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(Article begins on next page)



REVIEW

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Host matrix metalloproteinases in cerebral malaria: new kids on the block against blood–brain barrier integrity?

Manuela Polimeni¹ and Mauro Prato^{2,3*}

Abstract

Cerebral malaria (CM) is a life-threatening complication of *falciparum* malaria, associated with high mortality rates, as well as neurological impairment in surviving patients. Despite disease severity, the etiology of CM remains elusive. Interestingly, although the *Plasmodium* parasite is sequestered in cerebral microvessels, it does not enter the brain parenchyma: so how does *Plasmodium* induce neuronal dysfunction? Several independent research groups have suggested a mechanism in which increased blood–brain barrier (BBB) permeability might allow toxic molecules from the parasite or the host to enter the brain. However, the reported severity of BBB damage in CM is variable depending on the model system, ranging from mild impairment to full BBB breakdown. Moreover, the factors responsible for increased BBB permeability are still unknown. Here we review the prevailing theories on CM pathophysiology and discuss new evidence from animal and human CM models implicating BBB damage. Finally, we will review the newly-described role of matrix metalloproteinases (MMPs) and BBB integrity. MMPs comprise a family of proteolytic enzymes involved in modulating inflammatory response, disrupting tight junctions, and degrading sub-endothelial basal lamina. As such, MMPs represent potential innovative drug targets for CM.

Keywords: Cerebral malaria (CM), *Plasmodium*, Blood–brain barrier (BBB), Matrix metalloproteinases (MMPs), Inflammation

Introduction

Human malaria is a widespread infectious disease caused by *Plasmodium* protozoan parasites and is associated with high morbidity and mortality rates, resulting in 627,000 deaths among 207 million cases estimated in 2012 [1]. Human malaria is caused by five different *Plasmodium* species: *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi*. *P. falciparum* and *P. vivax* are the most common, correlating with the most severe forms of malaria and the highest death rate, whereas other *Plasmodium* species generally cause milder forms of malaria which are rarely fatal [1]. The majority of deaths occur among children under the age of five years living in sub-Saharan Africa, and in Southern/South-Eastern Asia and Central/

Southern America where mortality mainly affects adults. Additionally, occasional cases are observed in non-immune adult travelers from developed countries returning from these areas. Despite the intense efforts made by the research community and the Global Eradication program [2], no effective vaccines or adjuvant therapies are available for complicated malaria. It is projected that in the next few years the dramatic issue of drug-resistant malaria could become a serious threat [3–5].

P. falciparum is unique in that it causes mature infected red blood cells (iRBCs) to sequester and adhere to microvascular beds in numerous organs. A paradigmatic complication of *falciparum* malaria is cerebral malaria (CM), which develops after iRBCs sequester in the microvasculature of the central nervous system (CNS). Unlike the other human malarial parasites which rarely cause neurological dysfunction, *P. falciparum*-induced CM often leads to death or severe neurological sequelae [6]. Curiously, *P. falciparum* appears to remain in the vascular space without ever entering the brain parenchyma,

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in contrast to other encephalitis-causing pathogens, such as *Trypanosoma* spp. or *Toxoplasma gondii* [7], thus raising question of how intravascular *Plasmodium* parasites are capable of inducing such a devastating neural dysfunction in CM.

Recent evidence suggests that a compromised integrity of the blood–brain barrier (BBB) results in a subsequent increase in BBB permeability which enables toxic soluble factors released either by host or parasite to cross this barrier and exert neurological effects. This review focuses on CM pathophysiology and novel insights from animal and human models into the role of BBB functional impairment in CM. Finally, we discuss the emerging role of host matrix metalloproteinases (MMPs), a family of proteolytic enzymes related to inflammation and BBB damage in CM, opening the possibility for discovery of new effective adjuvant therapies for CM.

Pathophysiology of cerebral malaria

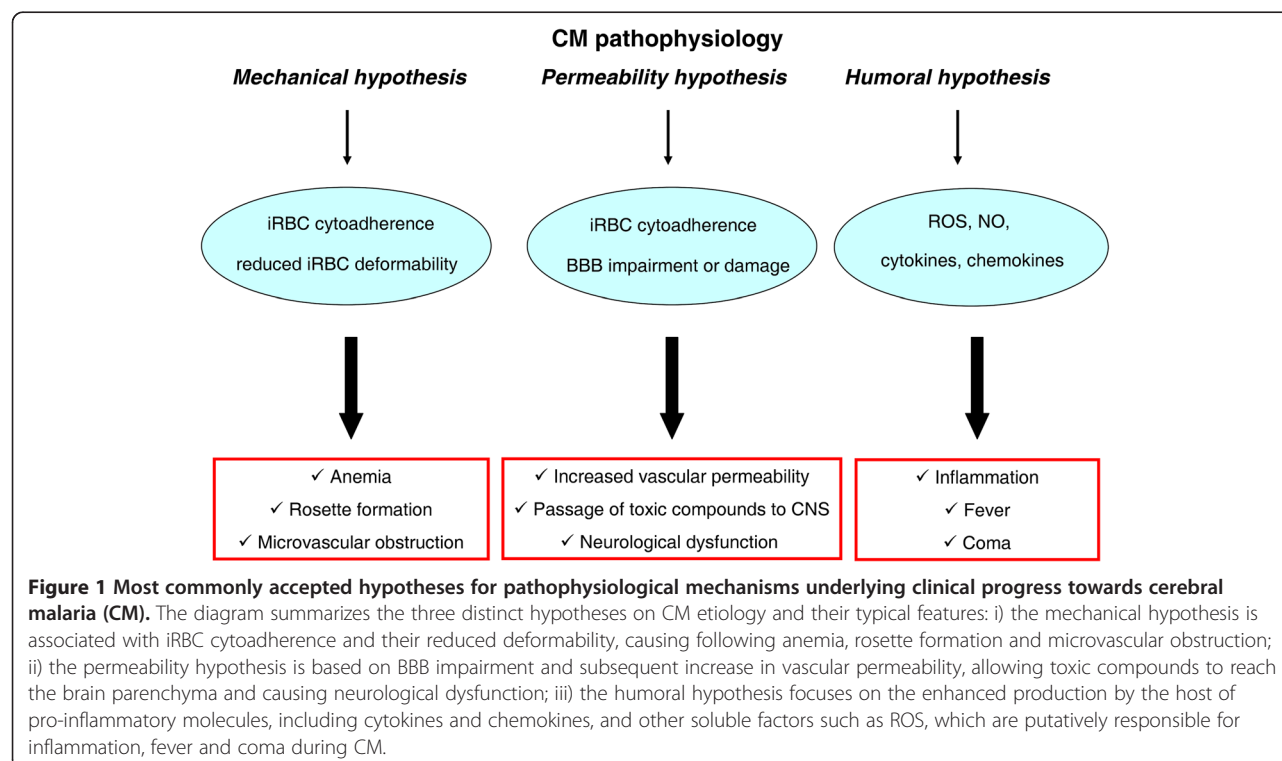
CM appears as a diffuse encephalopathy commonly presenting with headache, agitation, frank psychosis, seizures and impaired consciousness, and occasionally with brainstem signs or focal neurological signs such as hemiplegia and cranial nerve palsies [8,9]. According to the World Health Organization (WHO) clinical criteria, CM is defined as a potentially reversible, diffuse encephalopathy causing a Glasgow coma score of 11/15 or less, often associated with fitting, in the absence of other factors that could cause unconsciousness such as coexistent

hypoglycemia or other CNS infections [10]. It is difficult to confirm diagnoses of CM in endemic areas because of overlapping infections such as bacterial meningitis in patients showing incidental malarial parasitaemia [11]. Children from areas endemic for malaria or non-immune adults traveling from developed countries are at higher risk for developing CM. On the contrary, CM is rarely encountered in >10-year-old patients who have been exposed to *P. falciparum* since birth. Mortality ranges from 15–30%, and 11% of children display neurological deficits upon discharge [12].

The pathophysiological mechanisms underlying CM are not fully understood so far. As seen in Figure 1 and discussed in the next paragraphs, there are currently three distinct theories on the etiology of CM typical features: i) the mechanical hypothesis; ii) the permeability hypothesis; and iii) the humoral hypothesis [4,9,13–16]. It is possible that these theories are all pieces of that puzzle that need to be combined as they likely constitute more complementary than alternative models [6,17].

Mechanical hypothesis

The mechanical hypothesis proposes CM is caused by a mechanical obstruction of the cerebral microvasculature, with coma resulting from impaired brain perfusion [9,14,18]. Such a hypothesis was made after one of the first pathological studies on human CM showed that brain capillaries were packed with iRBCs [18]. In the mechanical hypothesis, specific interactions between iRBCs



and vascular endothelium are thought to mediate sequestration of iRBCs within the brain resulting in removal from peripheral circulation [19-21]. The molecules involved in these interactions are parasite proteins expressed on iRBC surface, such as *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), and specific host receptors in the microvascular endothelium, including intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), thrombospondin, CD36, and E-elastin [22-25].

Cytoadherence and decreased pliability are the main mechanisms underlying vascular obstruction [9,17-21]. It is speculated that cytoadherence evolved as a mechanism for the parasite to evade triggering a host immune response and being cleared from the spleen. Cytoadherence is also beneficial for the parasite as to provide an optimal environment of low oxygen tension for parasite growth. Decreased deformability along with increased membrane stiffness and rigidity of iRBCs are due to changes in the cytoskeleton triggered by growing intracellular parasites. Cell deformability has been indicated as a predictor of anemia development [26], whereas cell rigidity correlates with a higher fatality rate [27]. Another phenomenon occurring along with iRBC sequestration is rosetting, characterized by iRBCs forming a flower-like cluster around a non-iRBC, making a tight rigid structure [28]. Rosetting is more frequent in patients with CM than in those with uncomplicated malaria. However, rosette formation has also been reported for other *Plasmodium* strains (*P. vivax* and *P. ovale*) which do not cause CM [29]. Since rosetting occurs in all manifestations of the disease, it is not associated with severity or clinical outcome of CM [30]. One question the mechanical hypothesis by itself does not explain is why most patients recovering from CM do not show any evidence of ischemic brain damage [12].

Permeability hypothesis

The permeability hypothesis proposes that BBB damage is the underlying mechanism of CM, such that a leaky BBB allows toxic compounds to enter the brain and cause neurological dysfunction [9,13-15,31]. Several animal CM models have confirmed that the BBB is disrupted and that cerebral edema is present in CM, although this is less evident in humans [15]. Nevertheless, iRBCs remain attached to endothelium, without entering the brain parenchyma [7,9]. Interestingly, Adams and colleagues have suggested that iRBC cytoadherence might activate secondary signaling events similar to those occurring in leukocytes [32]. These secondary signaling events are thought to cause functional alterations in the BBB, which could allow toxic compounds to pass into the CNS. These events might be reversible, therefore explaining why neurological manifestations are just transient in most cases and why a large number of recovering patients lack neurological sequelae [32].

Enwonwu and colleagues implicated histamine as one of these toxic molecules that enters the brain parenchyma after BBB impairment and contributes to the neurological manifestations of CM [33-37]. The authors observed altered neural histidine uptake in children with severe *falciparum* malaria providing an explanation for the enhanced cerebral production of histamine [33]. They also found increased plasma levels of histamine in severe malaria patients, further supporting their hypothesis [34]. Moreover, the involvement of histamine in CM has also recently been confirmed in a murine model [35-37]. In this study, histidine decarboxylase-deficient mice were unable to synthesize free histamine and did not develop CM after infection with *P. berghei* ANKA. These mice displayed preserved BBB integrity, were void of iRBC aggregation in the brain vessels, and did not sequester CD4⁺ and CD8⁺ T cells [36]. Further investigation of histamine receptors revealed histamine-1-receptor (H1R) and histamine-2-receptor (H2R) are associated with severe malaria development [37], whereas histamine-3-receptor (H3R) has a neuroprotective role [36].

Humoral hypothesis

The humoral hypothesis is a natural extension of the permeability hypothesis. This hypothesis suggests that host factors such as leukocyte-derived cytokines and chemokines can enter the brain parenchyma after increased BBB permeability, thus causing CM symptoms such as fever and coma [9,13,14,16,38-40].

Effector cells including T cells, NK cells, and monocytes, along with inflammatory responses mediated by cytokines such as tumor necrosis factor- α (TNF- α), lymphotoxin- α (LT- α), and interferon- γ (IFN- γ), are proposed to contribute to the development of murine CM [41-48]. However, the extent of their involvement and molecular mechanisms in human CM is still topic of debate [48,49].

CD8⁺ T cells have been reported to initiate BBB tight junction disruption and promote CNS vascular permeability under neuroinflammatory conditions [50-52]. Consistently, CD8⁺ T cell sequestration in cerebral microvessels and subsequent brain infiltration have been demonstrated in murine CM [43,44], where *Plasmodium* antigens can be cross-presented during infection by dendritic cells (DCs) [53,54] and brain endothelial cells in association with MHC class I molecules [55]. Recent human studies support the idea that malaria antigens can be transferred to endothelial cells [56]. However, it is currently unknown whether *Plasmodium*-specific CD8⁺ T cells are involved in the pathogenesis of human CM [57]. Furthermore, lymphocyte infiltration into brain parenchyma remains to be investigated [49].

TNF- α relevance in CM is also unclear. TNF- α involvement in murine CM was first proposed in 1987 [58]. Since then there have been numerous studies investigating

TNF- α levels in CM mice albeit the results are inconsistent. For example, some works confirmed the association of high TNF- α levels with murine CM [59-61], whereas others argued against such correlation, finding LT- α and IFN- γ as more suitable markers [44,47,62,63]. To reconcile such discrepancies, it has been proposed that low concentrations of TNF- α could enhance parasite killing, whereas higher concentrations might be associated with increased incidence of murine CM [46]. However, data on TNF- α also appear inconclusive in human CM studies. Indeed, clinical studies tend to exclude any association between CM and increased plasma, serum or CSF levels of TNF- α [64-67], although a few works have proposed a correlation in two different Asian populations [68,69]. Instead, in some of these studies, high CXCL10/IP-10 plasma levels and low angiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) in children with CM, predicted subsequent mortality [65,66,68]. Moreover, a protective role for IL-12 has been proposed in human CM [70,71].

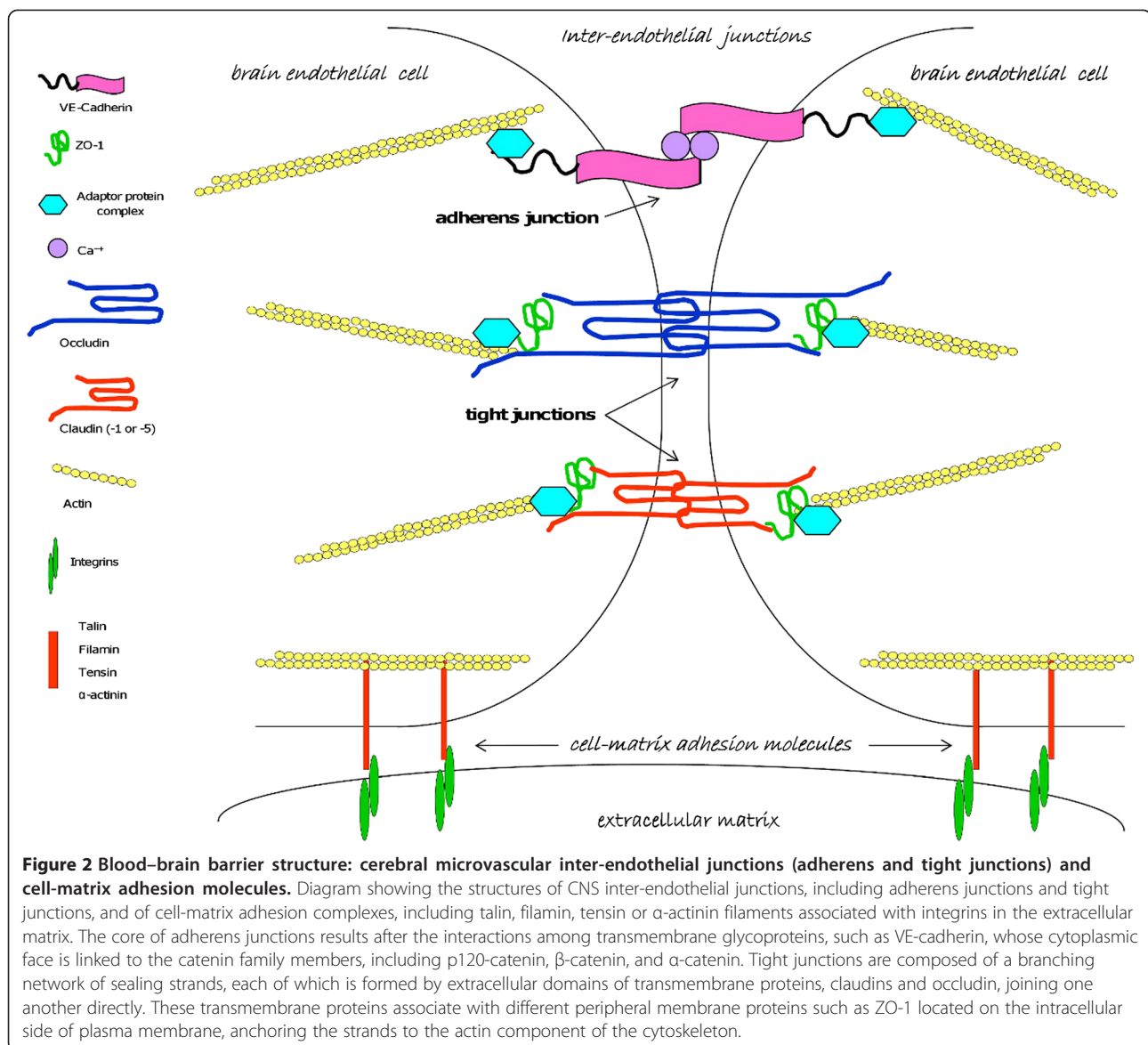
Among soluble factors involved in CM, a critical role for nitric oxide (NO) has also been suggested. It was hypothesized that NO levels correlate with disease severity, since the sequestration of iRBCs might contribute to CM pathogenesis by causing hypoxia, which is related to enhanced production of cytokine-induced NO, compensatory vasodilatation, and subsequent brain volume increase [39]. However, activation of inducible NO synthase (NOS) might also serve protective functions, since NOS inhibits the side effects of brain indoleamine 2,3-dioxygenase (IDO) and quinolinic acid accumulation [72], although IDO systemic distribution is independent of malaria disease severity [73]. In a study performed on Tanzanian children infected with malaria, the plasma levels of NOS-suppressing IL-10 increased with disease severity, suggesting that a reduced NO production may contribute to CM [74]. Moreover, a genetic single nucleotide polymorphism found in the NOS2 promoter region causes elevated NO production and was significantly associated with protection against CM in Tanzanian and Kenyan children [75]. In line with these observations, Anstey and colleagues demonstrated that decreased NO production was associated with endothelial dysfunction in human CM [76,77]. Similarly, van der Heyde and his group demonstrated that low NO bioavailability was associated with murine CM [78,79]. Interestingly, prophylaxis with inhaled NO in CM-sensitive mice significantly reduced systemic inflammation and endothelial activation by lowering TNF- α , IFN- γ , monocyte chemoattractant protein-1 (MCP-1), sICAM-1 and von Willebrand factor, and by increasing Ang-1 levels in peripheral blood [80]. The protective effect of exogenous NO on mouse CM appears associated with decreased brain vascular expression of inflammatory markers, resulting in attenuated endothelial

junction damage and facilitating blood flow [81]. Lastly, treatment with exogenous L-arginine, the substrate for NOS, recently proved to be safe in a pilot study on CM patients, although effective doses still need to be optimized [82].

Furthermore, during malaria infection both host and parasite undergo strong oxidative stress, which leads to increased production of reactive oxygen species (ROS) and subsequent protein and lipid peroxidation [83,84]. The co-existence of both parasite and erythrocyte is a matter of a delicate balance: low ROS concentrations seem to inhibit parasite growth, whereas larger amounts may damage vascular endothelial cells and increase vascular permeability [85]. Oxidative stress paradoxically has both a pathogenic and protective role in CM [86]. An anti-oxidant diet was shown to reduce BBB damage and counteract CM development in CM-sensitive mice [87], and anti-oxidant adjuvant therapy, provided at the initial stages of murine CM, prevented the development of persistent cognitive damage [88]. In contrast, NADPH-deficient mice were shown to develop CM despite the lack of ROS production, suggesting that ROS did not contribute to CM pathogenesis [89,90]. To reconcile such an apparent inconsistency, Linares and colleagues have recently shown that glutathione peroxidase and heme oxygenase-1 up-regulation cooperate to suppress superoxide dismutase, catalase, heat shock protein-70 and thioredoxin-1 down-regulation effects in murine CM, counteracting oxidative damage and maintaining redox equilibrium [91]. In human CM, ROS have been associated with a pathogenic role thus far. *In vitro*, ROS inhibition was shown to protect brain endothelial cells against *P. falciparum*-induced apoptosis and to decrease iRBC cytoadherence through ICAM-1 down-regulation and iNOS induction [92,93]. Consistently, in a recent clinical study performed on fifty Indian children with severe malaria, oxidative stress was associated with disease severity [94].

Blood-brain barrier impairment in cerebral malaria

The BBB is one of three main barrier defences protecting the CNS. It is constituted of cerebral vascular endothelial cells, which do not form a rigid structure, but rather a dynamic interface with a range of physical, biochemical and immune properties and functions, built from effective inter-cellular junctions and cell-matrix adhesion molecules, enzymes, and trans-endothelial transport systems [95]. In particular, BBB integrity is dictated by tight junctions between adjacent endothelial cells, forming a network of strands composed by several proteins, including junctional adhesion molecules, claudins (mainly -1 and -5) and occludin, which interact with cellular actin through cytoplasmic proteins such as zonula occludens-1 (ZO-1) [96]. Figure 2 depicts the structure of neural inter-endothelial tight junctions, along with cell-matrix adhesion complexes



including talin, filamin, tensin or α-actinin filaments associated with integrins. We will next discuss how the disruption of these molecules by host proteolytic enzymes such as MMPs could play a relevant role in CM pathophysiology.

BBB functional integrity and permeability are generally assessed by evaluating the passage of molecules from the blood into the cerebral-spinal fluid (CSF). BBB permeability is determined by size and charge of the molecules, and the presence of specific BBB receptors to aid in the transport of certain molecules. The importance of BBB physiology and pathology has led to the development of several BBB models to better investigate the physiological, anatomical and functional characteristics [97]. However, once again the current experimental data on

BBB status during CM are high variable among different model systems [98].

Phenotype of brain and non-brain endothelial cells co-cultured with *Plasmodium* iRBCs in vitro

As discussed below and summarized in Table 1, evidence showing differential phenotypes between neural and non-neural endothelial cells after co-culture with *Plasmodium* iRBCs comes from several *in vitro* studies [56,93,99-108].

First, the effects of *P. falciparum* infection were investigated in a BBB model of cultured primary porcine brain capillary endothelial cells (PBCECs) [99]. In this study, membrane-associated malaria antigens obtained from lysed *P. falciparum* schizont-iRBCs increased endothelial E-selectin and ICAM-1 expression, reduced the trans-

Table 1 Phenotype of endothelial cells after co-culture with infected red blood cells *in vitro*

| Endothelial cell type | <i>Plasmodium</i> strain | Evaluated parameters | Endothelial phenotype | Ref. |
|---|--|--|--|-------|
| Porcine brain capillary endothelial cells (PBCEC) | <i>P. falciparum</i> | - ICAM-1, E-selectin expression; - TEER; - tight junction expression | - increased ICAM-1 and E-selectin - decreased BBB function; - tight junction disruption | [99] |
| Human umbilical vascular endothelial cells (HUVEC) co-cultured with iRBC-fed peripheral blood mononuclear cells | <i>P. falciparum</i> from patients with uncomplicated malaria, severe malaria, or CM | mRNA expression of: - adhesion molecules (ICAM-1, VCAM-1, E-selectin); - tight junctions (occludin, vinculin, ZO-1) | - increased adhesion molecule mRNA (not CM-specific); - reduced tight junction mRNA (CM-specific) | [100] |
| TNF- α - or LT- α -activated human brain endothelial cell line (HBEC-5i) (with/without platelet co-culture) | <i>P. falciparum</i> | - permeability to 70-kDa dextran; - TEER; - endothelial microparticle release; - endothelial apoptosis | - increased BBB permeability; - decreased BBB function; - increased microparticle release; - increased endothelial apoptosis (all effects potentiated by platelets) | [101] |
| Human brain microvascular endothelial cells (HBMEC); HUVEC | <i>P. falciparum</i> | - ICAM-1 expression | increased ICAM-1 expression in HBMEC but not in HUVEC | [93] |
| HBMEC | <i>P. falciparum</i> | - electrical cell substrate sensing; - TEER | - reduced BBB function; - increased BBB permeability | [102] |
| Human dermal microvascular endothelial cells (HDMEC); human lung microvascular endothelial cells (HLMEC) (with parasite sonicates or iRBCs) | <i>P. falciparum</i> | - immunofluorescence staining of ZO-1, claudin-5, VE-cadherin; - observation of inter-endothelial gaps in monolayers; - evaluation of pro-inflammatory response, direct cellular cytotoxicity or cell death. | - loss in total protein content of claudin-5; - redistribution of ZO-1 from cytoskeleton to membrane and cytosolic/nuclear fractions; - minimal inflammation and death (all effects only with sonicates) | [103] |
| HBMEC | <i>P. falciparum</i> | - expression of transcriptome (including ICAM-1 and pro-inflammatory molecules) | - increased expression of ICAM-1 and pro-inflammatory molecules | [104] |
| HBEC-5i; immortalized human cerebral microvascular cell line hCMEC/D3 | <i>P. falciparum</i> | - immunofluorescent microscopy to evaluate malaria antigen presentation by endothelial cells; - TEER | - malaria antigen presentation by endothelial cells; - tight junction opening; | [56] |
| hCMEC/D3 | <i>P. falciparum</i> | - fluorescent permeability assay; - expression of cell adhesion molecules and tight junctions | - increased BBB permeability; - increased ICAM-1 expression; - cytoadherence; - altered ZO-1 distribution | [105] |

Table 1 Phenotype of endothelial cells after co-culture with infected red blood cells *in vitro* (Continued)

| | | | | |
|--|---|---|---|-------|
| TNF- α -activated subcutaneous fat tissue-derived EC from patients with uncomplicated malaria or CM | <i>P. falciparum</i> | - adhesion molecule expression (ICAM-1, VCAM-1, CD61, CD62-E) - microparticle production; - MCP-1, RANTES, IL-6 release ; - caspase-3 activation | - higher ICAM-1, VCAM-1, CD61; - enhanced microparticle release; - induced MCP-1 and IL-6 release; - higher caspase-3 activation (all effects CM-specific) | [106] |
| HBEC-5i | <i>P. falciparum</i> (various strains) | parasite strain selection assay based on cytoadherence | CM-associated cytoadherence | [107] |
| Murine brain vascular endothelial cells (MBVEC) murine lung vascular endothelial cells (MLVEC) | <i>P. berghei</i> ANKA (CM model); <i>P. berghei</i> K173 (non-CM model) | - study of cytoadherence mechanisms; | higher VCAM-1-mediated cytoadherence in CM model compared to non-CM model | [108] |

endothelial electrical resistance (TEER), and promoted the disruption of tight junctions, indicative of increased BBB permeability.

Consistently in various types of human brain endothelium, including HMBEC primary cultures and HBEC-5i or hCMEC/D3 cell lines, iRBCs were also shown to increase ICAM-1 expression [93,99,104,105], to reduce TEER [56,101,102], to alter tight junction expression and distribution [56,105], and to enhance BBB permeability to 70-kDa dextran [101]. Interestingly, platelets were suggested to play a key role in iRBC-dependent increase in BBB permeability, releasing microparticles and causing cell apoptosis in TNF- α - and LT- α -activated HBEC-5i [101]. In hCMEC/D3 cells, iRBC-increased cell adhesion and paracellular permeability correlated with ZO-1 disorganization, but the latter effect appeared mediated by parasite-induced metabolic acidosis, independent from cytoadherence [105]. Moreover, differential global gene expression in HBMEC after interacting with iRBCs revealed significantly up-regulated transcripts related to immune and inflammatory responses, apoptosis, cell-cell signaling, signal transduction and nuclear factor- κ B (NF- κ B)-activation cascade [104]. After co-culturing with iRBCs, the mRNA expression of neural endothelial pro-inflammatory chemokines (IL-6, CXCL-8/IL-8, CXCL-1/GRO- α , CXCL-2/MIP-2 α , and CCL-20/MIP-3 α) increased more than 100-fold, highlighting the strong inflammatory component and the active role of the endothelium in CM pathogenesis [104]. Furthermore, in TNF- α -activated subcutaneous fat tissue-derived endothelial cells, a model comparable to cerebral endothelium, *P. falciparum* iRBCs induced several CM-specific effects, including up-regulation of ICAM-1, VCAM-1, and CD61, enhancement of microparticle, MCP-1 and IL-6 release, and higher caspase-3 activation [106]. Increased levels of inflammatory cytokines may have direct systemic effects and adversely affect the clinical outcome by increasing the cytoadherence of infected RBCs to venular endothelium through up-regulation of adhesion molecules, such as ICAM-1 [93].

To assess the specificity of these effects for human cerebral endothelium, additional comparative studies were also performed using non-neural endothelial cells. Interestingly, *P. falciparum* iRBCs did not affect the expression and distribution of tight junctions (as measured by claudin-5 and ZO-1) and did not induce pro-inflammatory response or cell death in human dermal or lung microvascular endothelium, although parasite sonicates did [103]. Additionally, the up-regulating effects of iRBCs on ICAM-1 expression observed in HBMEC were not reproduced in human umbilical vascular endothelial cells (HUVEC) from healthy donors [93]. An increase in ICAM-1, VCAM-1, and E-selectin mRNA was found in HUVEC from patients with different degrees of malaria (uncomplicated, severe, or

CM) after co-culturing with iRBC-fed mononuclear cells, however such increase did not appear specific for CM. On the contrary, reduced mRNA levels of tight junction proteins (occludin, vinculin, and ZO-1) were strictly associated with CM [100].

Genetic differences between *Plasmodium* strains might also play a role in CM development. Indeed, it has been shown that different strains of *P. falciparum* display variable degrees of cytoadherence to HBEC-5i [107]. Additionally, *P. berghei* ANKA, a murine CM-associated *Plasmodium* strain, induces a higher VCAM-1-mediated cytoadherence compared to *P. berghei* K173 (non-CM strain) in either brain or lung mouse vascular endothelial cells [108].

Blood-brain barrier and in vivo animal models of cerebral malaria

Several *in vivo* animal models have reported alterations in BBB after exposure to *Plasmodium* parasites or malaria products such as hemozoin (Hz, malarial pigment) [47,109-124]. As summarized in Table 2 and described below, these studies provide insightful findings regarding BBB breakdown in animal CM models.

The first animal studies on BBB permeability in malaria date back to 1968, when Migasena and Maegraith demonstrated the movement of albumin across the BBB in *Macaca mulatta* monkeys infected with *P. knowlesi* [109-111]. However, *P. knowlesi* does not induce CM. As such, the rhesus monkey infected with primate malaria parasites, *P. coatneyi* and *P. fragile*, is considered to be a more valid primate model to study in the context of severe malaria with cerebral involvement [112-114].

Of the four species of rodent malaria parasites (*P. berghei*, *P. yoelii*, *P. chabaudi*, *P. vinckei*), only a few *P. berghei* strains can induce experimental CM in mice, with the ANKA strain being the most widely studied. Symptoms of experimental CM in *P. berghei* ANKA-infected susceptible mice include paralysis, ataxia, head deviation, convulsion and coma [98]. In *P. berghei* K173-infected mice an excessive movement of water, albumin and other proteins into the brain, as well as severe brain edema, microthrombosis, sludging of mononuclear cells, arteriolar spasms, scattered disturbances of the microcirculation, and occasional proliferation of gliocytes were observed, suggesting a progressive deterioration of BBB integrity culminating in endothelial lesions and haemorrhages [31,115-118]. Of note, mouse CM models present neurological signs (ataxia, hemiplegia and coma) similar to the clinical features reported in human CM [119].

In a recent work, Penet and colleagues presented the first *in vivo* magnetic resonance study of mouse CM, demonstrating BBB breakdown in CM. Multimodal magnetic resonance neuroimaging techniques (imaging, diffusion, perfusion, angiography, spectroscopy) of *P. berghei* ANKA-infected mice revealed vascular damage, including

Table 2 Evidence of blood–brain barrier (BBB) impairment in animal models with cerebral malaria (CM)

| Animal source | Plasmodium strain | Method to evaluate BBB integrity | Degree of impairment | Reference |
|--|--|--|---|-----------|
| Rhesus monkey (<i>Macaca mulatta</i>) | <i>P. knowlesi</i> | Examination of movement of proteins across the BBB by radiometric and fluorimetric methods | Increase of BBB permeability | [109-111] |
| Rhesus monkey (<i>Macaca mulatta</i>) | <i>P. fragile</i> | Electron microscopy, immunohistochemical analysis (CD36, thrombospondin, ICAM-1), formation of rosettes | Parasitized red blood cells sequestration and adherence to endothelial cells in the cerebral microvessels, neurological symptoms similar to humans | [112] |
| Rhesus monkey (<i>Macaca mulatta</i>) | <i>P. coatneyi</i> | Clinical observation | Anemia, coagulopathy, and renal and metabolic dysfunction | [113] |
| Rhesus monkey (<i>Macaca mulatta</i>) | <i>P. coatneyi</i> | Tissue samples from the brain (cortex and white matter of the cerebrum, cerebellum, and midbrain) collected for quantitation of mRNA expression of cytokines, adhesion molecules, and iNOS | Expression of pro-inflammatory and T helper-1 cytokines, adhesion molecules, and iNOS appears to predominate in the cerebellum of infected rhesus monkeys | [114] |
| A/J and CBA/H mice | <i>P. berghei</i> (ANKA) | Detection of the movement of the dye Evans blue, radioisotope labelled albumin and erythrocytes | Breakdown of BBB | [115] |
| mouse | <i>P. berghei</i> (K173) | Histochemical and histological evaluation of cerebral lesions and their distribution | Progressive deterioration of BBB integrity | [116-118] |
| CBA/T6, Balb/c and DBA/2 J mice | <i>P. berghei</i> (ANKA and K173) | Evaluation of neurological signs (ataxia, hemiplegia and coma) | Increased permeability of BBB | [119] |
| Mouse | <i>P. berghei</i> (ANKA) | Multimodal magnetic resonance techniques (imaging, diffusion, perfusion, angiography, spectroscopy). | BBB breakdown | [120] |
| CM- resistant BALB/c mice | <i>P. berghei</i> (ANKA) | Evaluation of pro-inflammatory cytokines produced | BBB breakdown | [121] |
| C57BL/6 and BALB/c mice | <i>P. berghei</i> (NK65) | Histopathological analysis of cerebral tissue | Increased permeability of BBB | [122] |
| TNF- α -and LT- α -deficient mice | <i>P. berghei</i> (ANKA) | Histochemical and histological evaluation | Neurological signs of CM, associated with perivascular brain haemorrhage in TNF- α -/- mice; completely resistant to CM in LT- α -/- mice | [47] |
| Mouse | <i>P. berghei</i> (ANKA) | Examination of the outcome of TGF- β and TNF- α production in the context of splenocyte apoptosis | Critical balance between TGF- β and TNF- α might have a key role in BBB breakdown | [123] |
| Different murine models: CBA/CaJ and Swiss Webster mice (CM sensitive), Balb/c and A/J mice (CM resistant) | <i>P. berghei</i> (ANKA) <i>P. yoelii</i> (17XL) <i>P. berghei</i> (NK65) and <i>P. yoelii</i> (YM) | Examination of histopathological alterations, BBB dysfunction, or neurological signs | CM related to the opening of paracellular-junctional and transcellular-vesicular fluid transport pathways at the neuroimmunological BBB | [124] |

BBB disruption and haemorrhages, major edema formation, reduced brain perfusion and ischemic metabolic profile, with reduced high-energy phosphates and enhanced brain lactate. These data strongly point to the coexistence of inflammatory response and ischemic lesions [120].

Other recent works illustrated a complex strain-dependent relationship between leukocyte recruitment, BBB permeability and chemokine production. Major pathological consequences of malaria arise from inappropriate or excessive immune response mounted by the host in an attempt to eliminate the parasite. In *P. berghei* ANKA-infected mice, inflammation of the cerebral microvasculature and leukocyte recruitment were clearly evident and found to be driven by production of pro-inflammatory cytokines (IL-12, IFN- γ) and CM development [121]. On the other

hand, *P. berghei* NK65-infected mice showed enhanced production of LT- α and several chemokines (CXCL-9/MIG, CCL-2/MCP-1, CCL-3/MIP-1 α and CCL-5/RANTES), but no neurological symptoms [47,122]. A complementary study performed on the same model proposed a concurrent role for Transforming Growth Factor- β (TGF- β) and TNF- α in promoting splenocyte apoptosis [123].

It should be noted that the cerebral microvascular tree contains two functionally distinct BBB: i) the physiological BBB, formed by capillaries 4–8 mm in diameter, consisting of a single layer of endothelia, gliovascular membrane, and astrocyte endfeet; and ii) the neuroimmunological BBB, formed by postcapillary venules 10–60 mm in diameter and encompassing two layers - the endothelium with its basement membrane and the glia limitans with

associated astrocyte endfeet - separated by the perivascular space [125]. The physiological BBB serves as a tight diffusion barrier for small solutes while the neuroimmunological BBB permits transport of macromolecules and diapedesis of immune cells [125]. In a very recent study comparing different mouse models of experimental CM (*P. berghei* ANKA infection), human CM-like histopathology (*P. yoelii* 17XL) and non-CM (*P. berghei* NK65 and *P. yoelii* YM), Nacer and colleagues observed that the physiological BBB in the experimental CM model remained intact, whereas regulated fluid transport across the neuroimmunological BBB led to brain swelling, intracranial hypertension, coma, and ultimately death due to dysfunction of respiratory centers in pons and the medulla oblongata as a result of brain stem compression [124]. Thus, they proposed that CM may occur in two steps: 1) induction of coma based on regulated, preventable and reversible opening of the neuroimmunological BBB; and 2) endothelial death-associated haemorrhaging, which is difficult to reverse by treatment and eventually fatal [124]. A similar mechanism for neuroimmunological BBB opening in human CM would explain the reversibility of coma with treatment, the scarce traces of tissue necrosis in surviving patients, and the different neurological outcomes of patients despite similar clinical presentation [6,8,9,13,102].

Blood-brain barrier and human studies on cerebral malaria

BBB functional impairment during human CM has been investigated in several clinical and *post mortem* studies [126-143]. Table 3 summarizes the most relevant results. Here, the investigations on human CM patients were performed using albumin CSF/serum ratio as an indicator of BBB integrity [126-128], by *post mortem* immunohistochemical analysis [129-135], or through brain imaging techniques [136-144]. Interestingly, the BBB seems to be more impaired in children than in adults. Moreover, it appears that African and Asian patients display a different degree of BBB damage, with BBB breakdown being more likely to occur in African than Asian populations.

One of the first studies on Asian patients was conducted in Thailand [126]. In this work, albumin CSF/serum ratios were higher in CM patients than in controls, but it did not correlate with coma and mortality. Thus, the authors concluded that their data did not support the idea that cerebral edema might be the cause of coma. More than a decade later, albumin and Immunoglobulins G plasma/CSF ratios were found to be only mildly impaired in Vietnamese patients, suggesting only minimal degree of BBB breakdown in few CM cases [127]. Therein, human CM appeared to cause only subtle functional changes in BBB integrity, with minimal intra-parenchymal inflammatory response compared with other neurologic infections, such as cryptococcal, tubercular, and acute bacterial meningitis [127].

Regarding African populations, a study on Zairean children showed no difference in CSF albumin compared to controls [128]. However, in Malawian children with CM, the activation of endothelial cells and macrophages, along with the disruption of endothelial intercellular junctions in vessels containing sequestered iRBCs, and subtle but measurable changes in albumin CSF *versus* albumin serum levels were observed. Nevertheless, negligible leakage of plasma proteins was still apparent [129]. In Kenyan children with CM, protein and amino acid levels in paired plasma and CSF samples were measured, showing that BBB was mildly impaired in some children with severe *falciparum* malaria [130]. However, this impairment was not confined to CM, as it was also reported in children with prostration-associated malaria and, to a lesser extent, in children with malaria and seizures. Evidence of intrathecal immunoglobulin synthesis in children with malaria was also observed [130]. Finally, data obtained in a recent work performed on Malawian children are consistent with the proposed link between iRBCs sequestration and intravascular/perivascular pathology in fatal pediatric CM, resulting in myelin damage, axonal injury, and BBB breakdown; however, no Hz-laden monocyte extravasation was found [131].

Pathological studies on *post mortem* samples of CM patients showed cerebral edema and raised intracranial pressure in 50% of West African children [132] but not in South Asian adults [133,134] or Malawian children [129]. Nevertheless, an important correlation between sequestration of iRBCs in the brain microvessels and the malaria-related encephalopathy was shown in Asian patients [133]. The adhesion of iRBCs to brain microvessels is mediated by specific receptors on the host endothelium, including ICAM-1, CD36 and CD31 [22-25]. Immunohistochemistry showed altered distribution of the cell junction proteins occludin, vinculin and ZO-1 in Vietnamese adults and Malawian children with CM [129,135]. Sequestration of iRBCs in cerebral microvessels was significantly higher in the brains of patients with CM compared with non-CM patients in all parts of the brain (cerebrum, cerebellum, and medulla oblongata), and was quantitatively associated with *pre mortem* coma [129].

In recent years, several imaging studies have been also conducted on the brains of CM patients during disease progress or after recovery [136-144]. Using magnetic resonance or computed tomography, several common features implicating BBB damage have been observed, including cerebral edema, increased brain volume, ischemia and large vessel infarcts, hemorrhagic cortical lesions, focal and multifocal atrophy, and limited CSF circulation [136-139,141-144]. Interestingly, magnetic resonance in a recently published case-report of a 37-year-old French patient with malaria travelling back from Equatorial Guinea, showed that he developed posterior reversible encephalopathy syndrome, which is characterized by diffuse symmetric signal-intensity

Table 3 Evidence of blood–brain barrier (BBB) impairment in human cerebral malaria (CM) patients

| Group type | <i>Plasmodium</i> strain | Number of patients per cohort | Method to evaluate BBB integrity | Degree of impairment | Reference |
|---|--------------------------|-------------------------------|---|--|-----------|
| Thai patients | <i>P. falciparum</i> | 157 | Albumin CSF/serum ratio | BBB intact | [126] |
| Vietnamese patients | <i>P. falciparum</i> | 20 | Albumin and Immunoglobulins G plasma/CSF ratios | Minimal BBB breakdown in a few cases of CM | [127] |
| Zairean children | <i>P. falciparum</i> | 21 | Albumin CSF/serum ratio | BBB not impaired | [128] |
| Malawian children | <i>P. falciparum</i> | 72 | Immunohistochemistry on autopsy brain tissues | Disruption of endothelial intercellular junctions and impaired BBB function | [129] |
| Kenyan children | <i>P. falciparum</i> | 100 | Protein and immunoglobulin CSF/serum ratio | Mild BBB impairment in some cases | [130] |
| Malawian children | <i>P. falciparum</i> | 50 | Immunohistochemistry on autopsy brain tissues | BBB breakdown | [131] |
| Nigerian children | <i>P. falciparum</i> | 61 | Examination of the possible risk factors for poor prognosis and studies on <i>post mortem</i> samples | Cerebral edema and raised intracranial pressure in 50% | [132] |
| Thai and Vietnamese children | <i>P. falciparum</i> | 65 | Studies on <i>post mortem</i> samples | Cerebral sequestration of <i>P. falciparum</i> -infected erythrocytes | [133] |
| Vietnamese patients | <i>P. falciparum</i> | 20 | Studies on <i>post mortem</i> samples | Heterogeneous cerebral edema and plasma protein leakage | [134] |
| Vietnamese adults and Malawian children | <i>P. falciparum</i> | 14 | Immunohistochemistry | Alteration of cell junction proteins occludin, vinculin and ZO-1 | [135] |
| Kenyan children | <i>P. falciparum</i> | 14 | Computed tomography | Cerebral edema and ischemia | [136] |
| French adults back from Cameroon, Niger, and Thailand | <i>P. falciparum</i> | 3 | Magnetic resonance | Hemorrhagic cortical lesions | [137] |
| Malian children | <i>P. falciparum</i> | 8 | Computed tomography | Diffuse atrophy with asymmetrical ventricle dilation, suggesting limited CSF circulation | [138] |
| French adult back from Equatorial Guinea | <i>P. falciparum</i> | 1 | Magnetic resonance | BBB breakdown | [140] |
| Malawian children | <i>P. falciparum</i> | 14 | Computed tomography | Fatal CM: cerebral edema, large vessel infarcts; Non fatal CM with neurological sequelae: focal/multifocal atrophy | [141] |
| Indian adults | <i>P. falciparum</i> | 4 | Magnetic resonance | Bithalamic infarctions with or without haemorrhages | [142] |
| Malawian children | <i>P. falciparum</i> | 120 | Magnetic resonance | increased brain volume; abnormalities in cortical, deep gray, and white matter structures | [143] |
| Malawian children | <i>P. falciparum</i> | 38 | Magnetic resonance | periventricular and subcortical T2 signal changes, atrophy, and focal cortical defects | |

abnormalities of white matter in the posterior circulation territory [140]. Since data from previous literature suggest one of the mechanisms of posterior reversible encephalopathy involves capillary leakage and acute disruption of the BBB, the authors concluded that this case-report supports the theory of BBB disruption as a key factor for CM development [140].

Blood–brain barrier impairment in cerebral malaria: some reflections upon the available studies

Clearly there is much discrepancy on the extent of BBB damage between animal and human models of CM. While

several studies performed on mouse CM models suggest a strong BBB breakdown [115-124], data on increased BBB permeability in human CM are somehow less evident, generally suggesting the occurrence of only mild BBB impairment, characterized by a relevant degree of tight junction disruption, but lacking molecule exchange between serum and CSF [126-135].

In this context, it should be noted that the relevance of murine CM models for studying CM pathophysiology has been a topic of big debate in the recent years [49]. Being clearly an inflammatory syndrome with local vascular endothelial activation, murine CM displays obvious

differences and some similarities to the clinical and pathological features of human CM, such as signs of vascular inflammation/damage [145]. A recurring issue concerns the degree of iRBC sequestration in the brain and other organs of *P. berghei* ANKA-infected mice. Although recent data find increased iRBC accumulation during murine CM in multiple organs including the brain [146], *P. berghei* infection is generally acknowledged to promote marked accumulation of leukocytes (particularly monocyte, macrophages and T cells), which is in stark contrast to human CM [147]. Thus, despite several processes shared either by murine or human CM, the changes in the endothelial cell microenvironment induced by cytoadherence and inflammation are not the same [104]. Additionally, mouse studies suggesting associations between high levels of cytokines and CM [58-61] have been recently challenged by works showing that high levels of pro-inflammatory cytokines such as TNF- α are poor indicators of human CM in African children [64-67]. Thus, future experimental studies on alternative animal models (non-human primates and other mouse models) are encouraged and urgently necessary to better understand the pathological processes underlying human infection [49].

Another interesting point emerging from clinical data is that the BBB appears more impaired in children than in adults [126-143]. Since CM often strikes children at a critical time in brain development, Hawkes and colleagues have nicely hypothesized that developmental changes in the cerebral vasculature may account for some of the differences in disease presentation and outcome between children and adults, including mortality, seizures and neurocognitive sequelae, rates of associated anemia and renal dysfunction, retinal vessel changes, frequency of ring haemorrhages, and inflammatory cell accumulation in brain microvessels [148]. To avoid any misunderstanding, it has been pointed out that the widespread belief amongst neurotoxicologists that BBB is immature or even absent in the newborn is contrasted by a substantial body of evidence supporting the concept of well-developed barrier mechanisms in the developing brain [149,150]. For instance, inter-cellular tight junctions between cerebral endothelial cells and between choroid plexus epithelial cells are functionally effective as soon as they differentiate [150]. Nevertheless, the brain develops within an environment that is different from that of the rest of the body, and the developing brain possesses a number of unique features not generally present in the adult [149]. Interestingly, certain genes coding for influx/efflux proteins are expressed at much higher levels early in development than in the adult, and there is physiological evidence that these transport systems are functionally more active in the developing brain [150]. How such differences between the pediatric and adult BBB can affect CM pathogenesis and correlate with enhanced

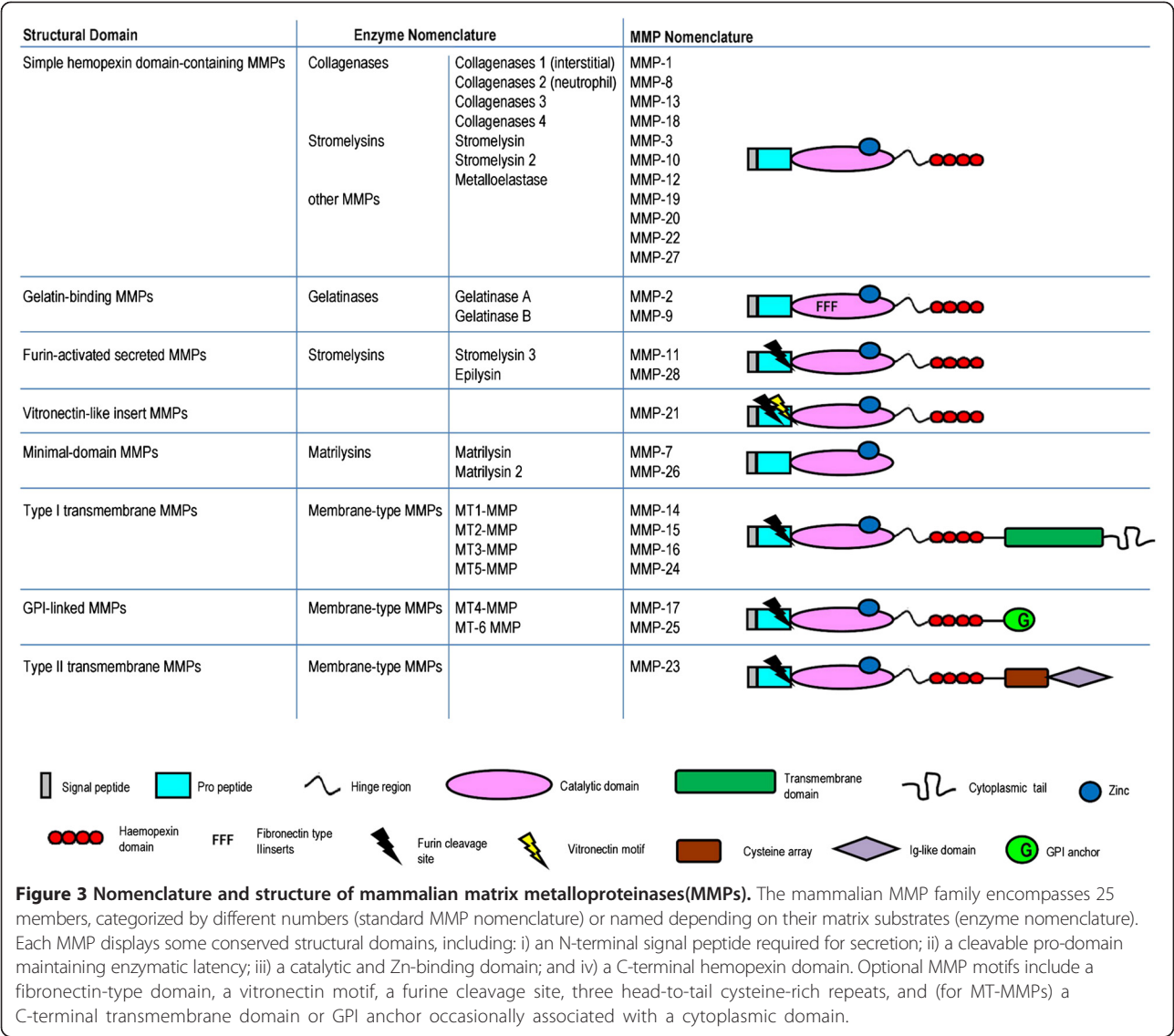
BBB permeability in pediatric CM is still unknown. Future research aimed at shedding light on this topic will certainly be useful.

Involvement of matrix metalloproteinases in cerebral malaria

In the last decade, experimental evidence implicated a specific family of host proteolytic enzymes known as MMPs in malaria pathogenesis [2,151-154]. MMPs are either secreted or membrane-bound zinc-dependent proteases, and their role is also related to the inflammatory response and the BBB function [155-163]. Members of the MMP family are produced by a broad spectrum of specialized cells, including fibroblasts, endothelial cells, lymphocytes, monocytes, macrophages, smooth muscle cells, glial cells, and neurons [155,158].

As detailed in Figure 3, the mammalian MMP family encompasses 25 members, categorized by different numbers or named depending on their matrix substrates. MMPs are evolutionarily conserved and tightly regulated. Conserved protein domains include an N-terminal signal peptide required for secretion, a cleavable pro-domain maintaining enzymatic latency, a catalytic domain, a Zn-binding domain, and (besides the minimal MMPs) a C-terminal hemopexin domain thought to be important for protein-protein interactions [155,157,160,162,163]. The active domain and the Zn-binding domain are essential for catalytic activity: upon pro-domain cleavage a Zn²⁺-ion becomes available to coordinate with a hydrolytic water molecule to enable nucleophilic attack of a substrate, and the enzyme is functionally active [164]. Additional MMP motifs include a gelatin-binding fibronectin-like domain, a serine-, threonine- and proline-rich collagen type V-like domain, a C-terminal transmembrane (TM) domain or GPI anchor, and in some cases a cytoplasmic domain [155,157,160,162,163]. MMP-2 and MMP-9 (also named gelatinase A and B) are further characterized by the presence of three head-to-tail cysteine-rich repeats within the catalytic domain reminiscent of the collagen-binding type II repeats of fibronectin and this domain is necessary for the binding and cleaving activities of these MMPs [161,165,166]. Six membrane-anchored MMPs (MT1- to MT6-MMPs) have a basic RX(K/R)R motif at the C-terminal end of their pro-domains. This motif is recognized and cleaved intracellularly by furin-like proteases. Four MT-MMPs (MT1- to MT4-MMPs) are anchored to the cell membrane through a type I TM domain while the other two MT-MMPs (MT5- and MT6-MMPs) are tethered to the membrane via a GPI moiety. An additional MT-MMP (MMP-23) has an N-terminal type II TM domain [162].

MMPs are regulated at multiple levels including transcription, translation, compartmentalization, secretion, activation, and inhibition by protein inhibitors. Most MMPs are found



at low levels and not constitutively transcribed, but are expressed after external induction by pro-inflammatory molecules, growth factors, NO, cell-cell interactions, cell-matrix interactions, UV radiations [155,157,159-162]. Several signalling pathways and transcription factors are known to regulate MMP expression, including mitogen-activated protein kinases (MAPKs), NF- κ B, and activator protein-1 (AP-1) [155,165,167]. After synthesis, MMPs are stored in inflammatory cell granules, which restrict their action [161]. Furthermore, MMPs are produced as inactive zymogens, referred to as pro-MMPs. Activation is achieved by various proteases (other activated MMPs and several serine proteases) or ROS that disrupt the interaction between the active site zinc atom in the catalytic domain and the conserved cysteine within the pro-domain. Pro-MMPs can be cleaved and activated through different mechanisms and in a context-specific manner. For example,

pro-MMP-9 is activated through a proteolytic cascade sequentially involving plasminogen, MMP-3 and MMP-1 [165]. The activation of proMMP-2 requires previous formation of a pro-MMP-2/tissue inhibitor of metalloproteinase-2 (TIMP-2)/MT1-MMP (MMP-14) multimeric complex [166]. In addition to pro-MMP-2 activation, the binding of TIMP-2 to MT-1-MMP and MT-3-MMP slows down the autocatalytic turnover of these MT-MMPs, paradoxically enhancing surface proteolysis further by stabilizing the pool of active enzyme at the cell surface [160,168]. Once MMPs have been released into the extracellular space or anchored to the membrane and activated, they are kept in check by their endogenous tissue inhibitors (four different forms, from TIMP-1 to TIMP-4). TIMPs inhibit MMP activity with relatively low selectivity in a 1:1 stoichiometric ratio. Interestingly, the ratio of MMP:TIMP can also influence activation mechanisms [157,167].

MMPs were originally discovered in tadpoles as the agents responsible for tail resorption during frog metamorphosis. Thus, they were first characterized as proteases involved in degrading structural proteins comprising the extracellular matrix (ECM) and sub-endothelial basement membranes [155]. However, MMPs are now known to have more sophisticated processes than mere ECM turnover. MMPs can also cleave a growing spectrum of other substrates, including cytokines, chemokines, growth factors, hormones, chemotactic and adhesion molecules, membrane receptors, intercellular junctions, as well as other proteases, including some hemostasis-related molecules and MMPs themselves, protease inhibitors, clotting factors, and anti-microbial peptides [169-171]. MMP-dependent cleavage can serve to activate, inhibit, process, release, shed, or reveal cryptic codes in the substrates they act on. Therefore, the once formidable proteolytic potential of MMPs is now realized to serve essential roles in promoting or inhibiting cell survival, proliferation, migration, invasion, hemostasis and inflammation in either physiological or pathological processes [159,167,169,170].

In physiology, MMPs are involved in diverse biological mechanisms ranging from wound repair to pregnancy [155,157,159,167]. In pathology, MMP dysfunction has been implicated in cancer, cardio-vascular diseases, emphysema, acute renal failure, ophthalmic pathologies, neuroinflammation, neurodegenerative disorders, autoimmune diseases [156-160,167,169,170] and, very recently, malaria [2,151-154].

Matrix metalloproteinases and animal models

In vivo mouse models of CM have recently implicated MMP dysfunction in disease pathology, although it should be kept in mind that experimental CM presents important differences compared to human CM, such as leukocyte sequestration in cerebral microvessels and subsequent migration into brain parenchyma [49]. An excellent study performed by Van den Steen and his group comprehensively investigated mRNA expression levels of MMPs and protein release or pro-enzyme activation in five different organs (brain, lung, spleen, liver, and kidney) from CM-sensitive C57B1/6 mice infected with *P. berghei* ANKA (CM model) or *P. berghei* NK65 (non-CM model) and CM-resistant Balb/C mice infected with *P. berghei* ANKA (CM-resistant model) [172]. Importantly, they observed enhanced expression and activation of monocytic (CD11b+) MMP-9 in brains of CM mice [172] specific to CM, as suggested by comparison with non-CM models, such as lung pathology [173]. Additionally, tissue-specific increases in mRNA expression were found for several MMPs, including MMP-3, -4, -8, and -13 in spleen, MMP-8, -12, -13, and -14 in liver, and MMP-8 and -13 in brain. All of these increases were more pronounced in the CM model. In a CM-resistant model, MMP-3 expression was significantly

enhanced, suggesting a protective role for this MMP in CM [172]. In another study, CM mice showed increased neural MMP-7 protein levels [151]. Interestingly, urokinase-type plasminogen activator (uPA) $-/-$ or urokinase-type plasminogen activator receptor (uPAR) $-/-$ knock-out mice with CM displayed enhanced survival and attenuated thrombocytopenia [174].

A parasite molecule, malarial pigment Hz - a lipid-bound ferriprotoporphyrin IX produced by *P. falciparum* after hemoglobin catabolism [175] - is proposed to play a role in experimental CM. Indeed, using a sensitive fluorometric method to determine Hz content in blood and tissue samples from mice infected with *P. berghei* NK65 (non-CM model) or ANKA (CM model), Sullivan and colleagues observed increased Hz levels in tissue correlating with the duration of infection, with neural Hz levels being higher in CM than non-CM mice, raising the possibility that Hz presence may be associated with cerebral pathology [176].

Interestingly, *in vitro*, Hz appears to play a major role in MMP dysfunction. Phagocytosis of Hz by RAW 264.7 rat macrophage cell line was shown to impair expression of several inflammatory molecules [177] and, after an early inhibitory peak, to increase the long-term mRNA expression of MMP-9 [178]. This effect was related to the lipid moiety of Hz, since lipid-free synthetic Hz (β -hematin) did not modulate MMP-9 expression. The Hz-dependent enhancement of MMP-9 transcription and protein release was mimicked by 4-hydroxy-2-nonenal (4-HNE) [178], a molecule generated by Hz from polyunsaturated fatty acids [179].

Matrix metalloproteinases and human studies

In vitro studies using human monocytes and endothelial cells [152,180-195] provide convincing and homogeneous evidence for Hz-dependent mechanisms underlying aberrant MMP-9 function (see Figure 4). In a series of works performed with human adherent or immunopurified monocytes from peripheral blood, the phagocytosis of free Hz or Hz-containing trophozoites enhanced MMP-9 mRNA levels, protein expression, and activity [180-186]. This observation was also investigated using THP-1 monocyte cell line [187]. Hz-fed monocytes display increased total gelatinolytic activity [186] and invasiveness [180] caused by MMP-9 - but not MMP-2 [188] - enhancement.

Increased MMP-9 function in human monocytes appears to be mediated by Hz-dependent over-production of several pro-inflammatory molecules, including TNF- α [180], IL-1 β [181], and CCL-3/MIP-1 α [184]. Further investigation revealed increases in MMP-9 [181], TNF- α [189,190] and IL-1 β [181,190], but not CCL-3/MIP-1 α [183,190], were dependent on the lipid moiety of Hz. These studies unveiled a major role for 15-HETE, a potent lipid peroxidation derivative generated by Hz autocatalysis.

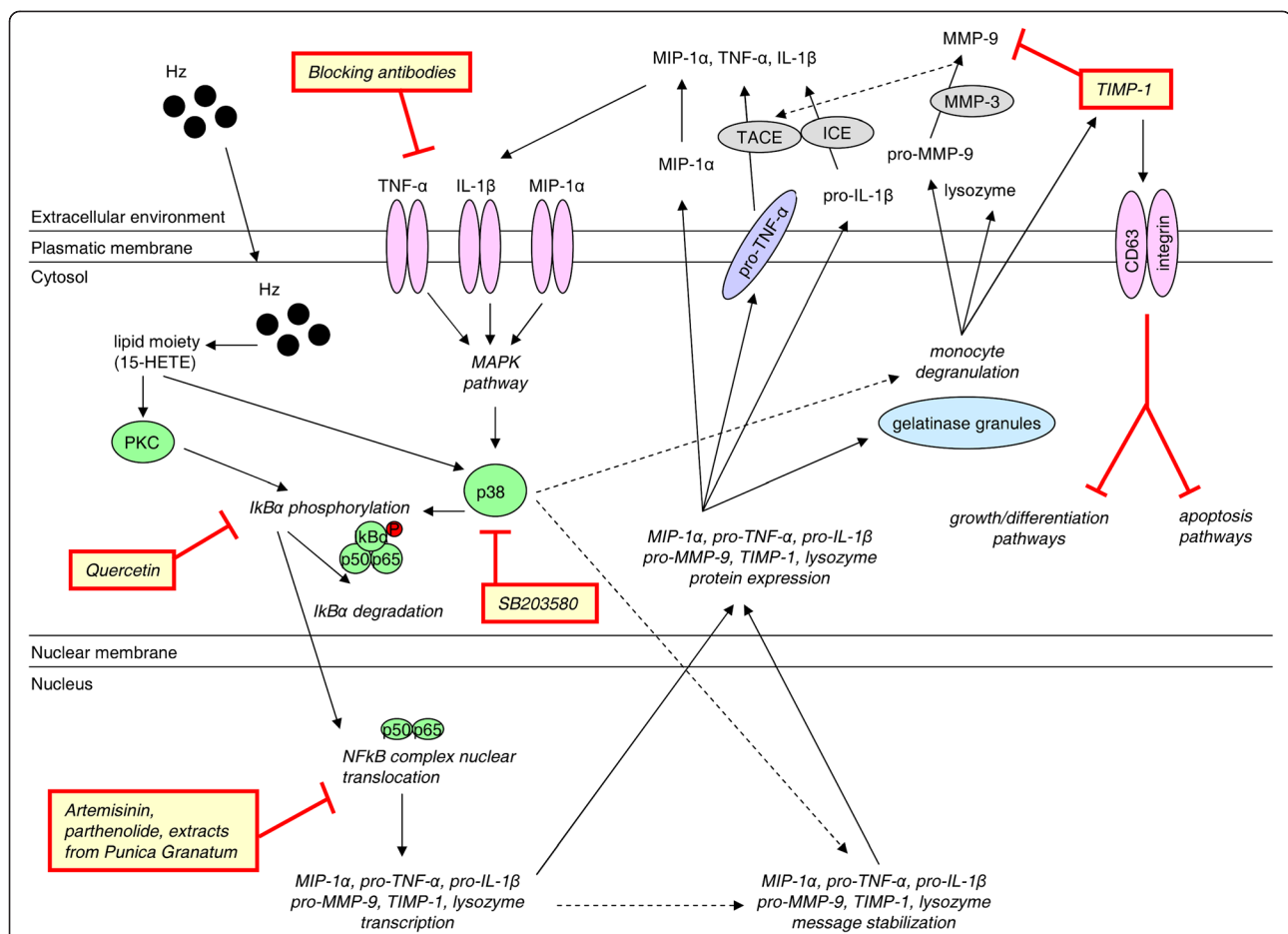


Figure 4 Principal mechanisms underlying Hz-dependent dysregulation of matrix metalloproteinase-9 (MMP-9) and related molecules in human monocytes.

The most relevant Hz-dependent mechanisms underlying aberrant MMP-9 function in malaria are based on current evidence from *in vitro* models of cultured human monocytes. After Hz phagocytosis, some lipoperoxidation products generated by Hz autocatalysis such as 15-HETE can promote early and late activation of PKC and p38 MAPK. These kinases have been associated with cytosolic I- κ B α phosphorylation and degradation, resulting in subsequent nuclear translocation of NF- κ B p50 and p65 subunits. Consequently, the transcription and protein expression of several pro-inflammatory molecules including TNF- α , IL-1 β , and MIP-1 α , and of some proteolytic enzymes or inhibitors such as lysozyme, pro-MMP-9, and TIMP-1 is enhanced. p38 MAPK can also promote monocyte degranulation, releasing pro-MMP-9, TIMP-1 and lysozyme into the extracellular environment. After secretion of these molecules, several proteolytic events can occur. Active MMP-9, generated from MMP-3 processing of the pro-enzyme, can further modulate TNF- α shedding from cell membrane in a similar manner as TACE, whereas ICE activates IL-1 β after cleaving its pro-peptide. Soluble TNF- α , IL-1 β , and MIP-1 α have been shown to play a key role in mediating Hz effects on MMP-9, lysozyme, and TIMP-1 production, possibly generating some auto-enhancing loops. Hz-enhanced MMP-9 could favour CM development through complementary proteolytic activities (see Figure 5). On the other hand, TIMP-1 is primarily referred to as a MMP-9 inhibitor, thus TIMP-1 Hz-enhanced levels could supposedly be protective. However, several MMP-independent functions such as inhibition of cell apoptosis and growth have been recently described for TIMP-1. Thus, Hz-enhanced TIMP-1 protein may play a role in prolonged survival of impaired Hz-fed monocytes, in their altered maturation to dendritic cells and in their reduced ability to coordinate erythropoiesis. Finally, enhanced plasma levels of human lysozyme have been depicted as a risk factor for severe malaria.

Hz was also causally related to increased TIMP-1 and lysozyme release from human adherent monocytes, two molecules stored in gelatinase granules along with MMP-9 [186,190-192]. Further studies also showed that Hz-induced monocyte degranulation was mediated by TNF- α , IL-1 β and MIP-1 α /CCL-3 [191,192] and dependent on Hz lipid moiety, suggesting a major role for 15-HETE [190]. The heme core of Hz was shown to bind MMP-9 hemopexin domain and to prime the activation of the zymogen

by other MMPs, such as MMP-3 [193]. The mechanisms underlying Hz-dependent enhancement of MMP-9, TNF- α , IL-1 β , CCL-3/MIP-1 α , TIMP-1 and lysozyme appear to involve NF- κ B activation, as suggested by results from parallel works performed with adherent monocytes from peripheral blood [182,186,190,191] and THP-1 cell line [187]. In these works, Hz-induced enhancement of MMP-9, TNF- α , IL-1 β , CCL-3/MIP-1 α and TIMP-1, as well as total gelatinolytic and lysozyme activity were abrogated by

using different NF- κ B inhibitors showing anti-malarial properties (quercetin, artemisinin, parthenolide, and extracts from *Punica granatum*). Moreover, results from experiments with SB203580, a known inhibitor of p38 MAPK pathway suggest that concurrent activation of p38 MAPK pathway seems to be mandatory for Hz- and 15-HETE-dependent increased MMP-9 [185] and related molecules TNF- α , IL-1 β , CCL-3/MIP-1 α , TIMP-1 and lysozyme [186,190,191]. On the contrary, ERK and JNK MAPK pathways do not seem to be activated by Hz [185].

Additional evidence on Hz-dependent MMP dysregulation is also derived from studies using human endothelial cells. In the human microvascular endothelial cell line HMEC-1, either free Hz [194] or Hz-containing iRBCs [195] induced the release of pro-MMP-9 and active MMP-9 proteins *de novo* without altering pro-MMP-2 basal levels. Interestingly, Hz also enhanced the protein levels of basal MMP-1 and MMP-3, two MMPs sequentially involved in pro-MMP-9 activation. Consequently, total gelatinolytic activity and cell invasion were increased. More evidence of Hz-triggered enhancement of MMP-9 protein release emerges from studies using human endothelial cells from large calibre vessels [152]. Similar to human monocytes, a role for the Hz lipid moiety also appears likely in endothelial cells, although the molecules responsible for these effects have not yet been identified [194].

Little evidence exists on the *in vivo* involvement of MMPs in human CM from organ autopsy studies or from fluid (blood, serum) analysis of patients with uncomplicated or severe malaria [65,196-201]. The little data that does exist are somehow conflicting and still incomplete.

The accumulation of pro-angiogenic uPAR [196], MMP-1 and VEGF [197] in Durck granulomas, along with the lack of anti-angiogenic endostatin/collagen XVIII in ring haemorrhage areas [198] has been observed in the brains of patients with fatal CM, suggesting that the proMMP-9 proteolytic machinery is activated in areas of intense parasite sequestration and vascular damage. Consistently, microarray analysis performed on whole blood from Kenyan children with severe malaria showed *P. falciparum* activation of the human MMP-9 gene [199].

However, sera of Gabonese and Ghanaian children with uncomplicated or severe malaria did not display altered MMP-9 levels [65,200]. Moreover, serum MMP-8 levels were elevated in Gabonese children with either uncomplicated malaria or CM [200], but not in Nigerian children with uncomplicated malaria [201]. Nonetheless, it has been argued that serum levels might not be an ideal source for reliable data concerning MMP levels, since MMP release from blood cells during sample processing might give a highly non-specific background result, thus hindering the ability to assess true concentrations of circulating MMPs [202].

Possible role of matrix metalloproteinases in pathophysiology and therapy of cerebral malaria

It is likely that MMPs play an active role in several steps during CM development as they can process a large repertoire of substrates [169-171], including pro-inflammatory molecules, tight junctions, and hemostatic factors likely involved in CM (see Figure 5 and Tables 4 and 5).

First, MMPs proteolytically cleave the pro-forms of many cytokines and chemokines reportedly enhanced in CM, including TNF- α , IL-1 β , CXCL-8/IL-8. As a result of cleavage, these molecules are shed, activated or functionally potentiated, respectively. Thus, MMPs could contribute to the uncontrolled inflammatory response typical of CM. Consistently, an *in vitro* study using adherent monocytes demonstrated a Hz-dependent pathological auto-enhancing loop established between MMP-9 and TNF- α [180].

Moreover, a growing number of inter-endothelial tight junctions, including occludin, ZO-1, claudin-1 and claudin-5 are known MMP substrates (e.g. MMP-2, MMP-3, MMP-7 and MMP-9), which raises the possibility that MMP activity on these substrates can enhance the permeability of endothelial barriers by destroying these junctions [203-209]. Therefore, enhanced MMP levels in malaria might facilitate BBB leakage. This hypothesis is consistent with data showing that Hz-containing iRBCs reduced human BBB permeability *in vitro* [102], and that Hz and iRBCs enhanced MMP-1, MMP-3 and MMP-9 produced by human microvascular endothelium [194,195].

Finally, MMPs can also influence hemostasis. Both tPA and uPA molecules, responsible for plasminogen conversion to plasmin, as well as tPA/uPA inhibitor PAI-1 can be processed by MMPs [210-212]. As a consequence, MMPs might affect fibrin degradation by plasmin, perhaps explaining thrombotic events that frequently occur during CM.

Interestingly, some molecules such as 4-aminoquinolines and artemisinins, which are currently used for primary therapy of uncomplicated malaria, have displayed MMP-inhibiting properties. Chloroquine treatment reduces MMP-9 serum levels in patients with systemic lupus erythematosus [213]. Artemisinin down-regulates MMP-2 levels in human melanoma cells [214] and MMP-1, MMP-2 and MMP-9 levels in mouse embryonic stem-cells derived from embryoid bodies [215]. Dihydroartemisinin inhibits MMP-2, MMP-9 and MMP-14 expression/activity in human fibrosarcoma cells [216] and MMP-9 expression in human umbilical vein endothelial cells [217]. Finally, artesunate down-regulates MMP-2 and MMP-7 expression in human non-small cell lung cancer [218]. However, it should be noticed that the drug concentrations used in such studies need to be verified and optimized for human clinical trials. This might explain why antimalarial drugs alone cannot prevent CM development.

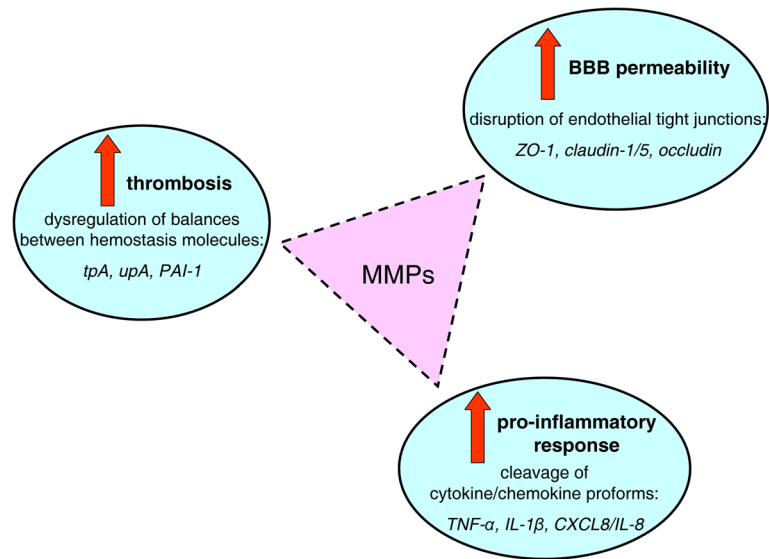


Figure 5 Multiple putative roles of matrix metalloproteinases (MMPs) in cerebral malaria (CM) according to their biochemical functions. MMPs could play an active role during CM development through several complementary mechanisms: i) by disrupting endothelial tight junctions after protein degradation of ZO-1, claudins and occludin, thus causing an increased BBB permeability; ii) by promoting TNF- α shedding, IL-1 β activation and CXCL8/IL-8 potentiation after proteolytic cleavage of their pro-domains, therefore inducing exacerbated pro-inflammatory response; iii) by processing some CM-associated hemostatic factors such as tpA, upA, and PAI-1, thus increasing the risk for thrombotic events.

It is intriguing to explore the idea of targeting MMPs with broad spectrum or specific MMP inhibitors as adjuvant therapy in CM. In the last two decades, a large number of synthetic MMP inhibitors have gone through clinical trials and largely failed as anti-cancer and anti-arthritis drugs due to serious long-term side effects, with only one currently commercially available [219-223]. Hopefully, using combinations of MMP inhibitors with antimalarials could justify lower therapeutic doses of both drugs, thereby reducing their potential side effects whilst still enhancing anti-MMP properties by drug synergy. To date, the effects of MMP inhibitors in CM remain scarce. *In vitro*, the use of a specific synthetic inhibitor of MMP-9 was shown to abrogate Hz-dependent

increase of TNF- α in human monocytes, suggesting that MMP-9 inhibition might be useful to counteract pathological inflammation in CM [180]. However, MMP-9 knock-out mice infected with *P. berghei* ANKA did not display any protection from CM development, probably due to the redundant functions of other MMPs which might compensate for the loss of MMP-9 [172]. On the contrary, treatment with broad-spectrum MMP inhibitor BB-94 significantly improved survival of CM mice [172].

Future research aimed at determining the exact role (protective or detrimental) of each MMP during malaria infections will be highly informative. Unfortunately, with the exception of a few cases, specific inhibitors against individual MMPs are currently lacking [224-226]. Some

Table 4 Cerebral malaria (CM)-associated brain inter-endothelial junctions and cell-matrix adhesion molecules known/hypothesized to be matrix metalloproteinase (MMP) substrates

| MMP | MMP substrate: junctions | Junction type |
|--------|--|---|
| MMP-1 | Collagen I/II/III/VII/VIII/X; Aggrecan; Entactin; Tenascin | Cell-matrix adhesion |
| MMP-3 | Collagen II/IV/IX/X; Claudin-5; E-cadherin; Elastin; Fibronectin; Laminin; Occludin; Selectin; ZO-1 | Cell-matrix adhesion; Adherens junctions; Tight junctions |
| MMP-8 | Collagen I/II/III/V/VII/VIII/X; Claudin-5; Laminin; Occludin; ZO-1 | Cell-matrix adhesion; Tight junctions |
| MMP-9 | Collagen IV/V/VII/X/XIV; Aggrecan; Claudin-5; E-cadherin; Elastin; Fibronectin; Laminin; Occludin; Vitronectin; ZO-1 | Cell-matrix adhesion; Adherens junctions; Tight junctions |
| MMP-12 | Elastin; Fibronectin; Laminin; Proteoglycans | Cell-matrix adhesion |
| MMP-13 | Collagen I/II/III/IV/V/IX; Aggrecan; Elastin; Fibronectin; Laminin; Tenascin | Cell-matrix adhesion |
| MMP-14 | Collagen I/II/III; E-cadherin; $\alpha_v\beta_4$ integrin; Aggrecan; Fibronectin; Laminin; Vitronectin | Cell-matrix adhesion; Adherens junctions |

Table 5 Cerebral malaria (CM)-associated pro-inflammatory molecules known/hypothesized to be matrix metalloproteinase (MMP) substrates

| MMP | MMP substrate: cytokine | MMP substrate: chemokine |
|--------|-------------------------|---|
| MMP-1 | IL-1 β | CCL-2/MCP-1/JE |
| | TNF- α | CCL-25/TECK |
| MMP-2 | IL-1 β | CCL-2/MCP-1/JE |
| | TGF- β | CCL-11/Eotaxin |
| | TNF- α | CCL-25/TECK |
| | | CXCL-1/GRO- α /KC CXCL-2/GRO- β /MIP-2 CXCL-12/SDF-1 |
| MMP-3 | proIL-1 β | CCL-2/MCP-1/JE |
| | proTNF- α | |
| MMP-7 | proTNF- α | |
| MMP-8 | | CCL-2/MCP-1/JE |
| | | CXCL-5/ENA-78/LIX |
| MMP-9 | IL-1 β | CXCL-1/GRO- α /KC |
| | IL2-R | CXCL-2/GRO- β /MIP-2 |
| | TGF- β | CXCL-4/PF-4 |
| | proTNF- α | CXCL-5/ENA-78/LIX |
| | | CXCL-10/IP-10 |
| | | CXCL-12/SDF-1 CCL-5/RANTES CCL-7/MCP-3/MARC CCL-17/TARC CCL-25/TECK |
| MMP-10 | | CCL-25/TECK |
| MMP-11 | | CCL-25/TECK |
| MMP-12 | | CXCL-3/GRO- γ |
| | | CXCL-9/MIG |
| | | CXCL-10/IP-10 CXCL-11/I-TAC |
| MMP-14 | proTNF- α | CXCL-8/IL-8 |
| | | CXCL-12/SDF-1 |

metalloproteinases are also produced by malaria parasites, for example to perform hemoglobin degradation [227]. Therefore, MMP inhibitors may not only influence host but also parasitic pathways. Another issue to be taken in account is represented by the effects of MMPs on other organs than brain. However, it should be noted that the adverse effects of MMP inhibitors documented in other pathologies such as cancer were associated with long-term treatment [220], whereas the time course of drug administration in CM therapy should be reasonably shorter, possibly limiting the development of side effects. A detailed analysis of the role of each protease in physiology and

pathology, along with the development of specific inhibitors, could yield novel insights to assess whether specific MMP inhibition might be considered as new adjuvant therapies.

Conclusion

As suggested by three complementary theories developed over the past century, CM might be a likely consequence of several concomitant phenomena, including iRBC sequestration in brain microvessels, enhanced BBB permeability, and release of pro-inflammatory molecules from host immune cells. Data from *in vitro* and *in vivo* studies suggest that a full BBB breakdown during CM is more likely to occur in mouse than in humans. In the latter case, the BBB appears only mildly impaired as a result of tight junction disruption. MMPs are host proteolytic enzymes involved in degradation of basement membranes, disruption of inter-endothelial tight junctions, and cleavage of a large spectrum of pro-inflammatory, membrane-bound and hemostasis-related molecules, and they may play a crucial role in CM. Further in-depth analysis of the involvement of MMPs in CM might help to design new adjuvant therapies. In this context, MMP inhibitors could prevent BBB leakage and reduce the exacerbated inflammatory response, thus reducing the high mortality rates of CM patients, along with the frequency of neurological sequelae in recovering patients.

Abbreviations

BBB: Blood-brain barrier (BBB); CNS: Central nervous system; CM: Cerebral malaria; CSF: Cerebral-spinal fluid; ECM: Extracellular matrix; Hz: Hemozoin; HR: Histamine receptor; 15-HETE: 15-hydroxyeicosatetraenoic acid; 4-HNE: 4-hydroxy-2-nonenal; iRBCs: Infected red blood cells; IDO: Indoleamine 2,3-dioxygenase; IFN: Interferon; IL: Interleukin; ICAM-1: Intracellular adhesion molecule-1; MMP: Matrix metalloproteinase; MCP-1: Monocyte chemoattractant protein-1; NO: Nitric oxide; NOS2: Nitric oxide synthase type 2; MAPK: Mitogen-activated protein kinase; NF- κ B: Nuclear factor- κ B; PfEMP-1: *P. falciparum* erythrocyte membrane protein-1; PBCEC: Porcine brain capillary endothelial cell; HBMEC: Human brain microvascular endothelial cell; HUVEC: Human umbilical vascular endothelial cell; ROS: Reactive oxygen species; TIMP: Tissue inhibitor of metalloproteinase; TEER: Trans-endothelial electrical resistance; TGF: Transforming growth factor; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor; uPA: Urokinase-type plasminogen activator; uPAR: Urokinase-type plasminogen activator receptor; VCAM-1: Vascular cellular adhesion molecule-1; ZO-1: Zonula occludens-1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors contributed equally in the process of creating the manuscript. Both authors read and approved the final manuscript.

Authors' information

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