Discussion
Despite appropriate case management and administration of effective anti-malarial drugs, case fatality rates for severe malaria remains unacceptably high (Dondorp et al., 2005). Several ancillary treatments have been tried in combination with conventional anti-malarial drugs (Dondorp et al., 2007; Looareesuwan et al., 1999; Taoufiq et al., 2008; Vogt et al., 2006; Wassmer et al., 2005) (Boggild et al., 2009). However, to date, there is little evidence to support the use of any of them in severe cases of *P. falciparum* infection. A greater understanding of the pathogenesis of severe malaria is essential for the rational development of novel intervention strategies to prevent and treat severe malaria.

The unique cytoadherence properties of *P. falciparum* are important parasite virulence factors leading to severe manifestation of malaria. Inhibiting cytoadherence by vaccines or drugs is regarded as a promising anti-severe malaria intervention. However, cytoadherence is a complex process involving several phenotypes, such as platelet-mediated clumping, rosetting and adhesion to endothelium. Moreover each cytoadherence phenotype is mediated by multiple receptor-ligand interactions. All cytoadherence phenotypes are not associated with severe malaria. In fact adhesion to CD36 is inversely associated with severe malaria. Thus, understanding specific receptor-ligand interactions associated with severe outcome of malaria is essential for developing effective interventions.

In this study we have identified gClqR, as a novel receptor for *P. falciparum* adhesion to endothelium and platelet-mediated clumping. We also report the identification of a *var* gene PFD1015c as a potential *P. falciparum* ligand for adhesion to gClqR. Moreover we report that *P. falciparum* adhesion phenotypes such as platelet-mediated clumping and adhesion to gClqR may have an important role in pathophysiology of severe falciparum malaria.

gClqR is a 32 kD protein, that serves as a receptor for the globular head of complement component C1q. It is also reported to bind to hyaluronic acid and referred to as hyaluronic acid binding protein1 (HABP1) as well. gClqR is synthesized as a pre-protein of 282 amino acids. The N-terminal 73 amino acids are cleaved to yield a mature protein, which is highly acidic with a calculated pI of 4.6. The 73 amino acid pre-sequence is predicted to have signal sequence as well as a mitochondrial targeting sequence. It has only one cysteine at residue 186. As a result it does not have any intra-chain disulphide bonds. gClqR also does not form inter-chain disulphide bonds as evident from its migration corresponding to a 32kD protein.
Discussion

on non-reducing SDS-PAGE. However in GPC three peaks corresponding to gC1qR monomer, dimer and trimer are observed. The gC1qR amino acid sequence contains three consensus N-glycosylation sites at residues 114 (NGT), 136 (NNS), and 223 (NYT); a PKC phosphorylation site at residue 207; a tyrosine kinase recognition site at position 268; and a myristylation site at position 252. Interestingly the mature gC1qR molecule does not have a predicted transmembrane domain or a consensus GPI anchor site. The crystal structure revealed that gC1qR has seven consecutive antiparallel β-strands flanked by one N-terminal and two C-terminal α-helices. Three monomers form a doughnut-shaped quaternary structure with a partially covered channel of 10 Å (Jiang et al., 1999).

gC1qR is expressed on the surface of diverse cell types including endothelial cells, platelets and dendritic cells. gC1qR has also been localized inside nucleus and mitochondria. gC1qR is a multi ligand binding protein and its known binding partners include kininogen, vitronectin, cC1qR, splicing factor SF2, Hepatitis C virus core protein and Staphylococcus aureus protein A. gC1qR is used as a cell surface receptor by Staphylococcus aureus and Listeria monocytogenes for host cell entry (Braun et al., 2000; Peerschke et al., 2006) and suppression of immune function by Hepatitis C virus (Kittlesen et al., 2000; Yao et al., 2004).

We confirmed localization of gC1qR on the surface of various human endothelial cells such as HBMEC, PBMEC and HUVEC. Given its localization on endothelial cells, and multi-ligand binding abilities we hypothesized that gC1qR may serve as a receptor for P. falciparum adhesion to host endothelial cells.

We have shown that P. falciparum field isolates and laboratory strains can adhere to gC1qR (Table 3.2). About 50% P. falciparum field isolates and laboratory strains tested adhere to gC1qR. Adhesion prevalence for gC1qR was lower than adhesion prevalence for ICAM1 (about 60%) and CD36 (100%). To detect expression of gC1qR by flow cytometry, we used mouse sera raised against gC1qR that specifically recognizes gC1qR in cell lysates of HBMEC, HBMEC and platelets (Figure 3.5). Using flow cytometry, we confirmed that gC1qR is expressed on the surface of HBMEC, PBMEC and HUVEC (Table 3.1). Upon TNF-α stimulation of endothelial cells, surface expression of gC1qR does not change. This is contrary to the surface expression of ICAM1 which gets upregulated upon TNF-α stimulation.

Selection of P. falciparum IGH-CR14 and 3D7 for adhesion to gC1qR allowed separation of gC1qR adherent parasites IGH-CR14+ and 3D7+ from gC1qR
non-adherent IGH-CR14+ and 3D7-. Selection of IGH-CR14 for adhesion to gClqR resulted in increased adhesion of IEs to HBMECs (Table 3.3), suggesting that these parasites use gClqR to adhere to human endothelial cells. Indeed, r-gClqR, as well as mouse sera raised against gClqR, blocked the adhesion of IGH-CR14+ to HBMEC, confirming that \textit{P. falciparum} IGH-CR14+ use gClqR as a receptor for adhesion of IEs to endothelial cells (Figure 3.8). The demonstration that \textit{P. falciparum} IEs can use gClqR as a receptor to adhere to microvascular endothelial cells suggests that adhesion to gClqR may play a role in parasite sequestration \textit{in vivo}.

Previous studies using gClqR specific monoclonal antibodies have suggested that gClqR is expressed on the surface of activated platelets (Peerschke et al., 2003). Here we show by flow cytometry that gClqR is expressed on the surface of resting platelets and surface expression of gClqR increases once platelets get activated (Table 3.4). Given its presence on surface of platelets, we explored if \textit{P. falciparum} can use gClqR as a receptor for platelet mediated clumping. IGH-CR14+ which adheres to gClqR but not to CD36, formed clumps in the presence of platelets. Formation of clumps by IGH-CR14+ was inhibited by soluble gClqR and mouse sera raised against gClqR. These observations demonstrate that \textit{P. falciparum} IEs can use gClqR as an alternative receptor to bind to platelets and form clumps.

Previously reported \textit{P. falciparum} ligands that mediate cytoadherence are almost exclusively PfEMP1s. Increase in the levels of adhesion to gClqR upon selection of \textit{P. falciparum} IGH-CR14 and 3D7 for adhesion to gClqR suggests that \textit{P. falciparum} variant surface antigens mediate adhesion to gClqR (Table 3.3 and 3.5). Binding experiments with IGH-CR14+ and IGH-CR14- IEs using r-gClqR, showed gClqR binds to membrane fraction of IGH-CR14+ IEs that contains PfEMP1 (Figure 3.10). This observation suggested that \textit{P. falciparum} ligand for gClqR may be a member of PfEMP1 family. Indeed real time PCR confirmed that at least 5 var gene transcripts are up-regulated > 10 folds in 3D7+ as compared to 3D7-, suggesting that PfEMP1 encoded by these var genes may be involved in adhesion to gClqR. Expression of var gene PFD1015c transcript was >30 fold higher in 3D7+ as compared to 3D7- (Figure 3.11). We selected PFD1015c for further studies. Bioinformatics analysis predicted four adhesive domains in PFD1015c. gClqR binding experiments with PFD1015c adhesive domains however showed that biotinylated gClqR did not bind to any of the four domains expressed on COS cell surface (Table 3.6). There can be several possible reasons for which we could not
identify the PfEMP1 domain that binds gC1qR. Firstly, we might have been working with a wrong \textit{var} gene. Although we have picked-up the \textit{var} gene which was most upregulated in 3D7+ as compared to 3D7-, the other \textit{var} genes which were comparatively less upregulated in 3D7+ may encode the principal PfEMP1 mediating adhesion to gC1qR. At transcript levels PFD1015c was most upregulated, but at protein levels it may not be as high. Moreover, PfEMP1 encoded by PFD1015c may not have the highest binding affinity to gC1qR, as compared to other less upregulated \textit{var} genes in 3D7+. Secondly, our method to study the receptor ligand interaction may not be correct. Biotinylation may destroy epitopes on gC1qR involved in binding to parasite ligand. Thirdly, multiple domains of PFD1015c may be involved in binding to gC1qR. And lastly, post translational modification in COS cells may destroy the gC1qR binding epitopes on PFD1015c domains.

The presence of gC1qR on human endothelial cells and platelets, and its involvement in adhesion of IEs to HBMEC and platelet mediated clumping suggest that cytoadherence to gC1qR may have an important role in pathophysiology of severe malaria. Particularly, presence of gC1qR on brain endothelial cells, suggests that adhesion to gC1qR may lead to severe malaria complications involving CNS, such as malaria associated coma, multiple seizures and/or prostration.

To evaluate the role of various cytoadherence phenotypes in pathogenesis of severe malaria, we carried out an age and gender matched case control study in children presenting with severe and uncomplicated malaria at Manhica Health Research Centre, Manhica Mozambique. The 92 patients participating in this study were representative of severe falciparum malaria as seen in Manhica (Mozambique) (Bassat et al., 2008), which is characterized by a high prevalence of prostration, severe anaemia and ARD. Overall, \textit{P. falciparum} isolates infecting Mozambican children were characterized by their predominant adhesion to CD36 (88\%), rosetting (69\%) and platelet-mediated clumping (65\%), while adhesion to ICAM1 (51\%) and gC1qR (47\%) were comparatively less frequent.

In our study patients, previous episodes of severe malaria were associated with manifestation of severe malaria (Table 3.7). Suggesting that host genetic factors, nutritional status and accessibility to prompt treatment may be important contributors in malaria severity.

Platelet-mediated clumping was the most prominent cytoadherence phenotype associated with severe malaria in this study (Table 3.10 and 3.11). Previous field
studies on clumping and malaria severity suffer from potential problems in experimental design and methodology, to the extent that it has been argued that the clumping may not be a causative agent of severe disease, specially because parasite densities seem to have a large effect on clumping (Arman et al., 2007; Arman and Rowe, 2008). To avoid the confounding effects of parasitemia, one of the criteria for enrollment of children into our study was to have high peripheral parasite densities (>400 parasites/µl). For this reason, there were no prominent differences in parasite densities of children with severe and non-severe malaria that could confound the association between clumping and malaria severity. Furthermore strong association between platelet-mediated clumping and severe malaria symptoms observed in this study may be partly because of the modified method used for platelet-mediated clumping assays, in which platelets are isolated in a manner that they are not activated. Previous studies have used platelets stored at 4°C (Arman et al., 2007; Chotivanich et al., 2004; Pain et al., 2001; Wassmer et al., 2008) which can dramatically alter normal physiological state of platelets (White and Krivit, 1967). If transfused such platelets are rapidly cleared from blood circulation (Hoffmeister et al., 2003). To avoid such effects on platelets, we have used platelets that were isolated and stored at room temperature in Hank's buffer. Interestingly, platelet-mediated clumping was not associated to adhesion of IEs to CD36 or gC1qR (Table 3.8), the two receptors that have been reported to be involved in platelet-mediated clumping, suggesting that other yet unidentified receptors might be involved in this adhesion phenotype.

Since pathogenesis may be quite different among the symptoms used to define severe malaria, we have also investigated for association of cytoadherence phenotypes with each severe malaria symptom. We found that prostration, the most prevalent severe malaria symptom in our study patients, was associated with platelet-mediated clumping (Table 3.10 and 3.11).

In addition, multiple seizures in severe malaria patients were associated with adhesion to gC1qR (Table 3.10). Seizures are commonly reported in children with cerebral malaria, occurring in over 60% patients after admission (Idro et al., 2007). Multiple and prolonged, seizures are associated with increased neurocognitive deficits and mortality (Molyneux et al., 1989; Waller et al., 2008). Although the precise causes of seizures are unclear (cerebral involvement, hypoglycemia or non-specific
consequences of fever), our results suggest that sequestration of parasites through adhesion to gC1qR might be an important mechanism leading to multiple seizures in *P. falciparum* infected patients.

Severe anaemia was associated with higher platelet-mediated clumping (Table 3.10), and a trend was observed for rosetting (Table 3.11), suggesting a role of these cytoadherence phenotypes in the etiology of malarial anaemia. Destruction of UEs attached to IEs in rosettes may contribute to severe anaemia. Additionally, as yet unidentified regulatory mechanisms that monitor abnormal erythrocyte aggregates such as agglutinates or rosettes may down regulate erythrocyte production contributing to severe malarial anaemia. Severe anaemia is an important contributor to malaria related morbidity and mortality in children residing in sub-Saharan Africa. In Manhica, it is the most important risk factor for death in children younger than 8 months (Bassat et al., 2008). It remains to be seen if anti-adhesion interventions can have an impact in reducing severe malarial anaemia in endemic areas.

We did not find any evidence for association of in vitro adhesion to ICAM1 with severe malaria (Table 3.10 and 3.11). However, the adhesion to ICAM1 has been implicated in the pathogenesis of cerebral malaria (Newbold et al., 1997; Traore et al., 2000); postmortem studies have revealed upregulation of ICAM1 expression on the cerebral vascular endothelium in cerebral malaria (Silamut et al., 1999). We could not evaluate the role of gC1qR in cerebral malaria due to low number of children with cerebral malaria in our study (n=3). A higher representation of isolates obtained from children with cerebral malaria might increase the power to detect an association with malaria severity. Interestingly we observed a significant association between adhesion to gC1qR and adhesion to ICAM1 (Table 3.9), which suggests that adhesion to both receptors might be mediated by similar mechanisms. Moreover, adhesion to gC1qR and ICAM1 were found to be strongly correlated to parasite densities. This association cannot be explained by higher detection of adhesion due to higher experimental parasitemia, as adhesion assays with purified receptors were done at the same level of parasitemia, but rather suggests that adhesion to ICAM1 and gC1qR might enhance parasite growth and survival by promoting immune evasion. Adhesion to all the three receptors; CD36, ICAM1 and gC1qR may help *P. falciparum* avoid splenetic clearance. However, CD36 adhering parasites may be rapidly cleared by other mechanisms such as phagocytosis by immune cells (Patel et al., 2004). This may explain absence of association between adhesion to CD36 and parasitemia.
Although not statistically significant, there was a consistent trend towards higher levels of adhesion to CD36 in parasites isolated from children with uncomplicated malaria than from children with severe malaria (Table 3.11). This observation is in accordance with previous reports (Newbold et al., 1997; Rogerson et al., 1999; Traore et al., 2000), and suggests that adherence to CD36 could be an indicator of a less pathogenic infection. This interpretation is supported by experimental evidences showing that CD36 is not expressed on HBMEC or PBMEC, that mutations causing CD36 deficiency are associated with susceptibility to severe malaria (Aitman et al., 2000) and that a significant proportion of CD36 binding Plasmodium berghei parasites sequester in the adipose tissue of the mice (Franke-Fayard et al., 2005). CD36-mediated adhesion to non critical sites of the body may allow the parasite to avoid splenic clearance without killing the hosts thereby providing better transmission opportunities. The propagation advantage of parasites adhering to CD36 would explain the high prevalence of cytoadherence phenotype in P. falciparum isolates. Alternatively, adhesion to CD36 may make IEs prone to phagocytosis by immune cells such as macrophages (Patel et al., 2004), and protect from severe manifestation of malaria. Our observation suggest that anti-adhesion therapies should be considered with caution, as blocking adhesion to CD36 may potentially lead to selection of parasites with affinities for other receptors which might be detrimental to the host.

To our knowledge, this is the first study to report an association between malaria associated prostration and multiple seizures with cytoadherence. Prostration was the most common severe malaria manifestation in our study patients. Pathogenesis of prostration and multiple seizures may have systemic or central nervous system (CNS) involvement (Idro et al., 2007). Association of platelet-mediated clumping and adhesion to gC1qR with prostration and multiple seizures, respectively, allows us to speculate that sequestration of parasites in the brain may be among the underlying causes of these symptoms.

Several limitations of the study should be kept in mind when interpreting the data on cytoadherence phenotypes of field isolates. First, samples were collected at admission, and clinical definition of disease took into account symptoms at presentation. Some individuals presenting early with uncomplicated malaria may have gone on to develop severe malaria in the absence of treatment. So this study does not reflect on association of cytoadherence on long term progression of the disease.
Prospective studies are required to address such questions. Second, we have recruited children with no other probable cause of illness than *P. falciparum* malaria, although other underlying infections cannot be completely ruled out. Finally, adhesion to specific receptors may be underscored, as parasites were sampled from the blood, and not those sequestered in the tissues.

The results of this case control study have shown that malaria severity is associated with platelet-mediated clumping and adhesion to gC1qR. Thus, the cytoadherence of *P. falciparum* may be an important virulence factor. The potential for inhibiting or reversing cytoadherence with antibodies or receptor analogs should be actively explored. Our observations suggest the need for studies with larger sample sizes to better assess the role of platelet-mediated clumping, rosetting, adhesion to ICAM1, CD36 and gC1qR in severe malaria.