable of binding.
to the vascular endothelium under normal circulatory flow. Furthermore, the juxtaposition of these accessory proteins with the red cell membrane skeleton also greatly reduces red cell deformability and dramatically alters their membrane mechanical properties which, in turn, compromise their ability to circulate. A recent ultrastructural study revealed fewer and smaller knobs at the membrane skeleton of *P. falciparum*-infected HbCC red cells and Hb AS red cells which may affect the amount or distribution of PfEMP1 or other antigens expressed on the red cell surface which could affect their ability to cytoadhere. This is an interesting hypothesis and could offer a possible mechanism for protection against severe disease in HbCC and sickle cell disease.

### 4.2 Rosetting

The binding of mature-parasite infected red cells to one or more uninfected red cells is a phenomenon referred to as rosetting (17). Clinical isolates show a varying degree of rosetting, with up to 70% of infected cells from some individuals forming rosettes. The size of individual rosettes can vary considerably and a single infected red cell may be surrounded by as many as 10-20 non-infected cells. The precise physiological advantage for rosette formation remains to be fully defined but it appears that it is likely that rosetting increases the efficiency of parasite invasion by targeting merozoites emerging from newly ruptured schizonts into closely juxtaposing red cells. It is interesting to note that numerous studies have shown a correlation between the tendency for uninfected cells to rosette in vitro and the severity of clinical disease. The type 1 complement receptor (CR1; CD35) has been identified as a receptor on the surface of non-infected red cells and binding to this antigen appears to be mediated through an interaction with the DBL-α domain of PfEMP1. It is highly likely that CR1 is not the only molecule with this function. Other molecules for which there is at least some experimental evidence that they can act as rosetting receptors include CD36 and several carbohydrate moieties, some of which are constituents of heparin, and others which are ABO blood group antigens.

### 5. Anaemia

Normochromic and normocytic anaemia is a common and frequently severe complication of malaria, particularly in young children and pregnant women, and in some endemic areas it can account for more than 50% of malaria-associated mortality. The pathogenesis of severe anaemia (defined as Hb <5g/dL) during malaria infection is not fully understood. Both increased destruction of infected red cells and decreased production of red cells in response to anaemia due to dyserythropoiesis appear to be involved. There is some evidence of accelerated
destruction of uninfected red cells but the mechanism by which uninfected red blood cells are destroyed has not been fully elucidated. Marked splenomegaly during acute infection reflects extensive sequestration of red cells by the spleen resulting in anaemia (18). The pathophysiology of severe malarial anaemia remains an important yet relatively neglected area of research and further studies to provide convincing explanations for the pathogenesis of this important complication of malaria are urgently needed.

6. Recent advances
The complete sequence of the malaria genome and the establishment of a transfection system for the red blood cell stages of \textit{P. falciparum} have set the stage for rapid progress in our understanding of the function of parasite proteins in the altered properties of infected red blood cells. It is anticipated these advances in combination with significant advances in our understanding of red cell membrane structure and function will offer opportunities for the discovery of new and urgently needed therapeutic targets for the treatment of malaria.

\textbf{Acknowledgements}

\textit{We would like to thank our colleagues and long time collaborators, Ross Coppel, Brian Cooke, and Kasturi Haldar, for teaching us about malaria and for their sustained and productive collaborative efforts with us over many years.}

\textbf{References}


Multiple Choice Questionnaire

To find the correct answer, go to http://www.esh.org/iron-handbook2009answers.htm

1. Malarial infection is a global health problem affecting a large number of individuals. How many humans are infected each year and how many deaths are attributable to malaria?
   a) 10 million people infected every year and 100,000 deaths
   b) 1 million people infected every year and 10,000 deaths
   c) 100 million people infected every year and 1,000,000 deaths
   d) 1 billion people infected every year and 1 million deaths

2. A number of inherited disorders have been implicated in offering protection...
against morbidity of malaria infection including sickle cell disease, thalassaemias, hereditary ovalocytosis, G6PD-deficiency. What level of protection do these disorders offer against malaria?
a) No protection
b) Partial protection
c) Complete protection

3. What are the common clinical manifestations of malarial infection?
a) Coma
b) Fever
c) Anaemia
d) All of the above

4. The causes of severe malarial anaemia are:
a) Increased destruction of infected red cells
b) Increased destruction of uninfected red cells
c) Decreased red cell production in response to anaemia due to dyserythropoietis
d) All of the above

5. What cell-cell interactions contribute to clinical manifestations of malarial infection?
a) Infected red cell adhesion to vascular endothelial cells
b) Auto-agglutination of infected red cells
c) Rosette formation of uninfected red cells with infected red cells
d) All of the above