EMERGING ROLES FOR HAEMOSTATIC DYSFUNCTION IN MALARIA PATHOGENESIS

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ABSTRACT

Severe *Plasmodium falciparum* malaria remains a leading cause of mortality, particularly in sub-Saharan Africa where it accounts for up to 1 million deaths per annum. In spite of the significant mortality and morbidity associated with cerebral malaria (CM), the molecular mechanisms involved in the pathophysiology of severe malaria remain surprisingly poorly understood. Previous studies have demonstrated that sequestration of *P. falciparum*-infected erythrocytes within the microvasculature of the brain plays a key role in the development of CM. In addition, there is convincing evidence that both EC activation and platelets play critical roles in the modulating the pathogenesis of severe *P. falciparum* malaria. In this review, we provide an overview of recent studies that have identified novel roles through which haemostatic dysfunction may directly influence malaria pathogenesis In particular, we focus on emerging data suggesting that von Willebrand factor, coagulation cascade activation and dysfunction of the protein C pathway may be of specific importance in this context. These collective insights underscore a growing appreciation of the important, but poorly understood, role of haemostatic dysfunction in malaria progression and importantly, illuminate potential approaches for novel therapeutic strategies. Given that the mortality rate associated with CM remains in the order of 20% despite the availability of effective anti-malarial therapy, development of adjunctive therapies that can attenuate CM progression clearly represents a major unmet need. These emerging data are thus not of only of basic scientific interest, but also of direct clinical significance.

KEYWORDS – Malaria, *Plasmodium falciparum*, von Willebrand factor; protein C
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INTRODUCTION

Severe Plasmodium falciparum malaria remains a leading cause of mortality in sub-Saharan Africa, where it accounts for up to 1 million deaths per annum, particularly in children under 5 years of age.\textsuperscript{1,2} Cerebral malaria (CM), is a life threatening complication of P. falciparum that develops in approximately 1% of infections and is characterized by a diffuse encephalopathy resulting in decreased consciousness and unrousable coma.\textsuperscript{3} Children typically present with ataxia, seizures and unrousable coma. Although effective anti-malarial drugs have been developed, CM is still associated with a case fatality rate of 15-20%.\textsuperscript{4} In addition, a significant proportion (10-20%) of children who survive CM suffer significant neurological sequelae which can include learning difficulties and memory impairment.\textsuperscript{4,5} In spite of this significant mortality and morbidity, the molecular mechanisms involved in the pathophysiology of severe malaria remain surprisingly poorly understood.

Previous studies have demonstrated that sequestration of P. falciparum-infected erythrocytes (IE) within the microvasculature of the brain plays a key role in the development of CM.\textsuperscript{6,7} This sequestration process, which involves the cytoadhesion of IE to endothelial cell (EC) surfaces, has been extensively studied and is mediated by parasite-related ligands such as P. falciparum erythrocyte membrane protein 1 (PfEMP1) which are exported to the IE surface.\textsuperscript{8,9} In addition, a number of specific EC surface receptors have been shown to modulate the adhesion of IE, including thrombomodulin, endothelial protein C receptor (EPCR), CD36, thrombospondin, intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), P-selectin and E-selectin.\textsuperscript{9} Importantly, expression of these EC receptors varies between different vascular beds, and is subject to regulation by inflammatory cytokines (e.g. TNF and interleukin-1).\textsuperscript{10,11} Consequently, inflammation and EC activation play critical roles in regulating the sequestration of IE within the brain.
microvasculature, which is a characteristic feature of CM.\textsuperscript{7,12} Although the pathogenesis remains poorly defined, it is likely that IE sequestration leads to obstruction of brain microvessels, reduced blood flow, and ultimately cerebral hypoxia. Interestingly, post mortem studies on patients with CM have suggested that platelets and leucocytes may also be sequestered within the cerebral microvasculature, and thereby contribute to vessel occlusion.\textsuperscript{7}

In addition to this mechanical obstruction of the cerebral microvasculature, significant evidence suggests that pro-inflammatory cytokines secreted in response to the presence of IE (including TNF and interferon-gamma) may also be important in modulating the development of CM.\textsuperscript{13} The relative importance of IE sequestration versus cytokine secretion in the aetiology of CM remains a contentious area that has previously been reviewed in detail.\textsuperscript{7,13,14}

Previous studies reporting diminished vascular nitric oxide (NO) bioavailability in individuals with CM compared to healthy individuals further underscore the importance of dysregulated EC activation in CM pathogenesis.\textsuperscript{15} Impaired NO production promotes enhanced EC activation and IE cytoadherence,\textsuperscript{16} and is associated with increased mortality in a murine CM model.\textsuperscript{17} Furthermore, reduced vascular NO bioavailability also promotes angiopoietin-2 release from ECs, which may in turn disrupt the angiopoietin-1/Tie-2-dependent signalling axis that maintains EC quiescence and vascular integrity.\textsuperscript{18,19}

In this review, we provide an overview of recent studies that have identified novel roles through which haemostatic dysfunction may directly influence \textit{P. falciparum} pathogenesis. In particular, we focus on specific emerging data suggesting that (1) von Willebrand factor (VWF), (2) coagulation cascade activation, and (3) dysfunction of the protein C pathway, may play a role in this context. As our understanding of the biological mechanisms underpinning these observations continues to progress, it seems likely that significant opportunities for novel therapeutic strategies may arise. Given the major morbidity and mortality associated
with CM, these emerging data are thus not only of basic scientific interest, but also of direct clinical significance.


Von Willebrand factor in malaria

VWF is a large plasma glycoprotein synthesized within EC and megakaryocytes.\textsuperscript{20,21} VWF circulates in normal plasma as a series of heterogeneous multimers and plays two essential roles in normal haemostasis.\textsuperscript{21} First, VWF mediates the adhesion of platelets to exposed collagen at sites of vascular injury.\textsuperscript{20} Second, VWF acts as a carrier molecule for procoagulant factor VIII (FVIII), thereby protecting it from premature proteolytic degradation and clearance.\textsuperscript{22} VWF synthesised within EC is either constitutively secreted into the plasma, or alternatively stored within intracellular organelles known as Weibel-Palade (WP) bodies.\textsuperscript{23,24} This stored VWF is enriched in high molecular weight multimers (HMWM), which is secreted together with other WP contents following EC activation.\textsuperscript{24} A series of recent studies have shown that plasma levels of VWF antigen (VWF:Ag) and VWF propeptide (VWFpp) are both markedly elevated in patients with severe \textit{P. falciparum} malaria.\textsuperscript{25-29} For example, Hollestelle \textit{et al.}, observed that plasma VWF concentration at presentation in Ghanaian children with CM was \textasciitilde3-fold higher than that observed in healthy age-matched controls.\textsuperscript{25} Interestingly, plasma VWFpp levels were also 3-fold increased in children with CM.\textsuperscript{25} Using the selective expression of ABO(H) blood group antigens on EC-derived VWF,\textsuperscript{25,30} Hollestelle \textit{et al.} demonstrated that the elevated plasma VWF:Ag levels in CM patients was predominantly derived from activated EC by showing that in blood group A cerebral malaria patients, blood group A antigen expression on VWF increased concurrently with plasma VWF:Ag. Interestingly, peak levels of plasma VWF:Ag and VWFpp levels in some children with CM were increased more than fivefold, exceeding those previously seen in fulminant vascular diseases such as thrombotic thrombocytopenic purpura (TTP).\textsuperscript{25,31}

Significantly increased plasma VWF:Ag and VWFpp levels have been confirmed in a number of other independent malaria cohort studies. Although these subsequent studies
recruited combinations of both adult and children from a variety of different geographical locations (including Indonesia, Bangladesh, Uganda and Malawi), all observed markedly elevated plasma VWF:Ag and VWFpp levels (approximately 3-5 fold) in patients with severe malaria compared to healthy local controls.\textsuperscript{32,33} Although the absolute VWF:Ag levels were more modest, significantly elevated plasma VWF:Ag was also seen in an Indonesian cohort of patients \textit{P. vivax} malaria.\textsuperscript{32} Collectively, these studies clearly demonstrate that markedly elevated VWF:Ag and VWFpp levels constitute consistent features of severe malaria infection. Importantly, in a study of fourteen healthy volunteers infected with \textit{P. falciparum}, de Mast \textit{et al.}, further showed that the increase in plasma VWF:Ag and VWFpp develops at a very early stage following the onset of blood-stage infection, when estimated levels of IE remain less than 0.001\%.\textsuperscript{34} The molecular mechanisms responsible for this early EC activation and WP body secretion following \textit{P. falciparum} infection have not been elucidated. However previous studies have shown that \textit{P. falciparum} does secrete a functional histamine release factor that may contribute to WP body exocytosis.\textsuperscript{35}

As well as the marked increase in plasma VWF levels in severe malaria, a pathological accumulation of ultra-large VWF (UL-VWF) multimers has also been reported by several groups.\textsuperscript{26,32} Importantly, these UL-VWF multimers demonstrate significantly enhanced binding to platelets compared to monomers and consequently are more efficient in modulating platelet aggregation. Furthermore, a significant proportion of plasma VWF in patients with \textit{P. falciparum} infection circulates in an active confirmation that facilitates binding to platelet GpIb.\textsuperscript{34,36} In addition, \textit{in vitro} experiments have demonstrated that platelet-decorated UL-VWF strings on the surface of activated EC can tether \textit{P. falciparum} IE under physiological shear stress.\textsuperscript{37} This interaction is modulated by PfEMP-1 on the surface of IE binding to platelet CD36. Given that previous studies have highlighted that platelet adhesion and
aggregation are important in the development of cerebral microvasculature occlusion in CM, these emerging findings regarding a putative role for VWF in malaria are of significant interest. In particular, these data raise the intriguing possibility that the early marked increase in plasma VWF levels may play a specific role in modulating malaria pathogenesis. This hypothesis is supported by observations from studies of children with severe malaria demonstrating significant correlations between VWFpp levels and other established biomarkers of malaria severity including plasma lactate. Furthermore, plasma VWF:Ag levels are also inversely related with both platelet count and overall survival. Interestingly, ABO blood group glycan determinants are expressed on both the N- and O-linked glycans of VWF. Moreover, plasma VWF:Ag levels are significantly reduced in blood group O compared to non-O individuals. In addition, group O VWF also demonstrates significantly enhanced susceptibility to proteolysis by ADAMTS13. Collectively these findings are of interest, since blood group O individuals are significantly protected against *P. falciparum* malaria.

Defining whether the early increase in plasma VWF levels, and/or the presence of UL-VWF multimers merely constitute biomarkers of EC activation, or whether VWF actually contributes to the pathogenesis of *P. falciparum* malaria is difficult to address in human studies. However recent studies from our laboratory using a murine model of experimental cerebral malaria (ECM) in which C57BL/6J mice are infected with *P. berghei* ANKA, provide further support for the hypothesis that VWF plays a direct role in malaria pathogenesis. Following *P. berghei* inoculation, a significant increase in murine plasma VWF:Ag levels was observed. Consistent with previous studies in children with CM, peak VWF:Ag levels were increased approximately 2.5 fold in mice with CM compared to uninfected controls. Also in keeping with the observations of the human volunteer studies, a
significant increase in murine plasma VWF:Ag levels was apparent from an early stage following *P. berghei* infection, prior to the development of significant blood parasitaemia levels. In addition, a pathological accumulation of UL-VWF was also observed in the plasma of mice inoculated with *P. berghei*. Finally, VWF/−/ mice were significantly protected against ECM.⁴⁸ Although the molecular mechanism(s) through which VWF influences malaria progression have not been elucidated, a number of plausible mechanisms may be proposed (Figure 1). First, as previously described, *in vitro* studies have demonstrated that platelet-decorated UL-VWF strings can tether IE under conditions of physiological shear stress.³⁷ Thus VWF may directly facilitate the cytoadhesion of IE to EC surfaces. Second, VWF also modulates leucocyte and monocyte adhesion to EC,⁴⁹,⁵⁰ and also has been shown to regulate leucocyte extravasation.⁵¹ Finally, murine studies have demonstrated a role for VWF in regulating blood brain barrier (BBB) permeability,⁵²,⁵³ which is important since previous studies have reported that BBB dysfunction is important in the pathogenesis underlying CM.⁵⁴-⁵⁶ Further studies will be required to fully understand how VWF influences malaria progression *in vivo*. Nonetheless, these emerging human and murine data suggest that VWF plays a novel role in malaria pathogenesis.

**ADAMTS13 in malaria**

UL-VWF multimers secreted from WP bodies normally undergo partial proteolysis on the EC surface by the zinc metalloproteinase ADAMTS13.⁵⁷,⁵⁸ ADAMTS13 cleaves at a specific peptide bond (Y1605/M1606) within the VWF A2 domain and thus prevents accumulation of UL-VWF multimers in normal plasma.⁵⁹,⁶⁰ The molecular mechanism(s) responsible for the presence of UL-VWF in patients with severe malaria infection has not been defined. However several studies have reported that plasma ADAMTS13 antigen and activity levels are significantly reduced in CM.²⁶,³²,³³ Interestingly, only modest reductions in ADAMTS13 have
been observed, with plasma levels generally remaining above 30% normal values.\textsuperscript{26,32,33} Previous \textit{in vitro} studies suggest that these ADAMTS13 levels should be adequate to maintain normal plasma VWF multimer distribution.\textsuperscript{61} Consequently, the significance of this modest reduction in plasma ADAMTS13 with respect to the accumulation of UL-VWF multimers in children with severe \textit{P. falciparum} malaria remains unclear.

Preliminary studies have suggested that ADAMTS13 activity may also be inhibited in plasma from \textit{P. falciparum} infected children.\textsuperscript{26} Although the physiological regulation of ADAMTS13 activity is not fully understood, a number of putative inhibitors have been described. These include thrombin, interleukin-6 (IL-6), thrombospondin 1, thrombin, and free plasma haemoglobin.\textsuperscript{62-65} Of these, plasma IL-6 and free haemoglobin levels have both been shown to be significantly elevated in children with CM.\textsuperscript{26} However, the increases in plasma IL-6 and haemoglobin levels in severe malaria are significantly below the threshold levels necessary to significantly inhibit ADAMTS13 activity \textit{in vitro}.\textsuperscript{26,62,63} In summary, it seems likely that multiple biological mechanisms (including marked acute EC activation and release of UL-VWF from WP bodies; significantly reduced plasma ADAMTS13 antigen levels; and inhibition of ADAMTS13 functional activity) contribute to the accumulation of abnormal UL-VWF multimers in patients with severe \textit{P. falciparum} malaria. Additional studies will be required to define the relative importance of these different mechanisms.
Coagulation cascade activation in malaria

Thrombocytopenia is a common finding in patients with *P. falciparum* malaria. The mechanisms responsible for this thrombocytopenia have not been fully elucidated, but a number of putative mechanisms have been proposed. In addition, a series of studies dating back to the 1960s have shown that coagulation cascade activation is also common in both children and adults with *P. falciparum* infection. Many of the older studies of coagulation factor levels in malaria included relatively small numbers, and enrolled patient cohorts with variable characteristics at presentation (including differences in age ranges, ethnicity, and malaria severity). Consequently, it is perhaps not surprising that variation in results was observed between the individual studies. Nevertheless, these older studies in combination with more recent reports, collectively support the hypothesis that *P. falciparum* malaria is associated with significant coagulation activation. In particular, significant elevations in plasma levels of thrombin-antithrombin (TAT) complexes, fibrin degradation products (FDPs) and D-dimers have been consistently reported. Although mild prolongations in both the prothrombin time (PT) and activated partial thromboplastin time (APTT) have been noted, plasma fibrinogen levels typically remain within the normal range. Interestingly, the degree of coagulation activation in malaria has been associated with disease severity and parasitaemia levels in several studies. Finally, in keeping with this state of coagulation activation, significant reductions in plasma anticoagulant factors (including antithrombin, protein C and protein S) have also been observed in patients with malaria.

Moxon et al recently utilized the International Society for Thrombosis and Haemostasis (ISTH) scoring algorithm to objectively assess the prevalence and significance of disseminated intravascular coagulation (DIC) in a cohort of 176 Malawian children presenting with CM. In order to further refine the CM diagnosis, all children with CM also underwent
ophthalmology review. Children with characteristic retinal changes associated with IE sequestration in the cerebral microvasculature (whitening, vessel changes and/or microhemorrhages) were defined as ‘retinopathy-positive’. In contrast, in cases without these retinal changes (‘retinopathy-negative’), other etiologies for the encephalopathy were considered. Using this systematic approach, plasma levels of D-dimers, FDPs, and fibrin monomers were all significantly elevated in children with retinopathy-positive CM compared to controls.\textsuperscript{73} In addition, an ISTH score consistent with overt DIC ($\geq 5$) was observed in 19\% of children with retinopathy-positive CM. Importantly, in this subgroup of children with retinopathy-positive CM and overt DIC, overall mortality was significantly increased (OR 3.068; $P = 0.035$). Despite the fact that thrombocytopenia, coagulation activation, and DIC are common in \textit{P. falciparum} malaria, clinical bleeding and/or thrombotic complications are rarely observed in affected children.\textsuperscript{73,78} Although the reported incidence varies considerably between different studies, haemorrhagic complications and thrombotic sequelae do appear more common in adult patients with severe malaria.\textsuperscript{70,79} For example, Clemens \textit{et al} observed significant bleeding (melaena, ecchymoses, haemoptysis and haematemesis respectively) in four of twenty-two adult Thai patients admitted with severe \textit{P. falciparum} malaria.\textsuperscript{70} In addition, other studies have reported pulmonary haemorrhage\textsuperscript{80} and intracranial bleeding in adult patients with severe malaria.\textsuperscript{81} Nevertheless, even in adult patients with severe \textit{P. falciparum}, the overall risk of developing overt clinical bleeding or thrombotic complications has been estimated at less than 5\%.\textsuperscript{70,79}

The molecular mechanism(s) through which \textit{P. falciparum} infection causes \textit{in vivo} coagulation activation remain poorly understood. However, Francischetti \textit{et al} have previously demonstrated that incubation of IE with microvascular EC \textit{in vitro} results in the induction of EC tissue factor (TF) expression.\textsuperscript{82} Interestingly, this TF expression on EC was
found to be dependent upon *P. falciparum* parasite developmental stage. Consequently, although significant TF expression on EC was observed following incubation with mature forms of parasitized red blood cells (late-trophozoites and schizonts), only minimal TF was detected after incubation with early-mid trophozoite stages. Furthermore, immunohistochemical studies on post mortem samples from CM cases has also demonstrated aberrant TF expression on EC within the cerebral microvasculature. In addition to the upregulation of TF expression, two independent previous studies have shown that *P. falciparum* infection leads to the expression of negatively charged phosphatidylserine (PS) on red cell membrane surfaces. Interestingly, when co-incubated with FXa, FVα, Ca^{2+} and prothrombin, these IE supported thrombin generation in vitro. Similarly, incubation of IE with FVIIIα, FIXa, Ca^{2+} and FX also resulted in significant FXa generation. In contrast, if the IE were replaced with normal red blood cells in these experiments, no thrombin or FXa generation was observed. A further mechanism by which *P. falciparum* infection may promote coagulation is by expression of procoagulant protein(s) that enter the bloodstream upon IE rupture and impact upon haemostasis. One such protein is the *P. falciparum*-specific histidine-rich protein II (HRPII). Although the specific purpose of HRPII is not well understood, recent studies indicate that it binds glycosaminoglycans (GAG) with exceptionally high affinity in the presence of zinc or copper ions. Consequently, HRPII-GAG binding significantly impedes GAG-mediated antithrombin inhibition of FXa and thrombin in plasma, and reverses the anticoagulant activity of heparin. The ability of HRPII to potently inhibit a crucial plasma anticoagulant process implies a potential mechanism by which *P. falciparum*-encoded proteins can directly modulate coagulation. Together, these findings suggest that *P. falciparum* triggers coagulation activation through multiple different pathways that include induction of TF expression on microvascular EC, the exposure of negatively-charged PS on
the surface of IE which enables assembly of the intrinsic tenase and prothrombinase complexes, and direct inhibition of key endogenous anticoagulants (Figure 2).

The significant coagulation activation and DIC observed in patients with severe *P. falciparum* has led to suggestions that haemostasis dysfunction may play a pivotal role in malaria pathogenesis.\textsuperscript{75,86,87} This hypothesis is supported by a number of lines of evidence. In particular, post mortem studies in both children and adults with fatal CM have demonstrated the presence of fibrin within the microvasculature.\textsuperscript{74,87} In addition, the degree of haemostatic dysfunction observed in patients with malaria has been reported to correlate with disease severity and peripheral blood parasitaemia levels.\textsuperscript{72,76} However, it is important to note that other studies failed to demonstrate significant fibrin deposition in CM cases at post mortem.\textsuperscript{74} Consequently, analogous to the previous discussion regarding a putative role for VWF in malaria, it remains unclear whether coagulation cascade activation is directly involved in modulating the pathogenesis of *P. falciparum* malaria, or whether instead this coagulation activation merely represents a secondary epiphenomenon. Although previous studies demonstrated no beneficial effect with heparin anticoagulation in patients with severe malaria,\textsuperscript{88} further studies will be necessary to determine whether other targeted coagulation cascade inhibition (e.g. specific FXa or FIIa inhibition with direct oral anticoagulant agents) may have a role to play.
Malaria and the protein C pathway

The protein C pathway is a crucial anticoagulant and anti-inflammatory pathway triggered in response to the generation of excess thrombin production during clot formation.\textsuperscript{89} Thrombin engages thrombomodulin (TM) on intact endothelium, where its substrate specificity is switched to favour activation of protein C rather than procoagulant substrates that include factors V, VIII, FXI, and fibrinogen. Protein C activation by the thrombin-thrombomodulin complex is accelerated by the presence of EPCR, which binds protein C with high affinity and presents it for optimal activation by the thrombin-thrombomodulin complex.\textsuperscript{90} Activated protein C (APC), in conjunction with its cofactor protein S, then restricts further thrombin generation by proteolytic degradation of activated cofactors V and VIII. Further to its anticoagulant activity, APC can also trigger myriad cell signaling pathways via cell surface activation of protease-activated receptor 1 (PAR1), PAR3, apolipoprotein E receptor 2, Tie2 and integrin $\alpha_M\beta_2$.\textsuperscript{89} Cell signaling by APC, though diverse, is united by its ability to initiate protective cellular responses upon exposure to pro-inflammatory, pro-apoptotic or toxic insult.\textsuperscript{91}

Previous studies have demonstrated that plasma protein C levels are significantly reduced in retinopathy-positive CM compared to individuals with non-severe malaria or non-malaria induced coma.\textsuperscript{73} Moreover, recent studies suggest vascular protein C pathway receptors can also bind PfEMP1, and thereby enable the cytoadhesion and sequestration of IE.\textsuperscript{92} In addition to early reports that suggested PfEMP1 can bind thrombomodulin via chondroitin sulfate moieties expressed near the TM membrane proximal region,\textsuperscript{93} EPCR has recently been described as a dominant target for PfEMP1 subtypes associated with severe malaria.\textsuperscript{92} Specifically, N-terminal cysteine-rich interdomain region (CIDR) domains $\alpha1.1$ and $\alpha1.4-1.8$ that form part of PfEMP1 bind to EPCR with high affinity (Figure 3). Interestingly,
individuals living in areas where malaria transmission is high have been reported to develop antibodies against the EPCR binding CIDRα1 domain subtypes early in life, which implies a key role for antibody generation against EPCR binding-CIDRα1 domains in generating effective immunity to severe malarial disease.94

Recent elegant crystallography studies of two sEPCR-bound PfEMP1 CIDRα1 domains (HB3var03 and IT4var07) have revealed the key structural requirements for PfEMP1 binding to EPCR.95 Interestingly, both CIDRα1 domains bind to sEPCR using a binding site that overlaps significantly with that of protein C/APC. Moreover, despite the considerable sequence diversity exhibited by the EPCR binding regions of each tested CIDRα1 domain, core structural features enabling hydrophobic interactions are retained, such that the sequence diversity that characterizes PfEMP1 does not impact negatively upon EPCR binding. Additional studies have suggested that other CIDRα1.1 and CIDRα1.4 domains, not previously characterized by crystallography, can also bind EPCR in subtly different conformations.96 Collectively therefore, CIDRα1 domains share a largely conserved EPCR binding mechanism, but sequence-specific differences between PfEMP1 subtypes may confer subtle modifications to binding site and affinity with the potential to mediate divergent effects on EPCR function.97

PfEMP binding to EPCR appears to contribute to malaria pathogenesis beyond IE cytoadherence. Recent in vitro studies indicate that PfEMP binding to EPCR limits protein C activation, EPCR-dependent PAR1 activation and PAR1-dependent protection of the endothelial cell barrier integrity.97,98 Consequently, PfEMP1 binding would be predicted to severely attenuate normal protein C pathway activity during P. falciparum infection. In addition, the significant pro-inflammatory response associated with severe malaria is likely to
induce further protein C pathway dysfunction. Soluble EPCR (sEPCR) and TM (sTM), presumably cleaved from the inflamed vascular surface, are significantly elevated in the cerebrospinal fluid of CM patients compared to non-malarial febrile patients. Moreover, reduced expression of both receptors has been reported in the subcutaneous microvasculature from individuals with severe *P. falciparum* malaria at sites of local IE cytoadherence. As observed in other inflammatory disease settings, diminished EC surface expression of EPCR and TM acts as a brake on the protein C pathway in responding to proinflammatory stimuli, leading to unregulated thrombin generation, local fibrin deposition and defective vascular anti-inflammatory activity. In this way, competitive blockade of EPCR availability to drive protein C activation and promote PAR1-dependent anti-inflammatory activity amplifies further protein C pathway dysfunction to enable further *P. falciparum*-mediated vascular damage.

Despite the development of effective anti-malarial therapies, CM is still associated with significant mortality. Consequently, adjunctive therapies to limit vascular dysfunction and slow disease progression are urgently required. In this setting, one attractive therapeutic target would be to utilize the dual anticoagulant and anti-inflammatory properties of APC. Recombinant APC has previously been utilized in the treatment of severe sepsis and is currently under evaluation to enhance the safety of thrombolytic stroke therapy. Given the thrombocytopenia and DIC associated with severe *P. falciparum* malaria, use of wild type (WT) APC may be associated with significant bleeding risk. Nevertheless, some cases studies have reported beneficial effects following the use of WT APC infusion in patients with severe malaria. Alternatively, signaling-selective engineered APC analogs with diminished anticoagulant properties may offer a safer approach. An alternative potential therapeutic strategy is to use the high affinity of EPCR for PfEMP1 CIDRα1 domains to create
recombinant sEPCR analogs that could prevent IE cytoadherence to the vessel wall. To this end, a bioengineered sEPCR analog containing an amino acid substitution (E86A) that has previously been demonstrated to ablate protein C binding was developed.\textsuperscript{98} Importantly, this E86A substitution does not attenuate the ability of EPCR to bind PfEMP1. Unlike WT sEPCR, sEPCR-E86A did not influence normal protein C function or APC anticoagulant and signaling activity, but bound to recombinant CIDR\(\alpha\)1 domains with similar affinity to WT sEPCR,\textsuperscript{98} suggesting a possible means by which this PfEMP1 cytoadherence strategy could be therapeutically targeted, without deleterious impact upon normal protein C pathway function.

Four EPCR haplotypes have been previously described.\textsuperscript{103} Individuals with the H3 EPCR haplotype possess the g.4600A>G allele that encodes a single amino acid substitution (S219G) within the EPCR transmembrane region. This S219G substitution in individuals with the EPCR H3 haplotypes is associated with a four-fold increase in sEPCR plasma levels.\textsuperscript{103} Recent studies have examined whether individuals with the H3 haplotype exhibit altered susceptibility to malaria. To date, studies have provided conflicting results. For example, a large Ghanaian association study failed to link EPCR H3 haplotype with malaria disease severity.\textsuperscript{104} In contrast, a recent Thai study found that elevated sEPCR plasma levels in individuals with H3 haplotype was associated with protection from cerebral, malaria.\textsuperscript{105} Evidently, larger studies are required to unequivocally determine whether EPCR haplotype and constitutively high sEPCR plasma concentration truly impacts upon malaria disease severity.
Conclusions

In conclusion, there is overwhelming evidence that both EC activation and platelets play critical roles in the modulating the pathogenesis underlying severe *P. falciparum* malaria. Although the molecular mechanisms have not been fully defined, the emerging data discussed in this review support the hypothesis that specific aspects of haemostatic dysfunction may also be important in malaria progression. Further animal and human studies will be essential in order to elucidate which of these haemostatic effects are directly involved in malaria pathogenesis, and which merely arise as secondary epiphenomena. In addition, the clinical utility of specifically targeting these novel molecular mechanisms with new therapeutic strategies will need to be defined. However, given that the mortality rate associated with CM remains in the order of 20% despite the availability of effective anti-malarial therapy, development of adjunctive therapies that can significantly attenuate CM progression clearly represents a major unmet need.
AUTHORSHIP

Contribution: All authors drafted the first version of different sections of the manuscript and all critically reviewed the final manuscript.

Conflict-of-interest disclosure:

R.J.S.P. has received honoraria from Octapharma and has received grant awards from Novo Nordisk and Bayer. J.S.O’D has served on the speaker’s bureau for Baxter, Bayer, Novo Nordisk, Boehringer Ingelheim, Leo Pharma and Octapharma. He has also served on the advisory boards of Baxter, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim and Pfizer. J.S.O.D has also received research grant funding awards from Baxter, Bayer, Pfizer and Novo Nordisk.

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FIGURE LEGENDS

Figure 1. Putative mechanisms through which VWF secreted from EC may influence the pathogenesis of *P. falciparum* malaria

At an early stage following *P. falciparum* infection, acute EC activation results in Weibel Palade body exocytosis. Consequently plasma VWF:Ag levels are significantly increased, and pathological UL-VWF multimers accumulate in the plasma. Whilst tethered on the surface of activated EC, UL-VWF can bind circulating platelets. These bound activated platelets further activate the underlying EC (see hashed line). In addition, CD36 expressed on the platelet surface of these platelet-decorated UL-VWF strings can bind to PfEMP1, thereby facilitating the cytoadhesion and sequestration of IE. This VWF-modulated IE cytoadhesion may be of particular importance in the cerebral microvasculature where there is no constitutive CD36 expression on EC. Once again, IE cytoadhesion further stimulates EC activation and dysfunction. Finally, in addition to being important in modulating platelet and IE recruitment, VWF can also bind to neutrophils and monocytes, both of which can enhance EC damage resulting in enhanced EC permeability.

Figure 2. *P. falciparum* infection causes coagulation activation through multiple different mechanisms.

(a) Procoagulant effects: *P. falciparum* infection causes early EC activation, and platelet activation. Furthermore, IE induce tissue factor (TF) expression on EC surfaces and on monocytes. This aberrant intravascular TF expression in combination with FVIIa results in initiation of the extrinsic coagulation pathway. In addition, *P. falciparum* infection leads to expression of negatively charged phosphatidylserine (PS) on the red cell surface. This PS enables the assembly of the intrinsic tenase and prothrombinase complexes, thereby enhancing coagulation amplification. Activated clotting factor proteases (notably FXa and
thrombin) generated through *P. falciparum*-induced coagulation activation interact with specific EC surface including PAR1 and thereby initiate downstream intracellular signaling, which ultimately results in enhanced EC activation, damage and apoptosis.

(b) Attenuation of normal anticoagulant effects: In addition to the specific procoagulant effects described above, *P. falciparum* infection further promotes coagulation activation by downregulating normal endogenous anticoagulant pathways. EC surface expression of thrombomodulin (TM) and the endothelial protein C receptor (EPCR) are both reduced, likely due to cytokine-enhanced shedding. Moreover, plasma levels of sTM and sEPCR are both increased. Together, these effects combine to lead to a significant reduction in generation of anti-inflammatory and cytoprotective activated protein C (APC) on the EC surface. Finally, release of histidine-rich protein II (HRPII) following spontaneous IE lysis significantly inhibits the anticoagulant effects of antithrombin (AT).

**Figure 3.** EPCR binding PfEMP subtypes utilise CIDRα1 domains with overlapping EPCR binding sites to protein C/APC to restrict protein C pathway function.

(a) PfEMP subtypes expressing domain cassettes DC8 and DC13 that include CIDRα1.1 and CIDRα1.4-1.8 domains bind EPCR with high affinity. EPCR binding CIDRα1 domains appear to utilise a similar binding mechanism and binding site to that of protein C/APC (blue). As CIDRα1 domains are significantly larger than the the EPCR binding region of protein C/APC, EPCR binding-CIDRα1 domains make additional extended contacts with EPCR via loops containing amino acid residues 22-25 and 44-47 (pink). However, despite the significant overlap, amino acid residues that include Glu-86 (yellow), have been identified that are crucial for protein C/APC binding, but not PfEMP1. Accordingly, recombinant variants of sEPCR in which Glu-86 has been substituted have been proposed as a potential
therapeutic strategy to competitively impede EPCR-binding PfEMP subtypes enabling IE
cytoadherence to the vasculature, preventing blockade of (b) EPCR-dependent protein C
activation and (c) PAR1 cytoprotective signalling by APC.
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Figure 3

(a) Overlapping PC/APC and CIDRα1 binding sites

(b) CIDRα1-specific binding loops

Protein C activation inhibited

EPCR-dependent APC signaling blocked
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