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EPCR and malaria severity: the center of a perfect storm

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Abstract

Severe malaria due to *Plasmodium falciparum* infection causes nearly half a million deaths per year. The different symptomatology and disease manifestations among patients have hampered understanding of severe malaria pathology and complicated efforts to develop targeted disease interventions. Infected erythrocyte sequestration in the microvasculature plays a critical role in the development of severe disease and there is increasing evidence that cytoadherent parasites interact with host factors to enhance the damage caused by the parasite. The recent discovery that parasite binding to endothelial protein C receptor (EPCR) is associated with severe disease has suggested new mechanisms of pathology and provided new avenues for severe malaria adjunctive therapy research.

Keywords

EPCR; Malaria; *Plasmodium*; protein C; cytoadhesion; PfEMP1

Severe malaria: a complex disease

Even though it has been more than a century since Laveran discovered that *Plasmodium* is the causative agent of malaria, the fundamental pathogenic mechanisms underlying severe disease remain incompletely understood. *Plasmodium falciparum*, the most deadly of the human malaria parasites, accounts for approximately 95% of malaria deaths [1]. Infection with *P. falciparum* usually leads to mild febrile uncomplicated infection and severe malaria is estimated to ensue in less than 1% of cases [2]. Because most deaths from severe malaria occur within the first 24–48 hours of hospitalization there is a narrow window for treatment. Current treatment is focused on antimalarial drugs and supportive care for organ failure. Despite the introduction of quick and effective artemisinin-based antimalarial drugs that has improved survival [3, 4], malaria mortality in severe cases remains high, ranging from 6.1% in children younger than 10 years to 35.6% in patients older than 50 years old [5]. A better understanding of pathophysiological mechanisms may inform new adjunctive treatment strategies to improve clinical outcomes and reduce mortality rates.

Malaria is not a single disease [6]. Differences in disease manifestation, which include severe anemia, **cerebral malaria** (see Glossary), **placental malaria**, multi-organ failure, and **metabolic acidosis** increase the complexity of malaria pathology research. Severe malaria pathology is of a multifactorial nature whereby both parasite and host factors contribute to disease severity [7]. Additionally, the pattern of vital organ dysfunction differs based on host age [5, 8]. In both children and adults, endothelial dysfunction that results from infected erythrocyte (IE) sequestration is a central pathogenic mechanism [7]. Despite the variability of disease presentation in children and adults, recent findings suggest that disease mechanisms may be driven in part by parasite adhesion to **endothelial protein C receptor (EPCR)** [9, 10], a receptor involved in anti-coagulant and cytoprotective functions [11]. This review dissects the proposed mechanisms of disease pathology in severe malaria and discusses the role of parasite blockade of EPCR function as one of the central events in malaria pathology.

Severe malaria patients present age-related differences

The age of onset of severe disease is directly related to the parasite transmission intensity [12]. In areas of high transmission, such as sub-Saharan Africa, severe disease mostly occurs in children younger than 10 years old. In a Tanzania birth-cohort study, the risk of severe malaria was higher in early infections, but more than 50% of first episodes of severe malaria occurred after a second infection [13]. After repeated infections, malaria immunity builds and subsequent infections lead to mild or asymptomatic disease. In contrast, in South and Southeast Asia and South America, where transmission intensity is low, both children and adults are susceptible to severe episodes.

Severe malaria includes a broad array of symptoms that differ depending on patient age [14]. Adults have a higher mortality rate and more multi-organ involvement than children [3, 4]. Dondorp and colleagues elegantly described the differential age symptoms in a multicenter study in South and Southeast Asia [5]. Cerebral malaria and metabolic acidosis are present in both children and adults. Conversely, severe anemia was much more common in children 10 years and under, while jaundice, renal failure, and acute respiratory distress syndrome (ARDS) and pulmonary edema mostly occur in teenagers and adults. Respiratory distress (Kussmaul’s breathing) was common in both pediatric and adult severe malaria [5] and usually appears to compensate for metabolic acidosis. Of these complications, cerebral malaria and metabolic acidosis are associated with the greatest risk of death, and the combination of both is especially dangerous and increases mortality in both children [15] and adults [5]. Furthermore, severe episodes with multi-organ complications sharply raise the risk of death, increasing from 9.5% among patients with a single symptom, to 50% among patients that present more than five symptoms [5].

Parasite adhesion types and severe malaria

During blood stage infection, *P. falciparum* IEs sequester from blood circulation by binding within microvessels of the gut, brain, lung, skin, heart, and other tissues. IE sequestration in the microvasculature prevents splenic clearance and contributes to *P. falciparum* survival. The importance of sequestration to disease was first recognized more than a century ago by

two Italian pathologists, Marchiafava and Bignami, who proposed a mechanical obstruction theory of cerebral malaria based on autopsy findings of abundant IEs in brain microvasculature [16, 17]. *P. falciparum* mature stages can mediate binding to endothelial cells, uninfected erythrocytes (**rosetting**), and platelets (clumping) due to the expression of ***P. falciparum* erythrocyte membrane protein 1 (PfEMP1)** at the IE surface. PfEMP1 is encoded by a family of approximately 60 *var* genes per genotype expressed in a mutually exclusive fashion, leading to the display of a single PfEMP1 at the erythrocyte surface. This phenomenon provides distinct adhesion properties and contributes to parasite immune evasion.

EPCR-binding parasites and severe malaria

PfEMP1 proteins are highly polymorphic and composed of a series of adhesion domains, known as **Duffy binding-like (DBL)** and **cysteine-rich interdomain region (CIDR)** that confer binding properties to different host receptors [18]. Of these, a dichotomy in parasite binding to EPCR [10] and CD36 [19] is highly relevant to understand malaria pathogenesis. This binding dichotomy is determined by the type of PfEMP1 **head structure** (reviewed in [20]): CIDRα1 domains bind EPCR [10, 21] and CIDRα2-6 domains bind CD36 [22]. Infections dominated by CD36-binding parasites are associated with mild infections, while high transcription of EPCR binders is associated with pediatric severe malaria [23, 24], adult severe malaria [9], cerebral malaria [25] and **retinopathy-positive patients** [26]. EPCR-binding parasites tend to predominate in hosts with limited malaria immunity and antibodies against EPCR-binding domains (CIDRα1) are acquired more rapidly than those against CD36-binding domains (CIDRα2-6) [27]. The age of acquisition depends on transmission intensity [28] thus, potentially contributing to the acquisition of clinical immunity in Africa.

The discovery that *P. falciparum* binds to EPCR opened new insights into malaria pathogenesis as EPCR plays an important role in modulating blood coagulation, **endothelial activation** and barrier properties by enhancing the activation of protein C by the **thrombin-thrombomodulin** complex (Figure 1). **Activated protein C** (APC) exerts cytoprotective activities in endothelial cells by cleaving protease-activated receptor 1 (PAR1) at Arg46 and eliciting protective signaling pathways that promote an anti-apoptotic and anti-inflammatory response and strengthening endothelial barrier integrity. In addition, APC also exerts an anti-coagulation activity by inactivating FVa and FVIIIa, two cofactors that promote thrombin generation (reviewed in [29]). Conversely, thrombin has the opposite effect and contributes to a pro-coagulant state and converts fibrinogen into fibrin. Thrombin also cleaves PAR1 at Arg41 and promotes pro-apoptotic, proinflammatory and barrier disruptive signaling pathways in endothelial cells. Taking all into account, the finding that PfEMP1 and APC have an overlapping binding site [21] and compete for EPCR binding [30–32] suggests important linkages between parasite sequestration, coagulopathy and barrier disruption in severe malaria. In addition to mediating cytoadhesion, specific receptor interactions may allow sequestered IEs to respond to environmental changes in the microvascular environment (Box 1). Even though PfEMP1 heterogeneity confers varying extents of receptor blockade to APC [30, 32], a low level of blocking is sufficient to impair APC barrier-protective activity in primary human brain endothelial cell monolayers [9]. The blockade of EPCR function by IE cytoadhesion may be further exacerbated by the loss of

EPCR at sites of sequestration in pediatric cerebral malaria (CM) autopsies [33]. Notably, there is limited reduction in IE binding to primary brain endothelial cells in which EPCR expression has been reduced by siRNA silencing [30], suggesting that other accessory receptors compensate to mediate IE sequestration. However, parasite blockade and the loss of a key protective receptor, such as EPCR, might further exacerbate the deleterious effects of malaria pathogenesis.

Although the presence of an EPCR binding domain appears to be the one commonality among diverse PfEMP1 variants linked to severe malaria [24], the accompanying adhesion domains present in PfEMP1 can potentially bind to accessory endothelial co-receptors and contribute to malaria pathogenesis through unknown mechanisms. For instance, binding to intercellular adhesion molecule 1 (ICAM-1) is mediated by the DBLβ domain adjacent to the CIDRα domain [34–37], and at least some *P. falciparum* clonal lines present dual EPCR and ICAM-1 binding activity [38]. The multi-adhesive properties of PfEMP1 are likely important for adhesion strengthening [39] and potentially influence microvascular specificity. Our understanding of parasite tropism for brain and other microvascular beds is still in its infancy. Specific parasite subsets expressing a combination of domains called domain cassette (DC8) PfEMP1 (EPCR binders) and DC13 PfEMP1 (EPCR + ICAM-1 binders) were selected on human brain endothelial cells *in vitro* [40, 41]. However, brain endothelial panned parasites could also adhere strongly to other endothelial cells types [40, 42], suggesting that they may sequester inside or outside the brain microvasculature. In addition, the multiple recombinant domains present in DC8 and Group A PfEMP1 mediate binding to other endothelial niches through ICAM-1 [38] and other unknown receptors [40]. Because little is known about most sequestration sites, a better understanding of PfEMP1 multi-adhesive properties may explain differences in severe malaria symptomatology among patients.

Rosetting and severe malaria

Non-EPCR binding PfEMP1 variants have also been implicated in severe malaria. For instance, elevated rates of rosetting have been associated with severe malaria in Africa but not in Asia [18]. Rosetting parasites were more strongly associated with respiratory distress than impaired consciousness in a pediatric malaria cohort in Kenya [43], but it has also been associated with cerebral malaria in other studies [44]. The majority of rosetting PfEMP1 variants that have been characterized have atypical head structures (CIDRβ/γ/δ) that lack a CD36 or EPCR binding domain [45]. Binding to uninfected erythrocytes is mediated by the interaction of the DBLα domain in PfEMP1 with complement receptor 1, A and B blood group antigens, and heparin sulphate-like molecules on erythrocytes [46, 47]. Besides PfEMP1, it has been shown that other *P. falciparum* multigene families, such as *rifin* [48] or *stevor* [49] can mediate rosetting. Studies performed in African children showed that rosetting formation presents a high correlation with IgM-binding parasites [50]. PfEMP1 binding to serum proteins, such as IgM and α2-macroglobulin has been mapped to C-terminal domains (DBLε and DBLζ) [51–53]. It has been hypothesized that IgM and α2-macroglublin might cross-link PfEMP1 proteins to strengthen weak binding interactions to erythrocyte surface glycoproteins. Although rosetting parasites appear to have relatively limited binding activity for endothelial cells [54] they may play a role in microvascular

blocking [55] and tissue ischemia, possibly by blocking smaller diameter vessels or adhering within or upstream of congested vessels.

Parasite sequestration, microvascular obstruction and metabolic acidosis

Metabolic acidosis is a serious complication of malaria infection and is one of the strongest predictors of death in children [15, 56] and adults [57]. The pathophysiology is complex, but several factors can increase lactic acid production during falciparum malaria including hypovolemia, fever, severe anemia, and microvascular obstruction from sequestered IEs, rosetting, and reduced deformability of uninfected red blood cells [58]. Interactions between sequestration and microvascular congestion are also important for organ complications [59] (see below), and collectively, these processes lead to reduced tissue perfusion, hypoxia, and a shift to anaerobic metabolism [57]. Impairment of renal and hepatic function in severe adult malaria may compound the problem by decreasing the elimination of metabolic products [57].

The importance of acidosis in disease severity has prompted efforts to understand the contribution of microvascular obstruction to disease pathophysiology (Figure 2). It is difficult to measure the sequestered parasite burden in *P. falciparum* infection, but quantification of total **parasite biomass** (circulating and sequestered IEs) is estimated from plasma concentration of *P. falciparum* histidine-rich protein-2 (**HRP-2**), a parasite protein that is released to blood at the time of schizont rupture. Plasma HRP-2 levels are correlated with lactate levels in severe adult malaria [60], and predict the probability of disease deterioration [61], retinopathy-positive malaria [62], and disease severity and fatality in children and adults [63, 64].

Recent advances in imaging techniques allow a quantitative assessment of microcirculatory obstruction within infected patients. Orthogonal polarization spectral imaging has been applied to quantify the number of blocked capillaries in the rectal mucosa [65]. Studies performed in Asian adults have shown that around 84% of patients presented a median of 9–14% blocked rectal capillaries [60, 65, 66]. A higher percentage of blocked capillaries correlated with cerebral malaria, acute kidney failure, and multi-organ dysfunction. Moreover, blocked rectal capillaries were correlated with plasma lactate levels and were higher in patients that died (14.9%) than survivors (8.3%) [66]. Taken together, these reports suggest that microvascular obstruction associated with higher parasite burden is likely to be an important factor in elevated lactate levels in severe falciparum malaria (Figure 2).

Despite providing a good assessment of patient severity, orthogonal polarization spectral imaging does not reflect the parasite sequestration in organs directly affected by *P. falciparum*. To overcome this limitation, retinal imaging is used to study malaria retinopathy in cerebral malaria [67, 68]. The retina is a window to the brain microcirculation [69], as both tissues share embryologic origin. Ocular fundoscopy performed in malaria patients can reveal vessel color changes, macular and extramacular whitening, and the appearance of white-centered retinal hemorrhages. Several studies suggest that malaria retinopathy reflects pathological processes in the central nervous system. For instance, the severity of the retinopathy is associated with a higher risk of patient death [70] and parallels the extent of

parasite sequestration in retina and brain microvasculature [71, 72]. In addition, there is a correlation between retinal and brain hemorrhages and the presence of fibrinogen leakage in the retinal and brain microvasculature, indicative of barrier breakdown [73, 74]. The presence of blocked capillaries in the retina can be quantified using advanced approaches, such as fluorescein angiography. This novel technique showed that non-perfused capillaries overlapped with areas of retinal whitening observed by ocular fundoscopy [75]. 40% of retinopathy-positive patients present brain swelling assessed by magnetic resonance imaging (MRI) [68] and the degree of sequestration in the retina is associated with the degree of brain swelling [71]. The demonstration that retinal changes are directly associated with parasite sequestration provides valuable information about neurovascular pathology, and reveals that damage induced by parasite factors is one of the main contributors to cerebral disease.

As all parasite binding types can potentially contribute to microvascular obstruction, it is currently unclear whether EPCR-binding parasites make a greater contribution than other binding variants to microvascular obstruction, systemic lactic acid production, and metabolic acidosis. However, there is indirect evidence from both *var* transcriptional profiling and immunoepidemiological studies that EPCR binding variants tend to dominate infections of hosts with limited malaria immunity [9, 23, 24, 27, 28]. Moreover, the low expression of CD36 in brain endothelium suggests that EPCR-binding parasites might play a prominent role in microvascular obstruction and the pathology present in the retina and the brain.

Parasite sequestration, inflammation and organ complications

Histopathological observations in Southeast Asian adults and African children have suggested that both parasite and host factors might contribute to disease pathology (Figure 2). A large and systematic pediatric autopsy study in Blantyre, Malawi revealed two brain histological patterns: a) CM1 characterized by IE sequestration alone and b) CM2 characterized by IE sequestration in conjunction with ring hemorrhages, coagulation, and extra-erythrocytic **hemozoin pigment** (a byproduct of parasite hemoglobin digestion) [76, 77]. The Blantyre autopsy study has also revealed potential interplay between sequestered parasites, intravascular brain monocytes and platelets in pediatric cerebral malaria pathophysiology [78]. Furthermore, the presence of intravascular brain monocytes and platelets is increased in HIV^{+} cases compared to HIV- cases [79], suggesting there may also be interactions between HIV and falciparum malaria in CM pathophysiology. Although African children with fatal CM have shown more evidence of intravascular monocytes, platelets, and **fibrin** than adults [80], brain intravascular monocytes are occasionally present in adult CM studies [81]. Taken together, brain histopathology has revealed a range of monocyte inflammation that remains confined to the vascular space. In addition to the localized damage that inflammatory cells might cause, high systemic concentrations of proinflammatory cytokines, such as tumor necrosis factor α (TNFα), interferon-γ (IFN-γ), and IL-6 are found in plasma of cerebral malaria patients. Whereas higher systemic levels of TNFα have been associated with pediatric cerebral malaria fatality [82, 83], a recent study found that HIV coinfection blunted the proinflammatory cytokine response but did not affect parasite density or disease outcome in pediatric CM cases [84]. These findings point to complexity in severe malaria disease mechanisms

A much smaller series of autopsies have examined the organ histopathology associated with ARDS or acute renal failure in adults. In both ARDS and kidney failure, histopathology shows sequestered IEs, as well as intravascular inflammatory cells (predominantly monocytes in kidneys [85] and neutrophils in lungs [86]). However, the relative contribution of parasite sequestration and cellular infiltrates to kidney and lung organ dysfunction remains unclear.

Monocytes and fibrin clots are also prominent in placental malaria histopathology together with massive IE sequestration [87], raising the possibility of related mechanisms of organ injury/pathology in the brain, kidney, lung, and placenta [88]. In placenta malaria, monocytes are recruited secondary to sequestered IEs [87]. In cerebral malaria, it has been hypothesized that CM1 represents a more rapidly progressive disease that is associated with less microvascular injury (e.g. hemorrhages) and higher parasite burdens in the spleen (possibly due to clearance of dead and dying IEs) [89]. Conversely, CM2 has been hypothesized to represent a lower progressive infection which gives more time for host inflammatory cell recruitment and the development of microvascular pathology [89].

Parasite binding to EPCR and blockade of protein C/APC may additionally contribute to excessive proinflammatory and proadhesive signaling, by impairing an important regulator of inflammation. In addition, the lack of APC can lead to a pro-thrombin state that favors fibrin deposition and activates proinflammatory pathways that may promote monocyte recruitment to the microvasculature of the brain, kidney, and lung.

Endothelial activation and barrier disruption in severe malaria

Brain swelling and raised intracranial pressure are common findings in children with cerebral malaria, revealing a key pathogenic mechanism in pediatric mortality [6, 90]. This association has been strengthened by the application of MRI in comatose children. Severe brain swelling was detected in 84% of children who died from CM complications compared to 27% of survivors [91], and the most likely cause of death was attributed to brain-stem herniation as a result of swelling [91]. While neuroimaging has clarified the critical role of brain swelling in pediatric mortality, the extent of brain swelling in adult CM is debated, and is the subject of current research [92].

Early autopsy studies suggested that *P. falciparum* sequestration was associated with a loss of endothelial integrity and changes in the distribution of tight junction proteins [93]. The mechanisms that contribute to brain swelling are incompletely understood, but may involve distinct processes including i.) vasogenic edema (blood brain barrier (BBB) disruption), ii.) cytotoxic edema (cellular edema of neurons and astrocytes), iii.) increases in cerebral blood volume and changes in cerebral blood flow due to microvascular congestion, or iv.) fever, anemia, and seizure.

Central to the regulation of microvascular barrier properties is the balance of pro-barrier and barrier-disruptive signaling. Nitic oxide (NO) is a major factor contributing to vasodilation through relaxation of vascular smooth muscle [94] and disturbances in NO production may contribute to endothelial dysfunction in severe malaria. Both severe pediatric [95] and adult

cases [96] present lower levels of NO, probably due to hemolysis of uninfected erythrocytes and the rupture of IEs. Rupturing erythrocytes release free-hemoglobin [97] that acts as a NO scavenger, and arginases that decrease the availability of L-arginine, the substrate for NO synthesis [96] (Figure 2). In addition, hemolysis also increases the plasma concentration of asymmetric dimethylarginine (ADMA), a NO synthase inhibitor in adults [98]. Significantly, adult severe malaria patients present lower indexes of reactive hyperemia tonometry (RH-PAT), a measure of vascular endothelial function driven by NO [96]. Patients recovered endothelial function after two days of antimalarial treatment [99], suggesting a relationship between parasite sequestration, NO bioavailability, and endothelial function.

Low NO bioavailability and widespread endothelial activation during falciparum malaria [100] may further perturb endothelial barrier properties. For instance, plasma levels of angiopoetin-2 (Ang-2) are increased in severe malaria of children and adults. Both Ang-2 and Ang-1 signal through the same endothelial receptor, Tie-2 and exert opposite functions. While Ang-1 mediates vascular protective effects by increasing endothelial barrier function, inhibiting vascular inflammation and preventing apoptosis, Ang-2 is secreted from **Weibel-Palade bodies** (WPB) during endothelial activation and promotes endothelial leakiness and inflammation. In a physiological state, NO inhibits the exocytosis of WPB from endothelial cells and the release of Ang-2 to the blood. In adults with severe malaria, increased Ang-2 plasma levels were associated with a decrease in NO bioavailability, higher lactate plasma concentrations, and patient mortality [101]. Notably, pediatric studies also showed that Ang-1 and Ang-2 levels can discriminate children with cerebral malaria [102] and retinopathy-positive cases [103], and can predict the level of damage in the retina [104]. Both microvascular obstruction and systemic endothelial activation have been linked to tissue hypoperfusion in malaria, and may make independent contributions to plasma lactate levels in malaria [66]. For all these reasons, NO has been proposed as a candidate for severe malaria adjunctive therapy (Box 2). However, a recent clinical trial did not show a reduction in Ang-2 levels and mortality in patients that received inhaled NO [105].

An additional consequence of IE sequestration is that parasite factors released during IE rupture are in close proximity to endothelial cells and may further contribute to the dismantlement of the BBB. For instance, extracellular *P. falciparum* histones [106] and soluble HRP-2 [107] both compromise brain endothelial barriers and may contribute to pathology.

Consequently, numerous host and parasite factors (summarized in Figure 2) may converge to disturb endothelial barrier properties in congested microvessels, and these stimuli may be amplified in the presence of EPCR binding parasites. For instance, APC significantly upregulates the expression of Tie-2 and Ang-1 strengthening barrier properties [108], and consequently EPCR-binding parasites may contribute to increases in the Ang-2/Ang-1 ratio. In addition, PfEMP1 blockade of APC may increase barrier disruptive signaling, as APC inhibits the release of WPB and Ang-2 [109]. Moreover, parasite impairment of the APC-EPCR pathway may compromise regulation of both host and parasite factors that disturb barrier properties. For instance, APC counteracts barrier weakening signals from both thrombin and Ang-2, and it proteolytically inactivates *P. falciparum* histones, which induce endothelial activation and barrier dysfunction [106].

Coagulation and severe malaria

Several observations also point to alterations in coagulation in severe malaria (reviewed in [110]) (Figure 2). Although bleeding is rare in severe pediatric and adult malaria (<1%) [6], laboratory and histopathological findings suggest the induction of a procoagulant state in malaria. Recent studies have shown that the presence of disseminated intravascular coagulation is associated with fatal pediatric cerebral malaria [111]. In pediatric autopsies, 38–93% of brain microvessels contained fibrin [33]. Although fibrin staining was equally detected in cases with or without hemorrhages [33], microthrombi are present at the center of nearly all cerebral ring hemorrhages [76, 89]. Thrombi indicative of disseminated intravascular coagulation were also present in the pulmonary and renal capillaries of CM2 cases [89]. While fibrin is less prominent and thrombosis was absent in some adult autopsies [80], thromboses and ring hemorrhages were co-located in a histopathological study of military soldiers who died from falciparum malaria [112]. In addition, adult autopsy studies have shown the presence of ring hemorrhages and fibrin deposition in the brain [113].

Central to the regulation of coagulation is the balance between pro-coagulant factors (e.g. thrombin) and anti-coagulant factors (e.g. APC). Several findings suggest that *P. falciparum* IEs contribute to a procoagulant environment. For instance, IEs induce an increased expression of **tissue factor** in endothelial cells and the assembly of coagulation complexes [114], likely due to the presence of negatively charged phospholipids in the membrane of IEs. In addition, parasite blockade of protein C activation may further augment microvascular coagulopathy by limiting the inactivation of factors V and VIII in the thrombin generation cascade (Figure 1 and Figure 2).

The activated endothelium also contributes to the establishment of a procoagulant state by releasing **von Willebrand Factor** (VWF) from WPB. VWF is a large glycoprotein that carries procoagulant factor VIII and mediates the adhesion of platelets to the injured endothelium. Clinical studies have demonstrated an increase of VWF antigen and propeptide in children with either severe or cerebral malaria [103, 115]. In addition to increased VWF release, malaria severity is associated with a decrease in the elimination and processing of VWF, as cerebral malaria patients present lower concentrations of ADAMTS13, an enzyme responsible for VWF cleavage [116].

The thrombocytopenia found in the majority of patients might be caused in part, by platelet adhesion to the activated endothelium and VWF fibers. The role of platelets in malaria pathogenesis has been previously reviewed [117]. In vitro assays have shown that platelets can kill the parasite within the IE [118]. Conversely, activated platelets can directly potentiate IE damage to the endothelium [119], and indirectly enhance the recruitment of both IEs and monocytes through the release of microparticles [120]. Platelets bind to the activated endothelium or to VWF fibers, thus creating a possible bridge for CD36-binding parasites in the microvasculature of organs with low expression of this receptor, such as the brain [121]. Collectively, these observations make a strong case for the potential pathological significance of altered coagulation in disease pathology. Furthermore, EPCR-binding parasites promote a pro-coagulant state with an increased concentration of thrombin that contributes to the fibrin deposition present in the brain of cerebral malaria patients.

Concluding remarks

Severe malaria is a complex syndrome with a multifactorial origin caused by both parasite and host factors. Even with effective antimalarial drug treatments, fatality rates are high (9 to 15%) [3, 4], and many cerebral malaria survivors have long-term neurocognitive impairments. New treatment options beyond antimalarial drugs are needed to treat the disease pathology. The discovery that parasites from severe malaria patients preferentially bind to EPCR represents a breakthrough in malaria pathogenesis research because it provides a link between pathophysiological mechanisms and parasite cytoadhesion. Microvascular congestion may not only precipitate disease pathways, but parasite blockade of protective APC activities may exacerbate disease mechanisms.

Even though EPCR is a central component in severe malaria, we still do not fully understand how the various pieces fit together to drive disease outcomes (see Outstanding Questions). Under this complex disease scenario, advanced computational techniques, such as systems biology and machine learning analysis, hold great promise for disengtangling disease mechanisms and ranking their importance. As a proof of concept, we have described that both parasite biomass and the transcription of EPCR-binding PfEMP1 variants are important for adult severe malaria [9]. We predict that the use of advanced computational methodologies will accelarate research on the pathogenesis of severe malaria and will illuminate critical pathways that trigger severe malaria. This understanding will assist the development of targeted adjunctive therapies for severe malaria.

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Glossary

Activated Protein C (APC)
protein that plays a key role in regulating endothelial cell permeability, apoptosis, inflammation, and anti-coagulation

Cerebral malaria
a neurological complication of *P. falciparum* infection associated with adhesion of infected erythrocytes in brain microvasculature

Cysteine Rich Inter-domain Region (CIDR)
binding domain in PfEMP1 that mediates adhesion to a specific endothelial receptor; can be classified into CIDRα/β/γ/δ subtypes.

Duffy-Binding Like (DBL)
binding domain in PfEMP1 that mediates adhesion to a specific endothelial receptor; can be classified into DBLα/β/γ/δ/ε/ζ subtypes

Endothelial activation

procoagulant and proinflammatory state of endothelial cells in response to diverse stimuli or damage.

Endothelial protein C receptor (EPCR)
endothelial surface protein that enhances Protein C activation

Fibrin
insoluble protein present in blood clots in combination with platelets. Thrombin is responsible for fibrinogen polymerization into fibrin

Head structure (of PfEMP1)
tandem domains in the N-terminal region of PfEMP1 composed of DBLα-CIDRα/β/γ/δ

Hemozoin pigment
crystal produced by the polymerization of heme as a result of hemoglobin digestion by *Plasmodium* spp

HRP-2
P. falciparum blood stage protein released during IE rupture. It has a long half-life in blood and is used as a proxy for parasite biomass

Metabolic acidosis
condition that causes a drop in the blood pH. In severe malaria, it can be caused by kidney injury or by an excess of lactate due to tissue hypoxia

Parasite biomass
total amount of *P. falciparum* blood stage parasites. It comprises the circulating immature stages (measured by peripheral parasitemia) and the sequestered mature stages. The quantification of PfHRP2 is used as a proxy for parasite biomass

Placental malaria
Malaria disease syndrome associated with the adhesion of infected erythrocytes within the intervillous space of the placenta

***P. falciparum* Erythrocyte Membrane Protein 1**
key parasite cytoadhesion ligand expressed at the surface of IEs. PfEMP1 is composed of different arrangements of DBL and CIDR adhesion domains

Retinopathy-positive patients
patients with abnormal retinal vasculature, including vessel color changes, macular whitening and retinal hemorrhages

Rosetting
adhesion of IEs to uninfected erythrocytes. Rosettes are composed by at least one IE and two uninfected erythrocytes

Thrombin

pro-coagulant and proinflammatory serine protease that converts fibrinogen into fibrin and promotes blood clotting

Thrombomodulin
protein that reduces coagulation and endothelial activation by converting thrombin to an anti-coagulant enzyme that increases protein C activation

Tissue Factor
protein present in the subendothelial tissue that senses endothelial damage. It binds to factor VII and initiates the “extrinsic pathway” of the coagulation cascade.

Von Willebrand Factor
glycoprotein stored in Weibel-Palade Bodies and released after endothelial activation. It forms long strings and has an important role in coagulation because it binds to factor VIII and to platelets

Weibel-Palade Bodies (WPB)
membranous storage vesicles that are released after endothelial cell activation

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Box 1

Parasite Modulation of Endothelial Cells

The extreme polymorphism in the PfEMP1 family enables parasite evasion of host immunity, and recent evidence suggests that sequence variation may also result in variation in binding phenotypes. For instance, CIDRα1 domains bind EPCR with different affinities [10, 21] and vary in the extent of APC blockade [9, 30, 32]. Moreover, divergent functional outcomes of CIDR domain engagement were also observed on microvascular endothelial cells from the lung and brain [30]. These findings reveal a greater potential complexity in how CIDRα1-expressing parasites interact with endothelial cells. It is notable that PfEMP1 binding sites frequently overlap with functionally critical sites on cytoadhesion receptors, such as EPCR [21] and CD36 [122] . It may not be coincidental that parasites interact with host receptors that control important functional pathways regulating coagulation, inflammation, and endothelial barrier properties. Although speculative, it is possible that these host receptor interactions were evolutionary selected because they facilitate parasite survival by allowing a rapid response to localized changes in the microvasculature. An improved understanding of the molecular and cellular consequences of parasite cytoadhesion to endothelial receptors (either direct signaling pathways, competition with native ligands, or modulation of native ligand signaling) may inform disease mechanisms and assist in the design of adjunctive drug interventions for severe malaria.

Box 2

Parasite pathogenic factors and potential future adjunctive therapies

Parasite sequestration in the microvasculature might cause:

- Impaired microvascular flow, tissue hypoxia, and metabolic acidosis.
- Endothelial activation that promotes barrier breakdown and increased expression of endothelial receptors that subsequently will recruit other IEs, monocytes, and platelets.
- Decreased NO bioavailability and release of WPB that contain Ang-2 and VWF. Low NO and high Ang-2 blood concentrations contribute to endothelial barrier permeability.
- Increased expression of TF and conversion to a procoagulant state. Increased thrombin levels that contribute to endothelial barrier permeability.

Parasite blockade of the EPCR-APC interaction could adversely multiply the effects mentioned above because APC is an anti-inflammatory protease that prevents endothelial activation and leakiness and drives the vascular homeostasis towards an anti-coagulant state. Future strategies that might counteract EPCR-binding parasites:

- Vaccines to block the EPCR-IE interaction.
- Therapeutic anti-PfEMP1 monoclonal antibodies to disrupt the EPCR-IE interaction.
- Engineered thrombin that disrupt the PfEMP1-EPCR interaction [123].
- Engineered soluble EPCR to compete for IE binding [31].
- Therapeutic APC.
- Molecules that act downstream of the EPCR-APC pathway, such as compounds that target the sphingosine-1-phosphate (S1P) pathway.

Outstanding Questions Box

Are EPCR-binding parasites associated with severe disease because they inhibit a protective pathway and leave the cells more susceptible to parasite toxins (e.g. Pf histone and PfHRP2) and host factors, such as inflammatory or coagulatory molecules?

Are there common disease mechanisms in severe malaria associated with EPCR-binding parasites?

Can we design strategies to block the IE-EPCR interaction or counteract its effects?

Can disease mechanisms be reversed in hospitalized patients by restoring EPCR function or pathways downstream of APC-EPCR?

Do anti-CIDRα1 antibodies contribute to the relatively rapid acquisition of anti-disease immunity that protects against severe malaria?

Which are the unknown receptors that mediate binding and are they associated with disease severity?

What is the role of rosetting in severe disease?

Is cerebral sequestration always associated with EPCR binding parasites?

Are specific PfEMP1 associated with vital organ complications in adults (kidneys, lungs)?

Trends box

- Pediatric and adult severe malaria are associated with EPCR-binding parasites.
- Microvascular dysfunction of vasodilation, coagulation, and blood-brain barrier properties contributes to severe malaria pathology.
- Malaria-associated loss of EPCR combined with parasite impairment of the EPCR-APC interaction may promote coagulation, inflammation, and endothelial barrier breakdown.
- Strategies to disrupt the parasite-EPCR interaction or counteract its effects could lead to new adjunctive therapies.

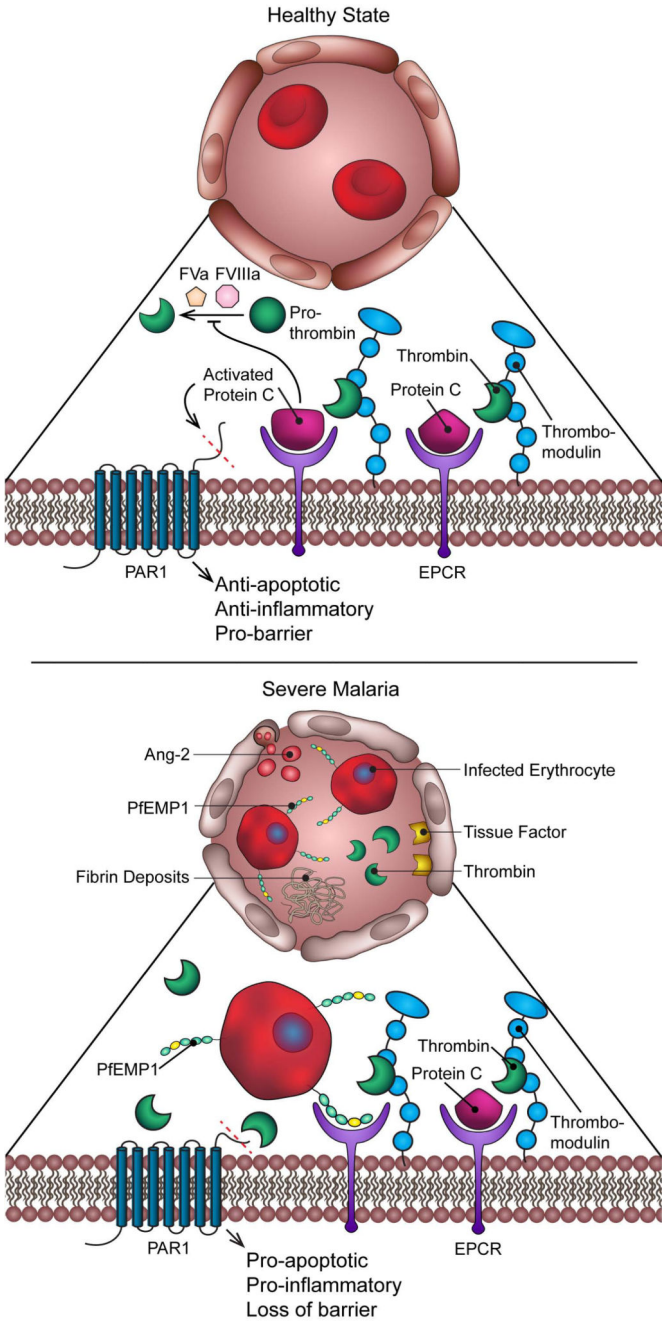

Figure 1. The role of EPCR in severe malaria

In healthy microvessels, EPCR promotes a quiescent state in endothelial cells (anti-coagulant and anti-adhesive) by enhancing the activation of protein C by the thrombin-thrombomodulin complex. APC elicits anti-apoptotic, anti-inflammatory and pro-barrier signaling pathways in endothelial cells by cleaving PAR1 at Arg46. Additionally, soluble APC inactivates the coagulation factors FV and FVIII leading to an anti-thrombotic state. In severe malaria, the parasite CIDRα1 domain (yellow) of PfEMP1 blocks the protein C binding site in EPCR. The lack of APC will cause an increase of thrombin formation and

eliminate a key negative feedback mechanism for regulating inflammation and coagulation. Thrombin elicits proinflammatory and barrier disruptive signals in endothelial cells by cleaving PAR1 at Arg41 and promotes the release of WPB and their contents (Ang-2, VWF) from activated endothelial cells. Additionally, thrombin cleaves fibrinogen to lead to fibrin deposition. Abbreviations: Ang-2, angiopoietin-2; APC, activated protein C; CIDRα1, cysteine-rich interdomain region α1; EPCR, endothelial protein C receptor; PAR1, protease-activated receptor 1; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; VWF, von Willebrand factor; WPB, Weibel-Palade bodies.

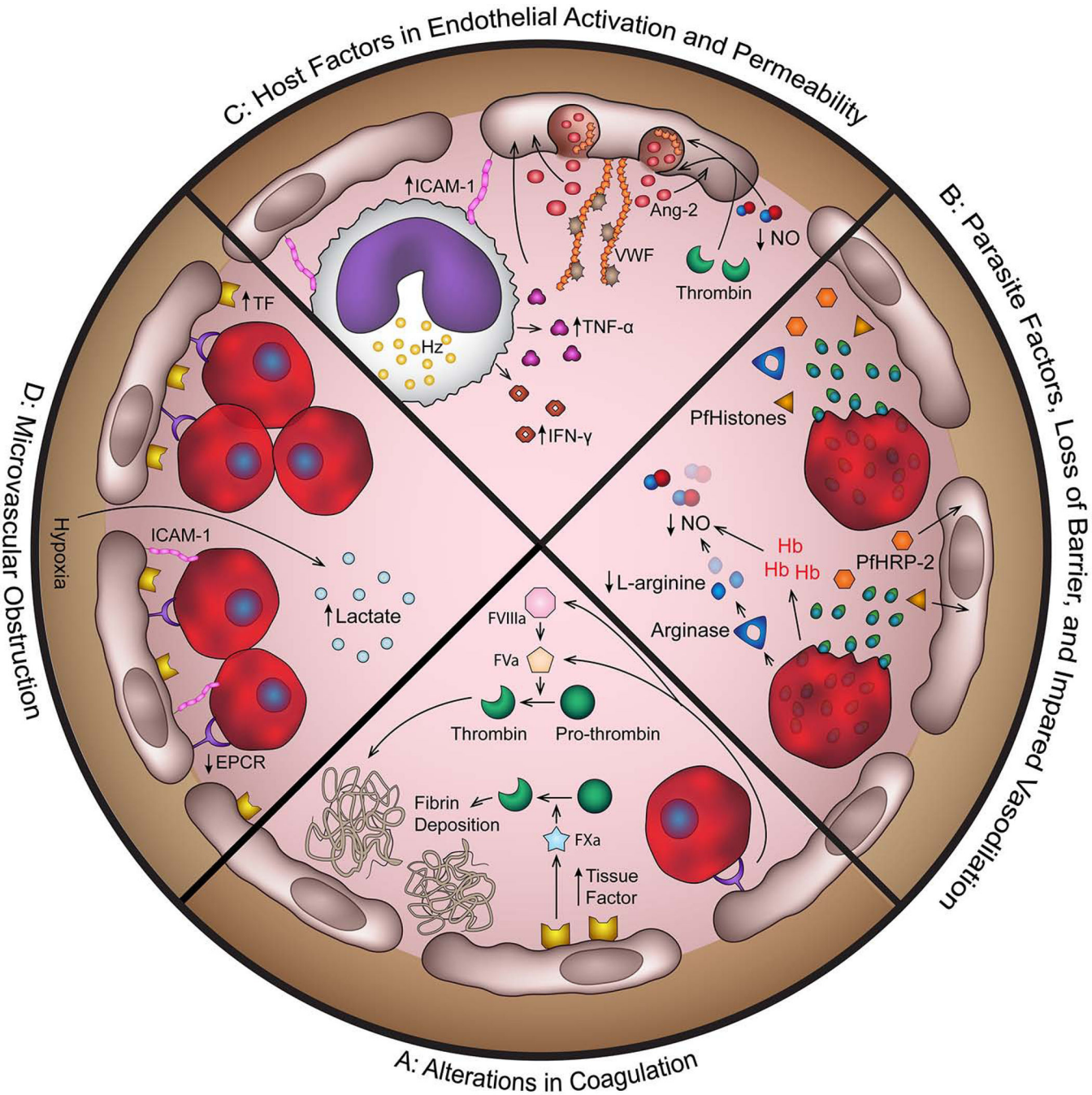

Figure 2. Schematic representation of the pathogenic events that contribute to severe malaria

(A) Alterations in coagulation. EPCR-binding parasites block the activation of APC. The lack of APC reduces the inactivation of FVIII and FV, and thereby promotes an increase in thrombin generation. Additionally, binding of IEs to endothelial cells induces an upregulation of tissue factor leading to a pro-thrombotic state. Thrombin causes fibrin deposition and enhances microthrombi formation. (B) Parasite factors that mediate loss of barrier properties and impaired vasodilation. Parasite toxins secreted during IE rupture, such as PfHRP-2 and extracellular *P. falciparum* histones, activate endothelial cells contributing

to loss of barrier properties. Rupturing erythrocytes release hemoglobin (Hb) and arginases leading to a decrease in NO bioavailability and impaired vasodilation. (C) Host factors that promote endothelial activation and permeability. Reduced bioavailability of NO and high concentrations of thrombin will increase the release of Ang-2 and VWF from activated endothelial cells. Both thrombin and Ang-2 promote endothelial activation and leakiness, and VWF contributes to a pro-coagulant state by recruiting platelets and coagulation factor (FVIII). Activated endothelial cells present an increased expression of endothelial markers contributing to the recruitment of monocytes. Sequestered monocytes present hemozoin pigment (Hz) due to phagocytosis of IEs and secrete proinflammatory cytokines, such as TNF-α and IFN-γ. (D) Microvascular obstruction. Microcirculatory blockages can result from a combination of sequestration and microvascular congestion. Reduced perfusion may potentiate the effect of inflammatory mediators and endothelial activation and lead to stronger IE binding or greater IE recruitment. Reduced tissue perfusion causes a shift to an anaerobic metabolism and metabolic acidosis. Abbreviations: Ang-2, angiopoietin-2; APC, activated protein C; EPCR, endothelial protein C receptor; IE, infected erythrocytes; IFN-γ, interferon-γ; NO, nitric oxide; PfHRP-2, *P. falciparum* histidine-rich protein-2; TNF-α, tumor necrosis factor-α; VWF, von Willebrand factor.