

Cerebral *Plasmodium falciparum* malaria: The role of PfEMP1 in its pathogenesis and immunity, and PfEMP1-based vaccines to prevent it

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Abstract

Malaria, a mosquito-borne infectious disease caused by parasites of the genus *Plasmodium* continues to be a major health problem worldwide. The unicellular *Plasmodium*-parasites have the unique capacity to infect and replicate within host erythrocytes. By expressing variant surface antigens *Plasmodium falciparum* has evolved to avoid protective immune responses; as a result in endemic areas anti-malaria immunity develops gradually over many years of multiple and repeated infections. We are studying the role of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) expressed by asexual stages of *P. falciparum* responsible for the pathogenicity of severe malaria. The immunopathology of *falciparum* malaria has been linked to cyto-adhesion of infected erythrocytes to specific host receptors. A greater appreciation of the PfEMP1 molecules important for the development of protective immunity and immunopathology is a prerequisite for the rational discovery and development of a safe and protective anti-disease malaria vaccine. Here we review the role of ICAM-1 and EPCR receptor adhering *falciparum*-parasites in the development of severe malaria; we discuss our current research to understand the factors involved in the pathogenesis of cerebral malaria and the feasibility of developing a vaccine targeted specifically to prevent this disease.

KEYWORDS

antibodies, cerebral malaria, immunity, PfEMP1, *Plasmodium falciparum*, vaccine

1 | INTRODUCTION

Infection with *Plasmodium falciparum* parasites causes the most severe form of malaria that is responsible for essentially all malaria-related deaths. The ability of *P. falciparum*-infected erythrocytes (IEs) to adhere efficiently to host vascular receptors sets this parasite aside from the other malaria parasites infecting humans, and is

generally considered an important reason why *P. falciparum* malaria is particularly dangerous.

IE adhesion is called sequestration when the IEs stick to tissue-bound receptors, rosetting when they stick to uninfected erythrocytes, and clumping when the IEs stick to each other. It can lead to circulatory disturbances, vascular occlusion, and inflammation. In all cases, the IEs interact with host receptors via members of

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parasite-encoded antigens displayed on the IE surface. These antigens belong mainly—if not exclusively—to products of several multigene families. Prominent among them—and by far the best studied—is *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by the clonally variant *var* gene family with approximately 60 members per parasite genome. This review focuses on PfEMP1, the putative role of this antigen family in the development of one of the most severe forms of malaria called cerebral malaria (CM) and in acquired immunity to CM, and finally on the prospect of a PfEMP1-based vaccine to prevent this often fatal complication. Before discussing each of these aspects it is necessary to recapitulate briefly the parasite life cycle, as it is important for appreciating the sections that follow.

1.1 | The parasite multiplication cycle

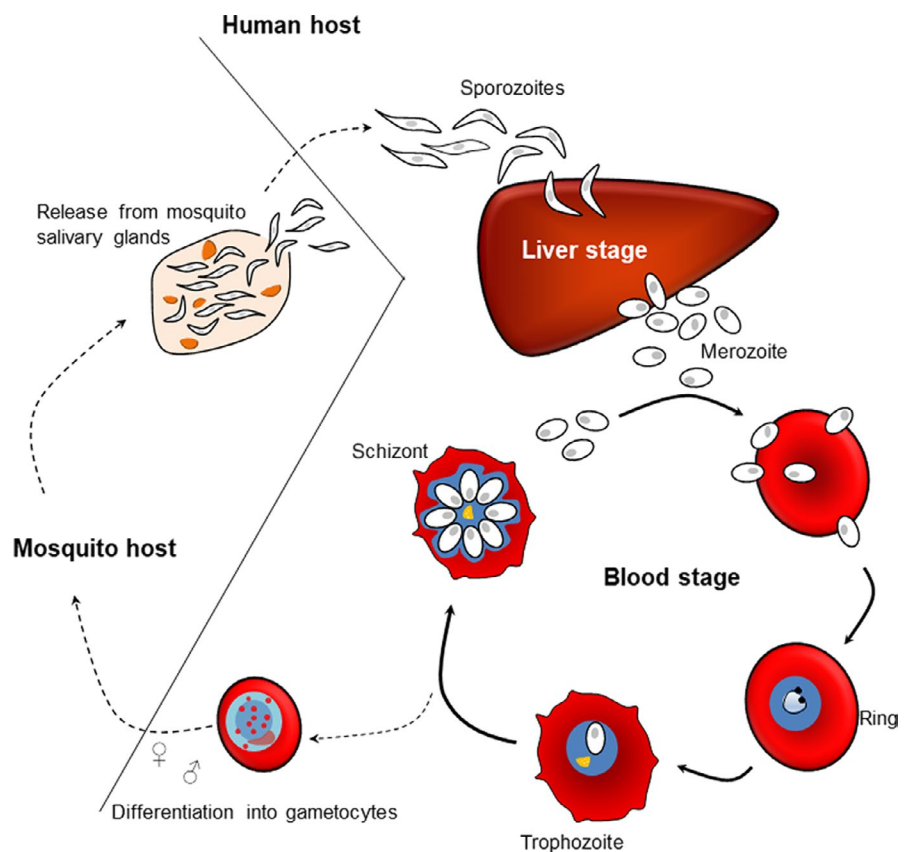
Plasmodium falciparum has a complex life cycle that involves two hosts (humans and *Anopheles* spp. mosquitoes), and several developmental stages in each host (Figure 1). The human part of the multiplication cycle, which is asexual, is initiated when a *P. falciparum*-infected female mosquito injects sporozoite-stage parasites into the skin while it is feeding for blood. The extracellular sporozoites rapidly transit via the peripheral circulation from the skin to the liver, where they infect hepatocytes. The liver stage is asymptomatic and lasts for approximately 1 week, during which time the intrahepatic parasite multiplies, resulting in a (pre- or extraerythrocytic) schizont that consists of at least 30 000 daughter parasites.

These, now called (pre- or extraerythrocytic) merozoites leave the infected hepatocyte and enter the blood circulation. The merozoites rapidly infect erythrocytes, an event that marks the beginning of the intraerythrocytic multiplication cycle. This part of the life cycle continues until the infection is controlled by either immunity or chemotherapy, or until the host dies. Each round of the intraerythrocytic cycle lasts approximately 48 hours. During each, the newly invaded merozoite rapidly transforms to a trophozoite (the early trophozoite is often called a ring-stage parasite, because of the prominent vacuole) that undergoes three to five mitotic divisions, resulting in a schizont. At the end of the intraerythrocytic cycle, the IE ruptures and the released (erythrocytic) merozoites rapidly invade new erythrocytes. Some of the newly invaded merozoites develop into male or female gametocytes rather than continuing the asexual multiplication cycle. The gametocytes do not divide, but remain inside the erythrocyte until taken up by a blood-feeding mosquito, where sexual reproduction and further asexual multiplication steps complete the parasite life cycle.¹⁻³

2 | THE *P. FALCIPARUM* ERYTHROCYTE MEMBRANE PROTEIN 1 ANTIGENS

The expression of PfEMP1 is largely (but not exclusively⁴) restricted to the intraerythrocytic blood stages of the infection, where these high-molecular weight proteins mediate IE adhesion to a variety of host receptors. Intracellular PfEMP1 can be detected a few hours

FIGURE 1 Life cycle of *Plasmodium falciparum*. Human infection with *P. falciparum* parasites is initiated when an infected female *Anopheles* spp. mosquito injects sporozoites during a blood meal. Sporozoites transit from the host peripheral circulation to the liver, where they infect hepatocytes. The liver stage is asymptomatic and lasts for approximately 1 wk. Eventually, the infected liver cells rupture to release extracellular merozoites into the host circulation. The merozoites invade erythrocytes, thereby initiating the asexual blood stage of the infection, which causes all the clinical symptoms of malaria. Once inside the erythrocyte, the merozoite undergoes a series of divisions (schizogony) over a period of 48 h, following which daughter merozoites are released to infect new erythrocytes. Some asexual parasites do not undergo schizogony, but develop into sexual precursors (gametocytes), which can be taken up by mosquitoes during a blood meal to complete the life cycle



after the merozoite has invaded an erythrocyte, whereas IE surface expression starts about 16 hours postinvasion.⁵ The PfEMP1 surface expression reaches a plateau about 8 hours later, and starts to decrease during the final hours of the 48-hour cycle,⁶ since export of PfEMP1 to the IE surface stops 30–36 hours postinvasion.⁷

2.1 | PfEMP1 structure

The members of the PfEMP1 family are high-molecular weight proteins (200–450 kD), encoded by approximately 60 two-exon *var* genes per haploid *P. falciparum* genome.⁵ The extracellular part of PfEMP1, which is encoded by exon I, is composed of cysteine-rich interdomain regions (CIDRs) and 2–10 Duffy-binding-like (DBL) domains. These DBL and CIDR domains can be divided into seven (α , β , γ , δ , ϵ , ξ , χ) and three (α , β , γ) main sequence classes, respectively, each with many further subdivisions.⁸ While exon I is characterized by extensive intraclonal (within single genomes) and interclonal (between genomes) sequence variation, the short transmembrane domain and the acidic intracellular terminal segment (ATS) are encoded by the relatively conserved exon II.⁸

The PfEMP1 CIDR domains are characterized by conserved cysteine-rich motifs,⁹ while the DBL domains are homologous to *P. falciparum* EBA-175 adhesive domains and to the Duffy-binding proteins of *P. vivax* and *P. knowlesi*.¹⁰ The DBL domains are composed of three structural subdomains (Figure 2A), which have a mixed helix-sheet structure (S1) or consist of helix bundles (S2 and S3).¹¹ The subdomains are held together by disulphide bonds between conserved cysteine residues,¹² whereas the α -helices of the CIDR and DBL domains are connected by flexible and/or ordered loops. The functional specificity of different PfEMP1 proteins often (but not always) depends on these highly variable loops.^{12–14}

Despite the sequence variability, PfEMP1 proteins can be grouped according to their chromosomal location, upstream promoter sequence (*ups*), and direction of transcription of the *var* genes encoding them.¹⁵ Group A (10 genes in *P. falciparum* 3D7), Group B (22 genes), and Group B/A *var* genes (4 genes) are all found in the subtelomeric regions of chromosomes. Group A genes are transcribed toward the telomere, whereas Group B and B/A *var* genes are transcribed toward the centromere (Figure 2B). Group C (13 genes in *P. falciparum* 3D7) and Group B/C *var* genes (9 genes) are typically found in internal regions of chromosome 4, 7, 8 and 12.^{5,8,15,16} All PfEMP1 subfamilies except two (Type 3 and VAR2CSA) have a head structure at their N-terminus that is composed of semiconserved DBL α domain and a CIDR domain.⁵ This is followed by a second and more diverse DBL-CIDR pair in most PfEMP1 proteins belonging to Group B, B/C, and C. Group A and B/A PfEMP1 proteins are composed of a total of 7–10 extracellular domains, including additional DBL domains upstream and/or downstream of the second DBL-CIDR pair (Figure 2C).

The combination of DBL and CIDR subtypes in different PfEMP1 proteins is non-random, and has led to the identification of 21 domain trains called domain cassettes (DCs).⁸ The DCs are defined as *var* gene sequences encoding two or more DBL or CIDR domains

with subclasses that can be predicted from each other, and they often predict the receptor specificity of the encoded PfEMP1 (Figure 2D).¹⁷ DC4 (DBL α 1.1/1.4-CIDR α .6-DBL β 3),¹⁸ DC8 (DBL α 2-CIDR α 1.1-DBL β 12-DBL γ 4/6), and DC13 (DBL α 1.7 and CIDR α 1.4)⁸ are the DCs studied most extensively.

Most PfEMP1 appear to be elongated and are rigid molecules with a zigzag shape and a length of about 30 nm,^{19,20} although VAR2CSA-type PfEMP1 assume a more compact and globular shape with a diameter of approximately 20 nm.^{21,22}

2.2 | PfEMP1 function

The primary function of the PfEMP1 proteins is to mediate adhesion of IEs to host receptors in the vasculature.^{17,23} This sequestration is vital to the parasites, as it allows mature IEs (misshapen and rigid because of the parasites growing inside them) to avoid the spleen passage, where they would be filtered and destroyed.^{24,25} A range of vascular surface proteins and carbohydrates can serve as IE adhesion receptors, including CD36,²⁶ intercellular adhesion molecule 1 (ICAM-1),²⁷ endothelial protein C receptor (EPCR),²⁸ oncofetal chondroitin sulphate (CSA),^{29,30} and ABO blood group antigens.³¹ The expression of IE adhesion receptors varies between different vascular beds, and is often regulated by cytokines.^{32,33}

Many different PfEMP1 proteins appear to have specificity for the same receptor, and this to some extent corresponds to the structurally defined PfEMP1 groups and domain subclasses mentioned above.^{8,15,16} Thus, subclasses of DBL β domains found in Group A, B, and C PfEMP1 bind ICAM-1.^{14,18,34,35} CIDR α 1 domains from Group A and B/A PfEMP1 bind EPCR,^{13,28} whereas Group B and C contain CIDR α 2-6 domains that bind CD36 (Figure 2D).^{36,37} Finally, Group E (VAR2CSA-type) PfEMP1, which appear responsible for IE accumulation in the placenta, have unique affinity for CSA.^{30,38,39} It is not very surprising that several of these large, multidomain proteins contain domains that can simultaneously mediate adhesion to several host receptors.^{14,35,40,41} Examples include PfEMP1 variants concurrently interacting with ICAM-1 and EPCR,^{14,41} with ICAM-1 and CD36,³⁵ or variants capable of distinct receptor-ligand interactions with endothelial cells and uninfected erythrocytes.⁴²

2.3 | Clonal antigenic variation controlling PfEMP1 expression

Aside from an exception of unresolved biological significance,⁴³ only one PfEMP1 variant is expressed on the surface of a given IE at any given time,⁴⁴ but the expressed variant may change from one 48-h cycle to the next. The ability to switch from the expression of one PfEMP1 variant to another (called clonal antigenic variation) acts as a key to the pathogenicity of *P. falciparum* parasites and is a major determinant of the characteristic chronicity of untreated infections.⁴⁵ The genetic processes governing clonal antigenic variation in *P. falciparum* parasites are complex, and will not be described here, as they have been recently reviewed in detail elsewhere.⁴⁶ However, the switching pattern does not appear to be fixed, but rather seems

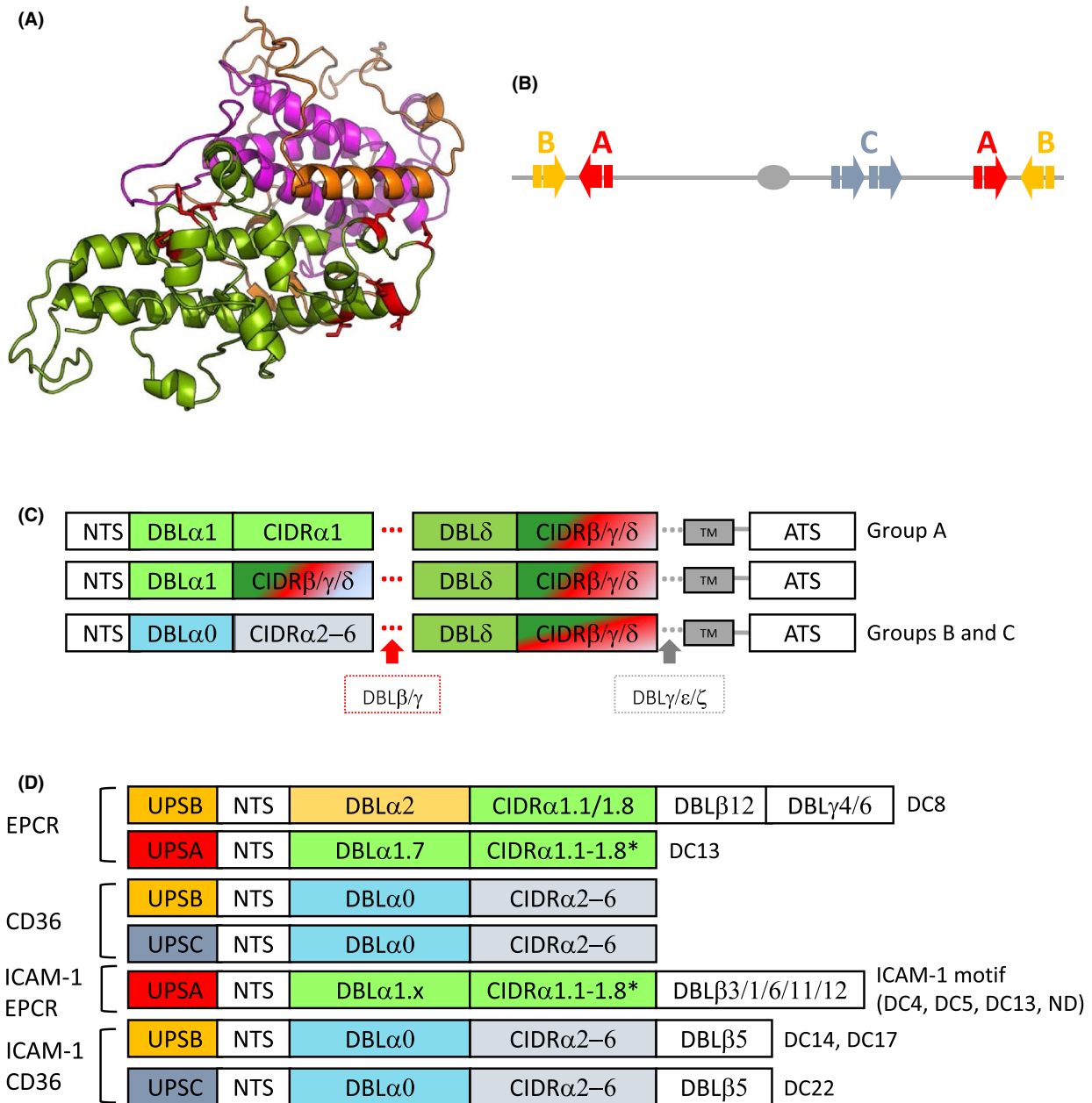


FIGURE 2 var genes and PfEMP1 structure. (A), Modeled structure of PFD1235w DBLβ. The structure consists of subdomain 1 (S1, orange) with mixed helix-sheet structure and two helix bundles; subdomain 2 (S2 magenta) and subdomain 3 (S3 green).¹² The ICAM-1 binding site of PFD1235w in S3 is indicated in red.^{14,18} (B), Group A and B var genes are located in subtelomeric regions of all chromosomes but are transcribed in opposite directions, whereas Group C var genes are found in central chromosomal regions. (C), PfEMP1 proteins are composed of different subtypes of DBL and CIDR domains. Groups B and C PfEMP1 predominantly have a four-domain structure, while larger PfEMP1 proteins have additional DBL domains following the first or second DBL-CIDR domains. (D), *Plasmodium falciparum* genomes encode tandem domain cassettes (DC) that are linked to different known adhesion phenotypes as indicated. DC8 is a chimeric gene between a group A and a group B var gene. Redrawn and modified from Hviid and Jensen¹⁷

to follow a loose hierarchy determined by variations in the intrinsic switching (on/off) rates of individual var genes.⁴⁷⁻⁵⁰

2.4 | PfEMP1 expression on the infected erythrocyte surface

The expression of PfEMP1 on the IE surface is confined to membrane protrusions called knobs.⁵¹ Formation of knobs involves

multiple host and parasite molecules in addition to PfEMP1, such as the parasite-encoded knob-associated, histidine-rich protein (KAHRP) which multimerizes into a five-molecule spiral cone-like structure linked to erythrocyte cytoskeleton spectrin-ankyrin complexes (Figure 3).⁵²⁻⁵⁴

The nascent PfEMP1 molecules are exported to the IE surface via a complex, multistep process that has recently been reviewed in detail elsewhere.⁵⁵ Their ATS domains bind to the cytoskeleton via

LyMP (a member of the PHIST [*Plasmodium* helical interspersed sub-telomeric] protein family (Figure 3)).^{53,56,57} Recent data suggest that the exported PfEMP1 molecules are delivered to the IE membrane away from the knobs, and then moved laterally and assembled into the knobs.⁵⁴

The knobs appear on the IE surface approximately 16 hours postinvasion, peak in density about 20 hours later, followed by a slight decrease in density toward the end of the intraerythrocytic 48-h cycle.⁶ Each knob appears to accommodate less than a handful of PfEMP1 molecules, which are expressed in a cluster near the tip of the knob.⁵⁸ The reason for the clustered and knob-confined display of PfEMP1 on the IE surface is not fully understood. However, it likely includes optimization for adhesion, as the surface knob density appears to vary with the PfEMP1 expressed and may be modulated by immunity, and because knob-less IEs generally have reduced PfEMP1 expression and do not adhere well.^{6,59-61} Consistent with that, disruption of the gene encoding KAHRP leads to disappearance of knobs, decreased PfEMP1 expression, and reduced IE adhesiveness.⁶²⁻⁶⁴ Disruption of the gene encoding LyMP causes a similar decrease in IE adhesiveness, although expression of both PfEMP1 and knobs remain at wildtype levels.⁵⁷

Altogether, these findings suggest that the overall IE adhesiveness is the net result of which PfEMP1 is expressed (quality), how much of it is expressed (quantity), and how it is expressed (topology). This conclusion is supported by studies linking the protective effects of haemoglobinopathies such as HbC, HbS, and α -thalassemia to an impaired ability of *P. falciparum* parasites to adequately remodel the erythrocyte cytoskeleton and display PfEMP1 in these aberrant erythrocytes.⁶⁵⁻⁶⁸

3 | CEREBRAL MALARIA

Cerebral *P. falciparum* malaria (CM) is defined by the World Health Organization (WHO) as deep and unarousable coma that persists for more than 1 hour after a seizure, irrespective of anticonvulsant medication, in a patient with peripheral *P. falciparum* parasitemia and without another cause of encephalopathy.⁶⁹ It is estimated that around 1% of children infected with *P. falciparum* develop CM, which has a very high mortality despite treatment, with up to 75% of deaths occurring within the first 24 hours postadmission.⁷⁰⁻⁷² CM is a leading cause of the estimated >400 000 deaths due to malaria each year⁷³ despite the fact that this clinical definition of CM may lead to misclassification in as many as one in four cases.^{74,75}

In sub-Saharan Africa, CM mostly affects children under 5 years of age who have only partial acquired immunity to *P. falciparum* infection. Several studies have shown seasonal and transmission intensity-dependent differences in the frequency of CM,^{76,77} suggesting that the level of acquired immunity is an important determinant of CM susceptibility.⁷⁸ This is supported by the finding that CM is mainly seen among older children and adults in Southeast Asia, where malaria transmission is less intense than in sub-Saharan Africa.⁷⁹

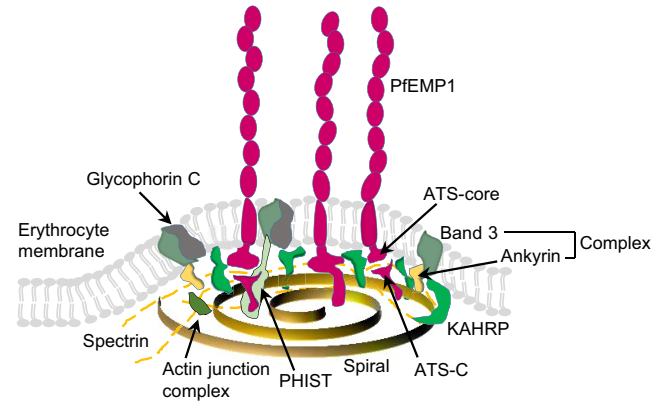


FIGURE 3 PfEMP1 presentation on knobs on the IE surface. KAHRP form the base of the knob complex. An average of three PfEMP1 molecules are located at the tip of each knob.⁵⁸ KAHRP binding to spectrin is necessary for the formation of the spiral structure, but KAHRP itself does not appear to be a component of the spiral.⁵⁴ PHIST protein may connect to PfEMP1 and the cytoskeleton.⁵⁶ Redrawn and modified from Cutts et al⁵³

There are significant differences in the pattern of vital organ dysfunction between African children and Southeast Asian adults with CM.^{70,80-82} In children, CM coincides with a period of rapid brain growth and physiologic changes of the blood-brain-barrier (BBB) that may account for some of these differences.⁸³ Although CM in children generally has lower mortality than in adults under otherwise comparable conditions, seizures are more frequent and rapid, and CM in children is more often associated with anemia and neurocognitive sequelae.^{83,84} Retinal vessel changes, ring hemorrhages, and accumulation of inflammatory cells in the brain microvasculature are also more frequent, and hypoglycemia is one of the most common concomitant complication in pediatric CM cases.⁸³⁻⁸⁵

In the following sections, we will focus on CM as it occurs among children in Africa because this is the population most affected, and also because most of the available knowledge about CM stems from studies of African children (Box 1).

3.1 | Subtypes of CM

Retinopathy, characterized by retinal hemorrhages, papilledema, retinal whitening, and vessel color changes, is the most specific clinical diagnostic sign of CM,⁸⁶⁻⁸⁸ because it directly reflects the cerebral sequestration of IEs and the pathological processes occurring in the brain.⁸⁸⁻⁹⁰ It thus allows distinction between children with coma caused by cerebral IE sequestration and those, whose coma has other causes but fulfil the abovementioned WHO criteria for CM. The number of retinal hemorrhages prior to death correlates with the density of hemorrhages in the brain at mortem.⁹¹ Retinopathy has therefore been suggested to reflect the spectrum of CM severity^{86,92,93} as patients with retinopathy have higher mortality than those without.^{94,95}

Box 1 Animal models of *Plasmodium falciparum* malaria

A detailed analysis of the intracerebral pathogenesis and pathology in CM comes primarily from postmortem studies. For this reason, infections of various strains of mice with *P. berghei* ANKA (PbA) is a popular in vivo model to dissect the mechanisms of CM. PbA infection can induce a condition known as experimental cerebral malaria (ECM), which recapitulates some—but certainly not all—features of CM. This has led to considerable scepticism regarding the utility of this murine model.^{364,365}

In both humans and mice, the clinical signs of CM progress from seizures, ataxia, and paralysis to coma and eventually death.³⁶⁶ However, although cerebral IE sequestration is a prominent feature of CM,³⁶⁴ it is a minor feature of ECM.^{367,368} Intravascular accumulation of platelets and immune cells has been observed in ECM, and CD8⁺ cells appear central to ECM pathogenesis.³⁶⁹⁻³⁷² The picture is less clear in CM, where some studies have reported infiltration of leukocytes and platelets within the brain microvasculature,^{96,173} whereas others did not find that.^{195,373} Intravascular accumulation of monocytes has also been reported, but there is little evidence of high numbers of CD8⁺ T-cell accumulation in human CM.

ECM is characterized by a prominent pro-inflammatory cytokine response with high levels of IFN- γ and TNF- α , which results in upregulation of activation markers including ICAM-1, VCAM-1, and E-selectin.³⁷⁴ Although TNF- α -deficient mice are also susceptible to PbA-induced ECM,³⁷⁵ ICAM-1 must be present for ECM to develop.³⁷⁶ Although inflammatory changes in the brain are lower in CM, elevated concentrations of circulating pro-inflammatory cytokines are characteristic.^{377,378} This contributes to a marked increase in the expression of endothelial cell adhesion molecules,^{106,144} and IEs and ICAM-1 co-localize in cerebral vessels postmortem.¹⁰⁶ Of particular relevance to the present text, none of the genomes of rodent malaria parasite encodes PfEMP1-like molecules, and we therefore direct interested readers to comprehensive ECM reviews elsewhere.^{379,380}

Pediatric CM can also be divided into two types based on clinical and pathological findings. CM1 (25% of cases) is characterized by IE sequestration in the cerebral microvasculature, rapid disease progression, limited organ pathology, and high splenic parasitemia. CM2 is characterized by more prolonged illness and parasite proliferation, with evidence of vascular brain pathology alongside endothelial dysfunction, activation of coagulation, ring hemorrhages, fibrin-platelet thrombi, and accumulation of monocytes in addition to sequestered IEs.^{75,82,96} The proportion of retinal capillaries with extraerythrocytic hemozoin predicts CM1 and CM2.^{75,97}

Increased intracranial pressure with brain swelling and perivascular edema are strongly associated with CM severity.⁸⁸ This is because the increased volume of the swollen brain can cause brainstem

herniation leading to death by respiratory arrest.^{98,99} These manifestations are partly due to breakdown of the blood-brain barrier.

3.2 | The blood-brain barrier

The BBB is vital for normal brain function and constitutes a physiological barrier that separates the brain and the cerebrospinal fluid from the rest of the body.¹⁰⁰ The BBB acts as a semipermeable cellular interface that tightly regulates the bidirectional transcellular molecular transport (of glucose, amino acids, transferrin, charged plasma proteins etc) between the blood and the brain parenchyma that is required to maintain cerebral homeostasis.

The BBB components include microvascular endothelial cells forming a continuous barrier through tight junctions, a basement membrane, pericytes, and astrocytes that are in direct contact with neurons and microglia. This composition is critical to minimize local inflammation and neuronal damage.¹⁰¹ Brain endothelial cells are structurally and functionally different from endothelial cells in other organs. In particular, they have intercellular tight and adherens junctions, which normally impede passive paracellular diffusion of small and large molecules and prevent infiltration of blood cells into the brain parenchyma.

Disruption of the BBB is common in diseases of the central nervous system.¹⁰² It is also a feature of CM,¹⁰³ where it is thought to be the result of endothelial inflammation in the brain, caused by accumulation and sequestration of IEs, leukocytes, and platelets.^{85,104,105} Focal loss of the endothelial intercellular junctions that are central to the maintenance of BBB integrity has been observed in vessels containing sequestered IEs.^{103,104,106-108} The finding of decreased transendothelial resistance and changes in the expression of proteins that make up these junctions in brain endothelial cells exposed to IEs in vitro is consistent with these observations.^{109,110} Numerous parasite and host factors that have been implicated in CM pathology can affect the permeability of the BBB. These include hemozoin-induced matrix metalloproteases (MMP), angiotensins, sphingosine-1-phosphate, nitric oxide, platelet-activating factor, several cytokines (IL-1 α , IL-1 β , IL-6, TNF α), and a number of other factors.^{91,111-117} As an example, MMP targets structural proteins of the basal lamina (fibronectin, laminin, heparan sulfate) and tight junction proteins (ZO-1, ZO-2, claudin-5), which is known to cause breakdown of tight junctions, increased paracellular leak, and opening of the BBB during ischemic and inflammatory insults. Another protein, histidine-rich protein-2 (HRP-2) that is released at the time of schizont rupture,¹¹⁸ can activate the innate immune system via NLRP3 inflammasome activation. The ensuing compromising of tight junction integrity and IL-1 β - and MyD88-dependent increased vascular permeability has been proposed to promote CM pathogenesis.¹¹⁹ In support of this, HRP-2 has been shown to line the endothelial walls of blood vessels,¹²⁰ particularly in retinopathy-positive CM patients.¹²¹ Once the BBB is disrupted, leukocytes, cytokines, chemokines, and soluble parasite products may enter the brain parenchyma to activate the microglia and damage astrocytes and neurons, causing neuroinflammation and coma.¹²²

3.3 | Endothelial activation

Endothelial inflammation is a characteristic feature of *P. falciparum* malaria and correlates with disease severity in general and CM in particular.¹²³⁻¹²⁷ The inflammation may be induced directly by IEs adhering to the endothelium, or indirectly by inflammatory host and parasite products (such as IE membrane components, HRP-2, etc).¹²⁸⁻¹³² However, activation may also occur independent of IEs as there is also evidence of generalized endothelial inflammation at sites devoid of IE sequestration.^{110,133-135}

Endothelial cells derived from Malawian children with CM have been shown to express particularly high levels of parasite and platelet receptors, to produce many endothelial microvesicles, release high levels of pro-inflammatory cytokines (including TNF α and IFN γ), and to be highly prone to undergo apoptosis.^{134,136,137} It seems likely that IEs may be involved, as they can induce apoptosis in primary brain endothelial cells, including cells from the brain,¹³⁸ and cellular apoptosis has been suggested to cause increased endothelial permeability.¹³⁸⁻¹⁴¹

Activated brain endothelial cells are known to express high levels of a number of potential IE receptors (ie, ICAM-1, VCAM-1, P-selectin, and E-selectin), exocytose Weibel-Palade bodies, release microvesicles, vascular endothelial growth factor (VEGF), and soluble cell adhesion molecules (ie, sICAM-1), and to show breakdown of tight junctions.¹⁴²⁻¹⁴⁶ Three bioactive molecules are released from the Weibel-Palade bodies, P-selectin (recruiting leukocytes), von Willebrand Factor (vWF; binding platelets), and Angiopoietin (Ang)-2. Ang-1 and Ang-2 are critical soluble regulators of endothelial activation and integrity, and levels of Ang-1 and Ang-2 have been described as reliable biomarkers of CM.¹⁴⁷ Ang-2 is a vessel-destabilizing molecule that increases vascular permeability and facilitates endothelial activation by counteracting the action of Ang-1 by displacing Ang-1 from the receptor.¹⁴⁸⁻¹⁵⁰ Ang-1 conversely mediates activation of the Tie-2 receptors on endothelial cells. This inhibits apoptosis, reduces expression of ICAM-1, VCAM-1, and E-selectin, promotes NO synthesis, and increases the expression of endothelial tight junctions.¹⁵¹⁻¹⁵⁴ It thus acts as a promoter of endothelial cell quiescence and survival. Release of Ang-2 from Weibel-Palade bodies increases the Ang-2/Ang-1 ratio and thus endothelial responsiveness. Increased concentration of Ang-2 with decreased levels of Ang-1 has been associated with development of severe malaria in several studies,^{113-115,147,155} and children with retinopathy-positive CM have higher levels of Ang-2, Ang-2/Ang-1, soluble Tie-2, von Willebrand Factor, VEGF, and sICAM-1, and lower levels of Ang-1, compared to CM patients without retinopathy.¹¹⁴ Both Ang-1 and Ang-2 are regulated by nitric oxide (NO) produced in the endothelium from L-arginine. NO causes vasorelaxation, downregulation of endothelial receptors, and reduces thrombosis.¹⁵⁶ The bioavailability of NO is reduced in CM and this has been associated with fatal outcome.¹⁵⁷⁻¹⁵⁹ Low NO stimulates Weibel-Palade body exocytosis and activation of endothelium, with increased Ang-2 release from endothelial cells and expression of ICAM-1 and VCAM-1.^{160,161} Impaired NO

production thus disrupts the ang-1/Tie-2-dependent signaling that maintains endothelial cell quiescence and vascular integrity.^{159,162} This in turn promotes enhanced endothelial cell activation and cytoadhesion of IEs.¹⁶³ All this notwithstanding, inhalation of NO was not found to reduce neurological defects or mortality in children with CM.¹⁶⁴

von Willebrand Factor is synthesized by the endothelial cells, and some of the synthesized vWF is constitutively secreted into plasma, but most is stored within Weibel-Palade bodies and only secreted following activation of the endothelial cell.¹⁶⁵ vWF, particularly as large multimers, show enhanced binding to platelets and efficiently modulates aggregation of platelets.¹⁶⁶ IEs can bind to platelets via P-selectin, C1q receptors, and thrombospondin receptor (CD36), leading to formation of IE/platelet clumps.¹⁶⁷⁻¹⁶⁹ The significance of this is indicated by the observation that children who died of CM had more platelet build-up in cerebral vessels than those dying of severe malarial anemia or non-malarial encephalopathy.¹⁷⁰ Platelet accumulation was particularly prominent at sites of IE sequestration. Platelet-mediated IE clumping is thus likely to aggravate microvascular obstruction in CM, and release of tumor growth factor β from platelet granules may furthermore cause apoptosis of brain endothelial cells.^{171,172} In addition, accumulation of platelets may enable transfer of CD36 to endothelial cells, thus potentially providing an additional IE receptor to brain endothelium, which normally expresses little or no CD36.¹⁷³ Overall, the marked increase in plasma vWF levels in patients with severe malaria is likely to contribute to severe malaria pathogenesis.^{114,115,166,174} Finally, activation of endothelium leads to increased shedding of microvesicles from the plasma membrane of cells.¹⁷⁵ Endothelial microvesicles have been found in very high concentrations in children with CM, and their levels correlate with disease severity.^{176,177} It has been proposed that these vesicles may contribute to excessive T-cell activation and the immune pathogenesis of CM, as they express the molecules required for antigen presentation and T-cell stimulation, such as β 2-microglobulin, MHC-II, CD40, and ICOSL.¹⁷⁸ In addition, increased concentrations of non-endothelial microvesicles have been observed in CM, where they may also contribute to pathogenesis. Thus, the number of platelet-derived microvesicles correlates with the depth of the coma and thrombocytopenia,¹⁷⁷ and extracellular IE-derived vesicles containing PfEMP1 (see below) can induce pro-inflammatory cytokines in human primary monocytes.^{179,180}

4 | PFEMP1 AND PATHOGENESIS OF CM

As mentioned above, *P. falciparum* parasites display PfEMP1 molecules on the surface of the erythrocytes they infect. From about 16 hours postinvasion, these high-molecular weight variant parasite proteins efficiently mediate adhesion of the IEs to a range of host receptors,¹⁸¹⁻¹⁸⁵ and this is the reason why only young, ring-stage IEs are present in the peripheral circulation. It has long been speculated that PfEMP1-mediated IE adhesion to specific

receptors in key tissues and organs is an important determinant of clinical outcomes of *P. falciparum* infection. This hypothesis has been confirmed in the case of placental malaria, where the selective accumulation of IEs in the intervillous space is mediated by VAR2CSA-type PfEMP1 with affinity for placental CSA.^{30,38,186} It is furthermore well established that protective immunity to placental malaria depends on acquisition of IgG to CSA-adhering and VAR2CSA-expressing IEs.¹⁸⁷⁻¹⁹⁰ These findings have raised the hope that other specific PfEMP1 variants and host receptors may play similar and decisive roles in other forms of severe *P. falciparum* malaria, not least CM.^{108,191-193}

4.1 | Sequestration of infected erythrocytes in the brain

IEs adhere to host endothelial receptors in the postcapillary venules of a number of organs, such as lungs, liver, intestine, brain, and the placenta.^{194,195} This tissue-specific sequestration causes circulatory disturbances and inflammation, and single- and multi-organ pathology such as renal, liver, lung, and placental dysfunction, and CM.¹⁹⁶⁻²⁰¹ Sequestration probably evolved to allow mature IEs, deformed by the parasites inside, to avoid destruction in the spleen.²⁴ Identification of the parasite ligands, not least the specific PfEMP1 variants, which mediate IE sequestration in particular tissues—and the host receptors they bind to, has thus been a long-standing and important research priority. CM research is no exception.

Many studies have reported links between severe malaria (including CM) in children and infection with parasites transcribing *var* genes encoding PfEMP1 proteins from Group A and B/A.²⁰²⁻²¹¹ Other studies have narrowed the list of candidate genes to those having specific sequence signatures and/or encoding PfEMP1 variants with well-defined receptor specificity.^{28,209,210,212-217} ICAM-1 (CD54) was recognized as an endothelial IE receptor early on,²⁷ and it has long been suspected to be important for the selective accumulation of IEs in the brain of CM patients.^{106,133,144,218} In line with this, contact with IEs can lead to endothelial upregulation of ICAM-1.^{128,129,134,219} Furthermore, it has been reported that *P. falciparum* parasites isolated from African children with CM bind preferentially to ICAM-1 in vitro.²²⁰ However, the opposite has also been reported,²²¹ and isolates from Asian adult malaria patients do not appear to show preferential adhesion to ICAM-1.^{222,223} Finally, some studies have failed to find evidence of high ICAM-1 expression in the brains of fatal CM victims.^{220,221} Taken together, a complex picture regarding the relationship between ICAM-1-specific IE adhesion and CM pathogenesis emerges, although most of these data suggest that CM in (African) children is quite different from CM in (Asian) adults.⁸³

IE adhesion to ICAM-1 is mediated by PfEMP1 variants that can also bind to either EPCR or CD36.^{14,34,35,224,225} The former of these groups, exemplified by the *P. falciparum* 3D7 PfEMP1 PFD1235w,¹⁸ shows a clear association specifically with CM.^{14,226-229} PFD1235w belongs to Group A, and contains the domain cassette DC4 (Figure 2D). The DC4 family was originally identified by a search for orthologs of the *pfcd1235w* gene in parasites from Ghanaian malaria

patients, inspired by the link between PFD1235w and severe malaria.^{202,230} The search resulted in a panel of genetically distinct parasites binding ICAM-1 via the DBL β 3 domain of the DC4-type PfEMP1 expressed on the IE surface.¹⁸ Sequence analysis of these domains identified a C-terminal ICAM-1-binding motif (I[V/L]_xN[E]GG[P/A]xYx₂₇GPPx₃H).¹⁴ The motif, which is also present in some Group A PfEMP1 proteins outside DC4 (including some DC5- and DC13-containing variants) and in a few Group B/A variants,^{14,18} is restricted to DBL β domains located immediately downstream of CIDR domains of the EPCR-binding subtype.^{13,14}

Endothelial protein C receptor is the cognate receptor for PfEMP1 proteins containing domain cassette DC8 or DC13.²⁸ DC13 is found among group A PfEMP1, whereas DC8 is found in Group B PfEMP1, and has evolved by recombination of ancestral Group A and B *var* genes.²⁰⁹ PfEMP1 variants containing DC8 or DC13 are common,⁸ and bind avidly to endothelial cells of lung, heart, and bone marrow.²³¹ DC8-containing PfEMP1 proteins tend to be among the first expressed in early childhood infections, indicating that they possess adhesion properties that confer a survival advantage to IEs in malaria-naïve children.²¹³ In addition, *P. falciparum* parasites obtained from African children and Indian adults with severe malaria—including CM—transcribe DC8- and DC13-encoding *var* genes at high levels.^{209,215-217,226,227} Their relevance to CM pathogenesis is further indicated by studies showing that IEs selected for adhesion to brain endothelial cells preferentially express these domain cassettes.^{213,214} Finally, expression of EPCR-binding PfEMP1 variants from Group A have been linked to brain swelling,²²⁸ which is a major contributor to mortality in pediatric CM.⁸⁸ The available evidence linking the EPCR-adhering IE phenotype to severe malaria in general, and to CM in particular, is not completely unequivocal.²³²⁻²³⁴ As an example, a study of Kenyan children with CM did not find evidence supporting particular enrichment of DC8- or DC13-containing PfEMP1 variants in children with retinopathy, a well-established indicator of CM, despite finding the expected association between CM and transcription of *var* genes encoding Group A PfEMP1.²³⁵

As mentioned above, not all PfEMP1 variants capable of binding ICAM-1 also bind EPCR. Indeed, all but one²³⁶ of the ICAM-1-binding DBL β domains identified prior to the discovery of DC4 in Group A were found in Group B and Group C proteins.^{34,35} Those PfEMP1 proteins appear to be under dual selection for adhesion to ICAM-1 and CD36, as they all contain a CD36-binding CIDR α domain upstream of the ICAM-1-binding DBL β domain.^{35,36,237} This is not surprising, as CD36 is a very common IE adhesion receptor and most non-placental *P. falciparum* isolates can bind to it.^{30,220,221} Affinity for CD36 is a feature of the majority of Group B and C PfEMP1 proteins,^{35,36} but is not found in the Group A and B/A PfEMP1 that dominate in severe infections and in individuals with limited malaria immunity.^{202,204,207,209,238} Rather, CD36 binding is associated with uncomplicated malaria,^{239,240} and appears to have evolved to mediate IE sequestration in tissues other than the brain, where CD36 is absent or only sparsely present.^{135,155}

Combining the above evidence, the adhesion phenotype that is most clearly related to CM is IEs expressing Group A PfEMP1

proteins (including DC4) that allow concomitant binding to both ICAM-1 and EPCR ("double binders").¹⁴ The association between severe disease (and in particular, CM) and IE affinity for either of these receptors alone is less clear. It is worth noting in that context that the EPCR-binding CIDR α 1 domain in DC8, and in some DC13, is not followed by an ICAM-1-binding DBL domain.^{13,14} Finally, although several molecules other than those already mentioned have been implicated as IE adhesion receptors, including some that appear to be expressed on brain endothelium,²⁴¹ none of them have been linked to disease severity.

4.2 | Rosetting and clumping

Infected erythrocytes do not only adhere to the endothelium, but also to surrounding uninfected or infected erythrocytes. The former type of such aggregates, called rosettes, were first reported in the *P. fragile*-infected monkeys.²⁴² The finding was quickly followed by studies demonstrating that the same phenotype was present in *P. falciparum*,^{243,244} and it appears that most species of malarial parasites are capable of inducing rosettes,²⁴⁵ which is a complex phenotype involving multiple parasite and host molecules.²⁴⁶ Thus, several erythrocyte molecules, including complement receptor 1, heparan sulphate, and the ABO blood group antigens, appear to be involved as receptors.²⁴⁷⁻²⁵⁰ On the parasite side, several ligands have been implicated, including members of several variant surface antigen families.^{251,252}

Infected erythrocytes isolated from patients with CM have been reported to form rosettes at significantly higher rates than IEs from patients with uncomplicated malaria.²⁵³⁻²⁵⁶ This difference might contribute to occlusion of cerebral microvessels by rosettes, to cerebral sequestration of IEs that express PfEMP1 variants that can bind to host receptors shared by cerebral microvessels and erythrocytes,^{257,258} and/or expressing PfEMP1 variants that allow binding to separate receptors on endothelium and on erythrocytes, respectively.⁴² With respect to the first of these possibilities, microvascular flow rates appear to be an important determinant for the clinical consequences of rosetting,²⁵⁹ similar to what appears to be the case for IE sequestration (see above). With respect to the last possibility, several PfEMP1 variants with capacity to bind to more than one receptor at the same time (mostly via different domains) have been described and linked to disease severity.^{14,40,260} However, while some studies have shown significant correlations between rosetting and severe malaria/CM,^{253,254,261-263} others have failed to find such a relationship.²⁶⁴⁻²⁶⁶ Although the reason for the discrepancy is not fully resolved, it may reflect genuine geographical differences between Africa (where the association is generally found) and Southeast Asia (where it usually is not). Similar geographic differences have been described for other receptor-specific adhesive IE phenotypes and malaria severity.^{220,222,267,268} If indeed such geographical variation exists, it may involve differences in the parasites, the hosts, and/or the transmission intensity. The relative contributions of these variables remain completely unresolved, however.

The receptor specificity of the PfEMP1 variant expressed is an obvious determinant of whether PfEMP1-dependent rosetting

occurs or not. However, soluble plasma factors that can bind to PfEMP1 may also be of pathologic significance. Thus, IE rosetting in vitro generally (but not always) requires the presence of plasma or serum in the assay, and several components have been implicated.²⁴⁶ The most studied of these is IgM, which can clearly enhance rosette formation.^{20,269,270} This function of IgM is independent of the antigen specificity of the antibody as it is mediated by the Fc rather than the Fab domains.^{20,271} It furthermore requires a pentameric IgM conformation,²⁷¹⁻²⁷³ probably because IgM augments rosette formation by cross-linking multiple PfEMP1 molecules, thereby enhancing their combined avidity for the erythrocyte receptor involved.^{20,274} Several of the approximately 60 PfEMP1 variants in a given genome contain domains that are able to bind IgM this way,^{275,276} but the potential of this finding for identifying PfEMP1 proteins involved in the pathogenesis of severe malaria in general, and of CM in particular, is largely unknown. A single study recently pointed to the protease-inhibitor α_2 -macroglobulin as another important cofactor of rosetting,²⁷⁷ but the generality and clinical significance of this finding remains unclear.

IEs can also bind to other IEs via platelets (thrombocytes); a phenotype referred to as clumping.¹⁶⁷ Clumping has been associated with severe malaria including CM in some, but not all studies.^{169,278,279} The platelet receptor involved appears to be gC1qR/HABP1/p32, and as this receptor is also present on cerebral microvascular endothelium, it provides a plausible link between clumping and C.¹⁶⁸ However, to our knowledge it is not known whether IE affinity for gC1qR/HABP1/p32 is mediated by PfEMP1 or whether this adhesion phenotype is significantly involved in the pathogenesis of CM.

4.3 | The role of blood flow on IE adhesion

Erythrocytes normally flow down the central line of a blood vessel,^{280,281} but the deformation and enhanced stiffness of IEs cause them to marginate, thus bringing them into contact with adhesion receptors on the endothelial surface.^{282,283} The distribution of cells, including IEs, in the blood stream is furthermore dependent on variables such as vessel diameter and plasma viscosity and flow rates.²⁸⁴ Flow not only affects margination of circulating cells, but can also lead to upregulation of endothelial receptors and cytokines in response to changes in shear stress. This is particularly true for ICAM-1, VCAM-1, and IL-1 β .^{285,286} Endothelial integrins are also sensitive to changes in blood flow and become activated in response to increased shear stress.^{287,288} Blood flow is thus an important parameter to consider in studies of IE adhesion. A variety of in vitro assays have yielded important insights in this regard.²⁸⁹⁻²⁹⁶ As an example, CD36-specific adhesion of normal and ovalocytotic IEs were similar in static assays, but were markedly different in assays conducted under physiologically more plausible flow conditions.²⁹⁷ With respect to CM-relevant and PfEMP1-specific adhesion, flow-based studies of adhesion of IEs expressing PfEMP1 variants that can mediate binding to both ICAM-1 and EPCR have revealed synergies that could not be discerned in static assays.^{14,298}

4.4 | Converging on the protein C pathway

In spite of the very significant morbidity and high mortality of cerebral *P. falciparum* malaria, the pathophysiology of the disease is only partly understood.^{104,191,299} A range of potential and non-exclusive pathogenic mechanisms has been proposed, such as circulatory obstruction by sequestered IEs, imbalanced cytokine responses, and endothelial dysfunction and loss of BBB integrity.^{105,107,108,300} The available evidence is slowly converging on a scenario where CM is the consequence of the impact of IEs expressing particular PfEMP1 variants on the protein C-dependent maintenance of the integrity of brain endothelium.^{17,301}

The protein C pathway is a crucial anti-coagulant and anti-inflammatory regulator of thrombin production during clot formation.³⁰² Normally (Figure 4), thrombomodulin on the endothelial surface binds thrombin and activates protein C to become activated protein C (APC)

in a process that is strongly promoted by EPCR (also known as activated protein C receptor).³⁰¹ The binding of APC to EPCR inhibits endothelial activation and TNF α -dependent inflammation, thereby limiting the opportunity for IE sequestration mediated by known PfEMP1 receptors such as ICAM-1, VCAM-1, E-selectin, and thrombospondin-1.³⁰³ The interaction also activates PAR-1, which has an anti-apoptotic effect that protects the endothelial barrier integrity.^{302,304}

It has been proposed that this delicate system of checks-and-balances may be upset by IEs adhering to EPCR, thereby preventing physiologically appropriate activation of protein C.^{301,305,306} The PfEMP1 proteins expressed by EPCR-adhering IEs bind EPCR near/at the site where protein C/APC normally binds. The IEs might thereby interfere with binding of the normal ligand and compromise protective APC-dependent maintenance of the BBB via PAR-1.^{13,307,308} The result would be excessive endothelial inflammation, thrombin activation, fibrin cross-linking, platelet activation, upregulation of ICAM-1

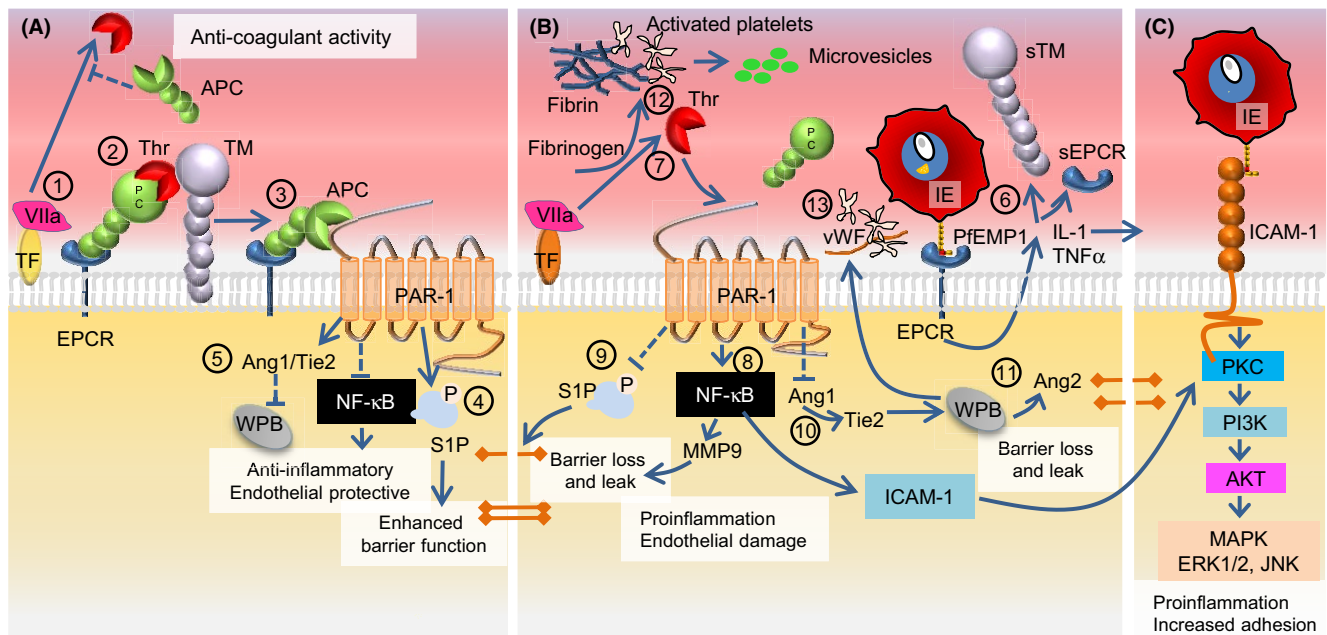


FIGURE 4 Linking the protein C pathway with EPCR- and ICAM-1-binding IEs in cerebral malaria. (A), Effects of EPCR in the absence of *Plasmodium falciparum*-IE. Thrombin (Thr) is produced by the interaction between tissue factor (TF) and circulating activated factor VII (VIIa) (1). Thrombin initiates the EPCR- and thrombomodulin (TM)-facilitated activation of protein C (APC) that then inhibits thrombin production (2). APC uses EPCR as a coreceptor for cleavage of proteinase-activated receptor 1 (PAR-1). The EPCR-APC activation of PAR-1 inhibits the nuclear factor- κ B pathway and exerts anti-inflammatory and anti-apoptotic activity (3). S1P signaling results in decreased endothelial permeability, and S1P production leads to enhancement of tight junctions and protection of endothelial barrier integrity (4). Angiopoietin-1 (Ang1) produced in response to the APC-PAR-1 interaction decreases Weibel-Palade body (WPB) exocytosis by occupying Tie2 (5). (B), The impact of infected erythrocytes expressing EPCR-bound PfEMP1 on the surface. The IE-EPCR interaction activates endothelial cells to release pro-inflammatory cytokines (IL-1, TNF α) that induce shedding of EPCR and TM from the endothelial surface and increases expression of ICAM-1 (6). The EPCR-IE interaction results in reduced levels of APC and increased thrombin generation with fibrin deposition (7). Increased levels of thrombin shift the PAR-1 response toward activation of the RhoA and NF κ B with increased surface expression of ICAM-1 on the endothelial cell (8). The shift in the PAR-1 response inhibits S1P release resulting in loss of tight junctions, and compromises endothelial barrier function by causing localized vascular leaks (9). A reduction in Ang-1 levels increases WPB exocytosis via Tie2 and production of von Willebrand Factor (vWF) and Ang2 (10). Increased levels of Ang-2 further increase WPB exocytosis and contribute to the loss of endothelial barrier integrity and leakage (11). Platelets become activated by thrombin and cytokines, which leads to production of platelet microvesicles (12). Thrombin and activated platelets combine to form thrombi (13). Strings of vWF and activated platelets form complexes, which like thrombi impair the cerebral circulation. (C), The increase in ICAM-1 (in panel B) allows IE expressing PfEMP1 with a shared DBL β ICAM-1 motif to adhere to the brain endothelium. A large proportion of the ICAM-1-adhering IEs might initially bind EPCR via their CIDR α 1 domains

expression, downregulation of EPCR, and endothelial leakage. This corresponds to the petechial lesions, fibrin clots, EPCR denuding, thrombomodulin deficiency, axonal injury, and brain swelling that have been reported in pathology studies of the brains of patients who died of CM.^{82,301,309-315} The lack of APC may furthermore allow induction of endothelial dysfunction via parasite and host soluble factors, such as histones, heme, and HRP-2, released locally when IEs rupture.^{316,317}

Brain microvascular endothelium may be particularly susceptible to the disruption of the protein C pathway by EPCR-adhering IEs because EPCR is expressed at low levels at this site as opposed to the high expression seen in arteries and veins.^{318,319} CM is associated with loss of EPCR in the brain, and increased levels of soluble EPCR have been reported and associated with CM mortality.^{301,320} Human genetic variability affects the level of soluble EPCR, and there is some indication that some variants may be associated with protection from severe malaria, including CM, conceivably by neutralizing IE adhesion to EPCR.²³² However, other studies did not detect such associations.^{233,234} Substantial variation also exists in genetic sequence of the CIDR α 1 domains in PfEMP1 that mediate binding to EPCR, although the EPCR-binding surface is largely conserved despite this variation.¹³ The variation nevertheless appears to impart differences in binding affinity that may affect how IE binding impacts normal EPCR function. The CIDR α 1.1 domains in DC8 thus affected APC and thrombin-induced permeability less than the CIDR α 1.4 domains of DC13.^{307,321} It is plausible that such diversity might contribute to the divergent pathophysiology of CM1 and CM2, where CM1 is characterized solely by IE sequestration, whereas CM2 also involves activation of coagulation and formation of fibrin clots and ring hemorrhages.^{75,82,96}

The impact of EPCR-binding IEs on the protein C pathway proposed above is consistent with features of CM pathogenesis, but most studies have found this IE adhesion phenotype to be associated with severe malaria in general, rather than with CM specifically.^{209,215-217,226,227} However, this lack of specificity can be explained if CM pathogenesis requires parasites that express PfEMP1 variants that bind both to EPCR and ICAM-1 (the above-mentioned “double binders”). Our hypothesis involves a pathogenic cascade (Figure 4), where IEs initially adhere to EPCR on non-inflamed cerebral endothelium.^{14,17} This activates the endothelial cells as described above, induces their release of pro-inflammatory cytokines, and increases their expression of ICAM-1.^{110,219,301} “Double binders” may directly exploit this inflammatory response by adhering to ICAM-1, and this has been associated with disruption of the BBB.¹³² In contrast, erythrocytes infected by parasites expressing PfEMP1 variants that only have affinity for EPCR are likely to dislodge, as EPCR is shed as a part of the inflammatory response.³⁰¹

5 | PFEMP1 AND IMMUNITY TO CM

In areas with stable transmission of *P. falciparum* parasites, susceptibility to clinical malaria is inversely correlated with age. Antibodies to

parasite antigens on the surface of IEs are important, even decisive, determinants of this relationship, including the gradually decreasing risk of developing severe malaria such as CM.³²²⁻³²⁵ Acquisition of this type of immunity following natural parasite exposure is remarkably slow, incomplete, and temporally unstable, characteristics that all point to variant antigens, and in particular to PfEMP1 as the primary antigenic target.^{17,326}

5.1 | Naturally acquired, PfEMP1-specific immunity

The variant-specific, PfEMP1-centric hypothesis of susceptibility to, and acquired immunological protection against, *P. falciparum* malaria hinges on the idea that the infecting parasites adapt to pre-existing and developing immunity by switching to variants that are not recognized by specific antibodies.^{327,328} Severe disease ensues when those variants enable IEs to adhere to receptors that are widespread, allow strong IE adhesion, are expressed in critical tissues, and/or have vital functions. This is more likely to occur in individuals with little or no acquired immunity, and to involve variants that are relatively conserved among different parasite clones. As immunity to initially virulent variants is acquired, the parasites are steadily forced to express variants that are less virulent (more diverse, less likely to mediate firm IE adhesion, less likely to bind to widespread receptors, and more likely to be expressed in tissues where the consequences of IE sequestration are less serious).

Variant-specific immunity is indeed acquired in the orderly fashion predicted by the above theory. Antibodies to relatively conserved (“common”) parasite antigens and protection from severe malaria are acquired first, followed by antibodies to more variant antigens associated with uncomplicated disease, and eventually by antibodies recognizing very diverse (“rare”) antigens expressed on the surface of IEs obtained from carriers of asymptomatic/subclinical infections.³²⁹⁻³³² Transcription of *var* genes and acquisition of PfEMP1-specific IgG follows this pattern, and shapes what PfEMP1 variants are compatible with parasite survival in a given semi-immune host.^{207,238,333} Thus, *var* gene transcription in children with limited pre-existing immunity and severe disease is skewed toward Group A,^{202,204,207} supporting the idea that these genes encode PfEMP1 proteins with adhesion specificities that are optimal for multiplication of the asexual parasites in non-immune hosts, and likely include binding properties that predispose to severe malaria. As children acquire Group A anti-PfEMP1 antibodies over successive infections, the proportion of *var* genes in Group B and C, encoding PfEMP1 variants associated with uncomplicated disease gradually increases.^{238,333-335} The clearest example of this type of relationship so far is the susceptibility to placental *P. falciparum* infection, and the acquisition of immunity to that particular form of severe malaria, as both depend on a particular type of PfEMP1 called VAR2CSA.^{187,188,336} However, there is every reason to assume that it applies to all forms of *P. falciparum* malaria, including CM.¹⁷

IgG antibodies to the Group A, DC4-containing PfEMP1 that can mediate IE adhesion to both ICAM-1 and EPCR are acquired early in life in areas with stable transmission of *P. falciparum*, and this acquisition is associated with protection from severe malaria, including

CM.^{202,337,338} The same applies to IgG specific for the CIDR domains that mediate IE adhesion to EPCR,^{214,339,340} although divergent evidence also exists.³⁴¹ A very recent study indicates that IgG to ICAM-1-binding PfEMP1 variants from Group B, which do not share the ICAM-1-binding motif with the Group A PfEMP1s (including DC4 variants) and that do not contain an EPCR-binding CIDR domain are acquired only when Group A (“dual binding”)-specific immunity is already in place.³⁴² This study thus further underpins the theory of a PfEMP1 hierarchy modulated by host immunity.

The degree of IgG cross-reactivity that transcends clone-specific variability in key antigens is of obvious importance. Encouragingly, naturally acquired antibodies capable of inhibiting the interaction between ICAM-1 and many ICAM-1-binding DBL β domain variants were recently observed.³³⁸ The prevalence of IgG specific for Group A ICAM-1-binding domains furthermore appears to be higher than those specific for EPCR-binding CIDR α 1 domains, suggesting that the former antibody specificity is more cross-reactive than the latter.³⁴¹

5.2 | PfEMP1-based vaccination and other PfEMP1-specific interventions against CM

An ideal malaria vaccine would elicit immunity that prevents infection of humans and leads to transmission elimination and global eradication of *P. falciparum*. While this goal is elusive at the present time, vaccines that prevent severe malaria illness, particularly in children and pregnant women, could constitute an alternative or interim strategy.

Current efforts to develop VAR2CSA-based vaccines to prevent placental malaria is the most advanced example of a PfEMP1-based approach to malaria vaccination, as several such vaccines are currently in clinical trials.³⁴³⁻³⁴⁷ However, it is conceivable that development of vaccines to prevent severe malaria in children, including CM, might be possible using a similar strategy.¹⁹³ An obvious goal would be vaccines eliciting a broadly reactive antibody response preventing, and ideally reversing, adhesion of IEs to ICAM-1 and EPCR, which appear as key receptors in CM pathogenesis. Vaccines designed to specifically target “double binders” would seem particularly attractive. Blocking of EPCR-specific adhesion of IE by vaccination with relevant CIDR α 1 domains would lead to protection not only against CM, but also against severe childhood malaria in general. Unfortunately, EPCR-binding CIDR α 1 domains show substantial interclonal sequence variation and experimental antibodies binding distant variants are difficult to generate and are rarely inhibitory.^{348,349} This reduces the likelihood of natural induction of cross-inhibitory IgG responses targeting the critical parts of CIDR α 1 domains. In contrast, the highly conserved ICAM-1 binding site of these “dual binding” PfEMP1 variants encourage future efforts to raise broadly cross-reactive IgG antibody responses against such molecules using their ICAM-1 binding DBL β domain as part of a strategy to prevent death due to CM.^{14,338}

However, many questions remain unanswered. Aside from the uncertainty regarding the feasibility of making a vaccine with

the desired qualities, it is unclear whether inhibition/reversal of ICAM-1/EPCR-specific IE adhesion would be sufficient to prevent CM. It is similarly unclear to what extent acquired clinical protection from CM requires neutralizing (IE adhesion-blocking) antibodies, as opposed to opsonizing antibodies leading to preferential phagocytic and/or cytotoxic removal of IEs expressing particularly pathogenic PfEMP1 variants.

While efforts to overcome the obstacles in the development of a PfEMP1-specific vaccine to protect against CM continue, other interventions should also be pursued. Unfortunately, adjunctive therapies (eg, glucocorticoids, anti-TNF α s, iron chelators, osmotic regulators, anti-oxidants, and glycosaminoglycans), aimed at protecting against CM-related brain damage and neuronal injury, have so far been unsuccessful.^{72,350,351} Furthermore, current anti-malarial drugs fail to reverse adhesion of IEs.³⁵² This notwithstanding, our improved understanding of molecular and cellular basis of CM pathogenesis might encourage novel adjunctive therapies aimed at dislodging sequestered parasite by interfering with PfEMP1-mediated IE adhesion. Along this line, soluble EPCR has been shown to inhibit adhesion of DC8-expressing IEs and endothelial cells in vitro.²⁸ The finding of a soluble EPCR variant that binds PfEMP1 without affecting protein C binding to EPCR supports the in vivo feasibility of this approach,¹³ although the very low off-rates of the PfEMP1-EPCR interaction may not allow release of IEs already bound.³⁰⁸ Finally, a monoclonal antibody has been reported to inhibit and reverse adhesion of IEs to ICAM-1, including antibodies that affect “dual binding” PfEMP1 variants.³⁵³ However, the associated cost of this intervention will probably prevent its use in clinical practice.³⁵⁴

6 | CONCLUSIONS AND THE WAY FORWARD

Our understanding of the molecular basis of the interactions between *P. falciparum* parasites and the humans they infect is progressing at a rapid pace. This is not least so for the tissue-, organ-, and receptor-specific adhesion of IEs that act as major contributors to the pathogenesis of *P. falciparum* malaria, including the development of severe complications such as CM.

The critical importance of PfEMP1 for parasite survival makes these antigens major immune targets, and acquisition of PfEMP1-specific antibodies indeed appears to be a central component of naturally acquired protection. Emulating and accelerating these responses through vaccination would therefore seem obvious. However, the flipside is that the importance of PfEMP1 to the parasites applies a strong selective pressure on them to evolve mechanisms to evade protective, PfEMP1-specific immunity. The most prominent example of this is clonal antigenic variation, which undoubtedly evolved to delay and frustrate the acquisition of PfEMP1-specific protective antibodies, thereby enabling chronic infections.^{17,326,355-358} It is an important issue to ascertain precisely how, and at what level(s), this undermining operates. While it seems clear that interclonal variation in functionally important

antibody epitopes is involved, it is essentially unknown to what extent clonal antigenic PfEMP1 variation is affecting important T-cell-dependent helper functions. Clonal antigenic variation apart, it is becoming increasingly clear that *P. falciparum* parasites have evolved a range of other strategies to evade PfEMP1-specific immunity. Examples are antigen topology and cloaking, interference with antigen presentation, and subversion of soluble host molecules.^{20,277,359-362}

In conclusion, much has been learnt from years of research on CM pathogenesis and immunity, although a lot remains to be known. Because this malaria complication is such an important part of the intolerable obstacle that *P. falciparum* malaria constitutes to health and to economic progress and equality, we are obliged to continue this research.^{193,363}

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CONFLICTS OF INTEREST

The authors declare no competing interests.

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