Vascular dysfunction as a target for adjuvant therapy in cerebral malaria

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Cerebral malaria (CM) is a life-threatening complication of Plasmodium falciparum *malaria that continues to be a major global health problem. Brain vascular dysfunction is a main factor underlying the pathogenesis of CM and can be a target for the development of adjuvant therapies for the disease. Vascular occlusion by parasitised red blood cells and vasoconstriction/vascular dysfunction results in impaired cerebral blood flow, ischaemia, hypoxia, acidosis and death. In this review, we discuss the mechanisms of vascular dysfunction in CM and the roles of low nitric oxide bioavailability, high levels of endothelin-1 and dysfunction of the angiopoietin-Tie2 axis. We also discuss the usefulness and relevance of the murine experimental model of CM by* Plasmodium berghei *ANKA to identify mechanisms of disease and to screen potential therapeutic interventions.*

Key words: cerebral malaria - vascular dysfunction - nitric oxide

Cerebral malaria (CM) is one of the most lethal complications of *Plasmodium falciparum* malaria, responsible for a large fraction of the 1,238,000 (95% confidence interval: 929,000-1,685,000) malaria-related deaths estimated for 2010 in 105 countries (Murray et al. 2012). The World Health Organization (WHO) defines CM as coma (incapacity to localise a painful stimulus or Blantyre coma score ≤ 2) persisting at least 1 h after termination of a seizure or correction for hypoglycaemia in the presence of asexual *P. falciparum* parasitaemia and without the presence of other causes of encephalopathy (WHO/CDC 2000, Trampuz et al. 2003). However, for practical purposes, any patient presenting with impaired consciousness and falciparum parasitaemia must be started on parenteral antimalarial drugs and transferred to an intensive care unit. Severe malarial anaemia, respiratory distress and hypoglycaemia are the most common concomitant complications in paediatric CM cases (Murphy & Breman 2001), whereas jaundice, acute renal failure and hypoglycaemia are the most frequent concurrent complications occurring in adult CM cases (Mohanty et al. 2003, Idro et al. 2005). Up to 75% of CM-related deaths occur within 24 h of admission which makes the initiation of antimalarial treatment right after confirmation of malarial parasitaemia a priority over further diagnostic work up (Idro et al. 2005). Recent studies showed that intravenous artesunate is superior to intramuscular artemether or intravenous quinine and it is currently recommended as the treatment of choice for CM (2.4 mg/kg bolus on admission, at 12 h and 24 h, thereafter once daily until oral medication can be taken reliably) (Dondorp et al. 2005, 2010). Despite the efficacy of intravenous artesunate, mortality by severe malaria in general, and by CM in particular, is still high even when clinical trials provide adequate patient care, as observed in the South East Asian Quinine Artesunate Malaria Trial (adults) and Africa Quinine Artesunate Malaria Trial (children) (Dondorp et al. 2005, 2010). In addition, 11% of children surviving CM present gross neurological deficits at discharge (most commonly spasticity, ataxia, hemiplegia, speech disorders and blindness) and up to 25% may sustain long-term cognitive deficits (Brewster et al. 1990, Boivin 2002, Carter et al. 2005, Boivin et al. 2007, John et al. 2008). Development of adjuvant therapies is therefore urgently necessary to modify the high rates of mortality and sequelae resulting from CM.

CM pathogenesis: a central role for vascular dysfunction - The pathogenesis of CM is still poorly understood. The histopathological hallmark of CM is the sequestration of parasitised red blood cells (pRBCs) in the brain of patients that died of CM (Pongponratn et al. 1991, 2003). Quantitative analysis of brain sections in necropsy studies of patients in Thailand and Vietnam showed that CM patients had more sequestered pRBCs in the brain than patients that died of other malaria complications. In addition, pRBC accumulation in the brain of CM patients was also higher than in other vital organs (Pongponratn et al. 1991, 2003). *P. falciparum* pRBCs adhere to endothelial cells through ligand-receptor interactions mediated by a family of variant antigens expressed by the parasites and exported to the surface

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of the RBC. The *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) is encoded by approximately 60 vargenes in the *P. falciparum* genome, but usually only one variant is expressed at a time by each individual parasite (Kraemer & Smith 2006, Scherf et al. 2008). These proteins have characteristic motifs that signal for their export to the RBC membrane, where they accumulate in protuberant structures known as knobs. Different PfEMP1 variants can interact with different receptors in endothelial cells. Several ligands have been identified, among them CD36 (Barnwell et al. 1989), ICAM1 (Berendt et al. 1989, Brown et al. 2013), thrombospondin (Roberts et al. 1985) and chondroitin sulphate (Reeder et al. 1999). A potential role for ICAM1 in pRBC sequestration in the inflamed endothelium of brain vessels has been described (Turner et al. 1994, Silamut et al. 1999) and patients that died of CM showed high expression of ICAM1 in the brain (Silamut et al. 1999). Inflammation indeed has been linked to sequestration and coagulation as critical factors for CM development (van der Heyde et al. 2006, Moxon et al. 2013) and recent work has shown that markers of inflammation and endothelial activation such as plasma levels of soluble ICAM1, C-reactive protein and angiopoietin-2 are elevated at hospital admission of children with CM and remain high even after successful treatment with antimalarial drugs (Moxon et al. 2014). More recently, it was demonstrated that severe malaria and CM are associated with the expression of specific PfEMP1 subtypes containing domain cassette 8 (Lavstsen et al. 2012, Bertin et al. 2013), whose binding to endothelial cells is mediated by endothelial protein C receptor, a host receptor involved in anticoagulation and endothelial cytoprotection and quiescence (Turner et al. 2013).

Adherence of large numbers of pRBC to the endothelium of brain post-capillary venules would plug the vessels, leading to mechanical occlusion, impaired blood flow with resulting ischaemia and tissue hypoxia (Marsh et al. 1996). In fact, microvascular congestion and pRBC sequestration were independent predictors of clinical diagnosis of CM (Ponsford et al. 2012). In addition, retinal angiography studies in children with CM showed the frequent occurrence of impaired perfusion, vessel occlusion and filling defects likely due to pRBC sequestration in the retinal microvasculature (Beare et al. 2009). Single photon emission computed tomography also indicated the occurrence of marked cerebral hypoperfusion in a CM patient (Kampfl et al. 1997, Beare et al. 2006). These findings evidence hypoxia and ischaemia as important components in the pathogenesis of CM. Mechanical obstruction of blood flow by adherent pRBCs has long been considered as the central mechanism leading to CM (Aikawa et al. 1987, Pongponratn et al. 2003, White et al. 2010, 2013). However, it is becoming increasingly clear that blood vessel function in CM is compromised well beyond occlusion. Spastic constriction of cerebral arterioles has been identified in CM patients (Polder et al. 1991). In addition, severe malaria patients show lower reactive hyperaemia-peripheral arterial tonometry (RH-PAT) index (a measurement of reactive vasodilation) than uninfected controls or patients with uncomplicated malaria (Yeo et al. 2007). The mechanisms behind vasoconstriction and vascular dysfunction in CM are not completely understood, although mediators such as nitric oxide (NO), endothelins and the angiopoietin-Tie2 axis seem to be involved (Wenisch et al. 1996, Yeo et al. 2007, 2008a, Dietmann et al. 2008, Conroy et al. 2009, Lovegrove et al. 2009, Jain et al. 2011).

NO in human CM - NO is a small gaseous molecule with a central role in the maintenance and regulation of vascular tone (Ignarro et al. 1987). NO is generated from the amino acid (aa) L-arginine (L-Arg) by three isoforms of the enzyme NO synthase (NOS). Two of the isoforms are expressed constitutively in endothelial cells [endothelial NOS (eNOS)] or in neurons [neuronal NOS (nNOS)], whereas inducible NOS (iNOS) is expressed in several cell types, mainly phagocytes, upon stimuli such as a pro-inflammatory cytokines (Forstermann & Sessa 2012). eNOS is the main isoform regulating vascular tonus systemically, nNOS being also important in the regulation of cerebral blood flow. NO overproduction by iNOS during pathological conditions such as sepsis may cause life-threatening hypotension (Boisramé-Helms et al. 2013). In physiological conditions, NO produced by eNOS in endothelial cells diffuses to the neighbouring vascular smooth muscle cells where it activates soluble guanylate cyclase (sGC) that, in turn, generates cyclic guanosine monophosphate (cGMP). cGMP activates protein kinase G, which causes reuptake of Ca++ and the opening of calcium-activated potassium channels. The fall in concentration of Ca++ ensures that the myosin light-chain kinase can no longer phosphorylate the myosin molecule, thereby leading to relaxation of the smooth muscle cell, causing vasodilation and increasing blood flow.

Low NO bioavailability has been described in several haemolytic disorders (Rother et al. 2005, Morris et al. 2008). Haemolysis causes gram quantities of haemoglobin to be released in the bloodstream. Cell-free plasma haemoglobin is a potent scavenger of NO and will deplete most of the NO that is produced by endothelial cells (Rother et al. 2005). More recently, it has been shown that RBC microparticles formed during pathological haemolysis also scavenge NO with similar potency to cell-free haemoglobin (Liu et al. 2013). In addition, RBC arginase is released in the plasma during haemolysis and will compete with NOS for the same substrate, L-Arg, depleting it (Rother et al. 2005). Finally, L-Arg transport across RBC membrane is impaired and arginase-mediated L-Arg consumption enhanced by free plasma haeme released from cell-free haemoglobin (Omodeo-Salè et al. 2010). Impaired NO bioavailability represents the central feature of endothelial dysfunction observed in haemolytic disorders (Morris et al. 2008). The consequences of impaired NO bioavailability include vasoconstriction, reduced blood flow, impaired reactivity of blood vessels to dilation stimuli, acquisition of a pro-inflammatory, pro-adhesive phenotype by endothelial cells, loss of endothelial cell-cell junctional cohesiveness, among other alterations.

A seminal study in Tanzania showed that plasma and urine levels of nitrite and nitrate (stable metabolites of NO) were inversely correlated with malaria severity in children (Anstey et al. 1996). This finding was con-

firmed later in patients with CM who showed decreased plasma and cerebrospinal levels of nitrite and nitrate (Dondorp et al. 1998). In vitro, inhibition of NO production by treatment of human microvascular endothelial cell with the NOS inhibitor L-N(G)-nitro-arginine-methyl-ester (L-NAME) enhanced *P. falciparum* pRBC adhesion, whereas addition of a NO-donor reduced adhesion (Serirom et al. 2003). Patients with CM showed low levels of L-Arg, the substrate for NO generation by NOS isoforms and hypoargininaemia was significantly associated with CM case-fatality (Lopansri et al. 2003). Severe malaria patients showed endothelial dysfunction characterised by lower levels of exhaled NO and lower reactive RH-PAT index than uninfected controls or patients with moderately severe malaria (Yeo et al. 2007). Endothelial dysfunction was also associated with elevated blood lactate and measures of haemolysis. Recovery of endothelial function in severe malaria was shown to be associated with recovery from hypoargininaemia and lactic acidosis and the median time to normal endothelial function was 49 h after the start of antimalarial therapy (Yeo et al. 2008b). Cell-free haemoglobin concentrations in patients with severe malaria were significantly higher than in those with moderately severe malaria or controls and were independently associated with lactate, endothelial activation and proinflammatory cytokinaemia (Yeo et al. 2009). In addition, plasma levels of asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of NOS, were shown to be elevated in adults with severe malaria. ADMA levels were associated with decreased exhaled NO and endothelial function and shown to be an independent predictor of mortality (Yeo et al. 2010a). In children with severe malaria, although ADMA levels were actually low, the L-Arg to ADMA ratio was even lower due to severe hypoargininaemia (Weinberg et al. 2014). Intravenous infusion of L-Arg increased the RH-PAT index and exhaled NO levels in patients with moderately severe malaria (Yeo et al. 2007) was shown to be safe (Yeo et al. 2008a) and to result in sustained plasma levels of L-Arg (Yeo et al. 2008d). However, in a recent randomised pilot study of intravenous L-Arg infusion in Indonesian adults with severe malaria, no effect on lactate clearance or endothelial NO bioavailability (RH-PAT index) was observed (Yeo et al. 2013). A clinical trial with inhaled NO in Ugandan children with CM is ongoing (Hawkes et al. 2011).

Endothelins in human CM - Endothelins are potent regulators of the vascular tone that also have mitogenic, apoptotic and immunomodulatory properties (Rubanyi & Polokoff 1994, Kedzierski & Yanagisawa 2001, Bagnato et al. 2011). These characteristics make endothelins important regulators of pulmonary and systemic arterial pressure, kidney function, angiogenesis, inflammation and adaptive immune function (Bagnato et al. 2011, Guo et al. 2012, Kohan et al. 2012). Three isoforms of endothelin have been identified (ET-1, ET-2 and ET-3), all are 21 aa peptides produced by the same metabolic pathways and act through the same receptors (Masaki 2004). However, each isoform is encoded by a different gene and has a different tissue expression (Saida et al. 2002). ET-1 is the best studied, the most abundant and is synthesised by vascular endothelial cells throughout the body as well as by a variety of other cells, including leukocytes, fibroblasts, vascular smooth muscle cells, neurons and astrocytes (Vignon-Zellweger et al. 2012). Endothelin precursors are called big-endothelins (big-ET-1, big-ET-2 and big-ET-3) which bind to the endothelin receptors with a much lower affinity (Hemsén et al. 1991). All three isoforms of endothelin are synthesised by the cleavage of their precursors by one of the three endothelin converting enzymes (ECE-1, ECE-2 and ECE-3). Once synthesised, endothelins can act through two transmembrane G protein-coupled receptors called endothelin receptor A (ETA) (Arai et al. 1990) and B (ETB) (Sakurai et al. 1990). ETA and ETB receptors can have synergetic or opposing effects depending on cell type, tissue type or physiological situation (Vignon-Zellweger et al. 2012). In addition, the distribution of ETA and ETB receptors varies in each vascular bed and they also can form homo and heterodimers, which increases the complexity of possible responses to endothelins (Caló et al. 1996, Evans & Walker 2008, Vignon-Zellweger et al. 2012). For example, ET-1 is classically considered a potent vasoconstrictor acting by increasing intracellular Ca2+ on vascular smooth muscle cells via ETA receptor binding (Wagner et al. 1992). However, ET-1 can also cause vasodilation by acting via ETB receptors on endothelial cells, which promotes NO release (Tsukahara et al. 1994). Hence, the actual knowledge suggests that the effects of endothelins may differ depending on the physiological or pathological situations (Bagnato et al. 2011, Vignon-Zellweger et al. 2012).

ET-1 has been implicated in the pathogenesis of a number of infectious, cardiovascular, cerebrovascular, renal, oncologic, chronic degenerative, inflammatory and auto-immune diseases (Edvinsson 2009, Bagnato et al. 2011, Guo et al. 2012, Kohan et al. 2012). Only two human studies analysing ET-1 during malaria infection are available to date (Wenisch et al. 1996, Dietmann et al. 2008). They showed that ET-1 and big-ET-1 were increased in the serum of patients with complicated *P. falciparum* malaria when compared with healthy controls. However, except for serum tumour necrosis factor-alpha levels and thrombocytopenia, these studies did not find a correlation between the serum levels of ET-1 or big-ET-1 and disease severity. In vitro studies showed that parasite derived lipid moieties present in the membrane of *P. falciparum*-infected RBCs and haemozoin (malaria pigment) can bind ET-1 (Basilico et al. 2010). The authors speculate that this unspecific binding could neutralise ET-1 activity by decreasing its bioavailability in the local microenvironment where pRBC sequester. However, the opposite could also be true. pRBC could be a source of ET-1, actually increasing its concentrations and bioavailability in sites of parasite sequestration contributing to the pathogenesis of falciparum complications such as CM. This could also explain the apparent decrease in serum ET-1 levels in patients with severe malaria when compared to uncomplicated malaria patients. These findings indicate that endothelins are involved in CM vascular dysfunction. However, the role of ET-1, ET-2 and ET-3 during human

CM needs to be further analysed. ET-3 is of particular interest as it is produced in the brain by endothelial cells and astrocytes and has been shown to cause brain ischaemia and inflammation (Ehrenreich et al. 1991, Filipovich & Fleisher-Berkovich 2008, Li et al. 2010).

Angiopoietin-Tie2 axis in human CM - Angiopoietin-Tie-2 axis dysfunction is involved in the pathogenesis of several vascular, infectious and oncologic diseases such as breast and lung cancer, hepatocellular carcinoma, atherosclerosis, pre-eclampsia, pulmonary hypertension, thrombosis, vasculitis and infections (Ahmed & Fujisawa 2011, Carrol 2011, David et al. 2013, Diaz-Sanchez et al. 2013, Fagiani & Christofori 2013). Angiopoietins (Ang) are ~70 kDa glycoproteins that critically regulate endothelial reactivity to angiogenic and inflammatory factors (Fiedler & Augustin 2006, Fagiani & Christofori 2013). Four distinct Ang have been described until now: Ang-1, Ang-2, Ang-3 and Ang-4. Ang-1, 3 and 4 are agonists of the receptor Tie-2 (acronym for tyrosine kinase with immunoglobulin and endothelial growth factor homology domains) (Davis et al. 1996, Fagiani & Christofori 2013). Ang-2 is a partial/weak agonist of Tie-2 that physiologically acts as a context antagonist of Ang-1 (Maisonpierre et al. 1997, Yuan et al. 2009). Tie-2 receptors are expressed mainly by endothelial cells, but they are also present in neurons, astroglial cells, pericytes and smooth muscle cells (Prapansilp et al. 2013). Ang-1 is incorporated in the extracellular matrix after its release by perivascular cells such as pericytes, vascular smooth muscle cells, platelets and fibroblasts (Davis et al. 1996, Xu & Yu 2001, Milner et al. 2009, Fagiani & Christofori 2013). Ang-1 mediated activation of Tie-2 receptors present on endothelial cells induces endothelial quiescence by inhibiting apoptosis, promoting NO synthesis, increasing the expression of endothelial cell tight junctions and reducing the expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin (Jones et al. 1999, 2003, Kim et al. 2001, Saharinen et al. 2008). Therefore, although not formally proven, it is widely believed that continuous Tie-2 activation by Ang-1 maintains vascular quiescence during healthy states (Fiedler & Augustin 2006, Fagiani & Christofori 2013). Ang-2 is synthesised by endothelial cells and stored inside their specialised granules, Weibel-Palade bodies, for rapid release upon stimulation by angiogenic or inflammatory mediators (Fiedler et al. 2004). As Ang-2 is a weaker partial agonist of Tie-2, in the presence of Ang-1, Ang-2 act mainly as a competitive antagonist inhibiting Tie-2 signalling by displacing Ang-1 from the receptor (Yuan et al. 2009). Hence, release of Ang-2 destabilises the endothelium via an autocrine mechanism increasing its reactivity to angiogenic and inflammatory stimuli, promoting vascular permeability and up-regulating ICAM-1 and VCAM-1 (Maisonpierre et al. 1997, Hammes et al. 2004, Fiedler et al. 2006). Taken together, these data indicate that the Ang-2/Ang-1 ratio determines the functional status of the vasculature (Fiedler & Augustin 2006). Physiologically, a low Ang-2/Ang-1 ratio controls vascular responsiveness and keeps endothelium homeostasis. However, following local or systemic endothelial-cell activation, Ang-2 is rapidly released from Weibel-Palade bodies increasing the Ang-2/Ang-1 ratio and the endothelial responsiveness. Ang-3 and Ang-4 are much less studied being orthologous proteins found in mouse and human, respectively (Fagiani & Christofori 2013). Both Ang-3 and Ang-4 are able to induce angiogenesis in vivo, but their role during inflammation is not understood (Lee et al. 2004).

It is well known that endothelial activation is associated with CM development (Grau & Craig 2012). As Ang are key regulators of endothelial responsiveness, deregulated levels of Ang-1 and Ang-2 systemically or locally in the brain could be involved in the pathogenesis of CM and other falciparum complications. Several human studies showed that decreased Ang-1 and increased Ang-2 levels and elevated Ang-2/Ang-1 ratio are associated with development of severe malaria (Yeo et al. 2008c, Conroy et al. 2009, 2010, 2012, Silver et al. 2010, Erdman et al. 2011, Lucchi et al. 2011, Prapansilp et al. 2013). These alterations in serum Ang-1 and Ang-2 correlate with disease severity, mortality and seem to be particularly more prominent in patients with CM than with other complications of malaria (Conroy et al. 2009, Lovegrove et al. 2009, Jain et al. 2011). In addition, serum levels of Ang-2 and the soluble version of Tie-2 receptor are associated with CM retinopathy in children (Conroy et al. 2010, 2012). Increased Ang-2 levels and Ang-2/Ang-1 ratio were shown to be better predictors of disease severity and mortality than Ang-1 in CM, indicating that the increase in endothelium responsiveness during CM may be due to increased Ang-2 rather than decreased Ang-1 levels (Conroy et al. 2009, 2010, 2012). Plasma levels of Ang-2, as well as of soluble ICAM1 and C-reactive protein, remain elevated, even days or weeks after antimalarial treatment, in children admitted with CM, indicating persistent inflammation and endothelial activation (Moxon et al. 2014).

Deregulation of the angiopoietin-Tie-2 axis, however, is not specific of CM. Increased serum Ang-2 levels are also present during *Plasmodium vivax* infection, a parasite species that classically does not cause severe disease (Yeo et al. 2010b, MacMullin et al. 2012). In addition, a recent study with Vietnamese adults failed to show differences in the brain expression of Ang-1, Ang-2 and Tie-2 on endothelial cells, astroglial cells or neurons between CM and non-CM controls presenting confirmed multi-organ dysfunction (Prapansilp et al. 2013). Although Ang-1 and Ang-2 expression in neurons was correlated with the incidence of haemorrhages, there was no correlation between the expression of Ang-1, Ang-2 and Tie-2 and pRBC sequestration in the brain. The authors conclude that although angiopoietin-Tie-2 axis dysfunction is related to severity and outcome, it is not a specific event in the brain during CM. These data suggest that disruption of endothelial quiescence seems to be necessary, but not sufficient to cause CM. Several approaches have been used to modulate the systemic levels of Ang-1, Ang-2 and the Ang-2/Ang-1 ratio in murine models of sepsis with some of them showing promising results such as the use of vasculotide, a synthetic agonist of Tie-2 (Fiedler & Augustin 2006). These approaches could be used in the murine model of CM to reveal the importance of each mediator during the course of the disease and potential adjunctive therapies for CM and severe malaria.

Experimental models of CM: a way forward to identify mechanisms of vascular dysfunction and evaluate therapeutic interventions - Studies of CM pathogenesis and therapeutics in humans are subjected to several constraints. Most studies on CM rely on *post-mortem* brain samples, which provide important clues for the understanding of its pathogenesis. Nevertheless, while this material provides evidence of the damage inflicted in fatal cases, it may be deficient in teaching its processes and mechanisms and cannot be compared with patients with more favourable outcomes (Looareesuwan et al. 2009). In addition, in most cases these samples are obtained from patients that received antimalarial treatment for hours or days and it is hard to measure how the intervention affected the observed alterations. Ocular *fundus* examination allows for the determination some aspects of pathology in live patients being particularly useful for improving the specificity of CM diagnosis (Lewallen et al. 1993, White et al. 2001). Also, it will possibly be valuable as readout for evaluation of potential novel therapeutic interventions. A few magnetic resonance studies have been performed and this is a promising approach to study CM pathogenesis in live patients, however this is an expensive resource largely unavailable in malaria endemic areas and its use may pose some ethical concerns (Looareesuwan et al. 1995, 2009, Potchen et al. 2012, Kampondeni et al. 2013).

There is no animal model that can perfectly mimic human CM. Non-human primate models of CM have been described, for instance a CM-like syndrome occurs in *Macaca mulatta* monkeys infected with *Plasmodium coatneyi* (de Souza & Riley 2002). However, this and other primate models are largely unavailable for systematic studies and therefore lack extensive characterisation. The best studied animal model for CM is the infection of susceptible mouse strains by the rodent parasite *Plasmodium berghei* ANKA, which induces a fatal neurological syndrome known as experimental CM (ECM) with clinical signs such as ataxia, convulsions, limb paralysis and coma (de Souza et al. 2010). The brain of mice with ECM show vascular plugging mainly by adherent leukocytes, diffuse microhaemorrhages, breakdown of the blood brain barrier (BBB) and increased expression of cell adhesion molecules such as ICAM1 (Martins et al. 2009, Cabrales et al. 2011, Zanini et al. 2011). The relevance of this animal model for the human disease has been heatedly debated recently (Carvalho 2010, Hunt et al. 2010, Rénia et al. 2010, Riley et al. 2010, Stevenson et al. 2010, White et al. 2010, Langhorne et al. 2011, Craig et al. 2012). The main criticism lies on the fact that in human CM the key histological finding is the occlusion of brain post-capillary venules by *P. falciparum* pRBC, whereas in ECM post-capillary venular occlusion is mediated mostly by adherent activated leukocytes. However, leukocytes are found in the brain of CM patients (Pongponratn et al. 2003) and pRBC accumulate in the brain of mice with ECM (Amante et al. 2010, Baptista et al. 2010, Franke-Fayard et al. 2010, Riley et al. 2010), although the pRBC/leukocyte proportion is different, being higher in human CM. Markers of brain inflammation such as high levels of ICAM-1 expression in the endothelium is observed in both conditions and necessary for sequestration. It has been claimed that adjunctive therapies in ECM are highly effective, contrary to what has been observed in human CM, but in the murine studies the therapies cited as "adjunctive" do not fit this definition (Carvalho 2010). Finally, a large amount of evidence showing several similarities between human and murine CM has not been considered (van der Heyde et al. 2006, de Souza et al. 2010, Hunt et al. 2010).

The murine CM model has been instrumental in studying the role of vascular dysfunction in the pathogenesis of CM. Several aspects of vascular pathology have been shown to mimic very closely the findings in the human disease. Contrary to initial hypotheses (Clark et al. 1991), studies in this model showed that NO deficiency rather than excess NO production was involved in ECM pathogenesis. Administration of NOS inhibitors such as L-N-monomethyl arginine and N omega-nitro-L-Arg or aminoguanidine L-NAME, even intracranially, did not modify disease outcome (Asensio et al. 1993, Kremsner et al. 1993, Favre et al. 1999). Mice deficient in either eNOS or iNOS were as susceptible to ECM as wild-type mice and wild-type mice with ECM were shown to display low NO bioavailability, with decreased levels of plasma and brain nitrite/nitrate (stable products of NO), decreased levels of L-Arg (the substrate for NO synthesis by NOS) and decreased brain cGMP levels (the downstream product of NO-stimulated soluble sGC) (Gramaglia et al. 2006). Mice with ECM also showed high levels of plasma cell-free haemoglobin, which was shown to scavenge NO and therefore being a major responsible for NO deficiency. Finally, administration of exogenous NO by means of the NO-donor molecule DPTA-NO or by inhaled NO gas to *P. berghei*-infected mice was shown to prevent ECM, decreasing inflammatory and endothelial activation markers and preventing BBB breakdown (Gramaglia et al. 2006, Serghides et al. 2011).

In ECM, mRNA expression of ET-1, ECE, ETA and ETB are markedly increased during infection (Machado et al. 2006, Lovegrove et al. 2007). In addition, the pharmacologic blockade of ETA receptors decreased the incidence of haemorrhages in the brain and seemed to increase survival when used in conjunction with artemether to treat mice with CM (Dai et al. 2012). High ET-1 levels could be responsible for the intense vasoconstriction and ischaemia that occur in the brain of mice with ECM (Kennan et al. 2005, Machado et al. 2006, Cabrales et al. 2010). Elevated concentrations of ET-1 also can lead to decreased NO production (Ramzy et al. 2006), increased synthesis of pro-inflammatory mediators by monocytes (Simonson 1993, Browatzki et al. 2005), increased expression of leukocyte adhesion molecules by brain endothelial cells (McCarron et al. 1993), dysfunction of the platelet anti-aggregating properties of neutrophils (Gómez-Garre et al. 1992) and disruption of the integrity of the BBB (Matsuo et al. 2001, Reijerkerk et al. 2012), alterations that are present in the brain of mice with CM (Gay et al. 2012, Grau & Craig 2012, Rénia et al. 2012).

Few studies have explored the role of Ang in ECM. Improved survival in ECM was associated with decreased endothelial activation (increased Ang-1, decreased Ang-2 and soluble ICAM-1) upon preventative

treatment of PbA-infected mice with inhaled NO, neuregulin-1 or with compounds that modulate the levels of sphingosine 1-phosphate or in mice deficient for the C5a receptor (Finney et al. 2011, Serghides et al. 2011, Kim et al. 2014, Solomon et al. 2014). Furthermore, PbA-infected mice treated early during infection with artesunate in combination with either inhaled NO or the peroxisome proliferator-activated receptor γ agonist rosiglitazone showed improved survival and cognitive function than mice treated with artesunate plus saline (Serghides et al. 2011, 2014). Treatment with rosiglitazone resulted in increased plasma levels and brain expression of Ang-1.

We developed an intravital microscopy methodology for studying in detail the features of cerebral vascular dysfunction and NO deficiency in CM pathogenesis and therapeutics using this mouse model (Cabrales & Carvalho 2010). For this, a chronic closed cranial window is surgically implanted in the mouse's parietal bone allowing access to the pial microcirculation over extended periods of time. *P. berghei* ANKA-infected mice showed marked decreases in cerebral blood flow at the time of ECM manifestation, due to reduced RBC velocities and to widespread cerebral vasoconstriction, similar to the vasospasm phenomenon observed after subarachnoid haemorrhages (Cabrales et al. 2010). The decrease in blood flow associated with decreased haematocrit lead to hypoxia and acidosis, with a marked decrease in oxygen tension in brain arterioles, venules and the perivascular tissue in mice with ECM (Cabrales et al. 2013). Administration of the NO-donor DPTA-NO was able to ameliorate, but not completely prevent the microcirculatory complications of ECM (Cabrales et al. 2011, Zanini et al. 2011). DPTA-NO improved cerebral blood flow mainly by preventing vasoconstriction, with a beneficial effect more pronounced in smaller than in larger arterioles. DPTA-NO also decreased brain vascular inflammation by decreasing the expression of endothelial cell adhesion molecules such as ICAM1 and P-selectin, resulting in decreased leukocyte and platelet adherence and in decreased vascular resistance to blood flow. DPTA-NO treatment also decreased vascular leakage and the incidence of microhaemorrhages. DPTA-NO is a potent NO-donor and in the dose used (1 mg/mouse) it releases large amounts of NO in a short period of time resulting in marked hypotension in normal mice (Gramaglia et al. 2006). Therefore, it is useful as a research tool, but has limited potential as a therapeutic intervention. Inhaled NO is an alternative with therapeutic potential and shown to be effective in both preventing ECM as well as an adjunctive therapy with artesunate (Gramaglia et al. 2006, Serghides et al. 2011). S-nitrosylated glutathione (GSNO) is a physiological NO-donor and NO reservoir and might have better therapeutic potential. GSNO indeed largely prevented ECM incidence (Zanini et al. 2012). At higher dose (1 mg/mouse), it even affected parasite growth decreasing parasitaemia and completely preventing ECM incidence. At lower doses it largely prevented ECM incidence without affecting parasitaemia. The higher dose, however, showed a hypotensive effect similar to DPTA-NO, which was not observed with the lower doses. Interestingly, mice with ECM showed marked depletion of brain glutathione levels, indicating increased oxidative stress, but GSNO administration had little or no effect on restoring proper glutathione levels. Other attempted approaches to increase NO bioavailability such as L-Arg and tetrahydrobiopterin (BH4) supplementation with or without arginase inhibition or nitrite administration did not result in protection from ECM, at least in the doses and schemes used (Martins et al. 2012).The phosphodiesterase-5 inhibitor sildenafil by itself did not protect from ECM either, however in combination with a lower dose of DPTA-NO (0.1 mg/mouse, also shown to be ineffective in preventing ECM) was able to increase survival of PbA-infected mice with milder effects on blood pressure than the higher dose of DPTA-NO (1 mg/mouse).

Interventions given early during infection, as the approaches described above, are informative about mechanisms of disease and provide evidence on the role of NO and vascular dysfunction in the pathogenesis of the neurological syndrome. However, from these experiments it is not possible to derive information about the therapeutic potential of interventions. This information can be achieved only by testing interventions in late-stage disease in combination with antimalarial drugs such as artemisinin derivatives, the mainstay treatment for CM in humans. A model to test adjuvant therapies for ECM was described in which mice with late-stage ECM, determined by well-defined clinical parameters such as motor scores and degree of hypothermia, were treated with different antimalarial drugs, showing that artesunate and artemether were the most effective in rescuing animals from death (Clemmer et al. 2011). This model has been used to evaluate the efficacy of adjuvant therapies. Administration of the dihydropiridine calcium channel blocker nimodipine, the drug used to prevent vasospasm in sub-arachnoid haemorrhage patients (Keyrouz & Diringer 2007), in combination with artemether improved cerebral blood flow and increased survival of mice with late-stage ECM when compared to artemether alone (Cabrales et al. 2010). An improved nimodipine delivery system using subcutaneous miniosmotic pumps resulted in efficacy in increasing survival while avoiding deleterious effects on cardiovascular parameters such as heart rate and blood pressure (Martins et al. 2013). Similar findings were obtained with glyceryl trinitrate (GTN). GTN is an NO-donor that requires enzymatic transformation in order to release NO. GTN applied to mice with late-stage ECM in the form of transdermal patches increased survival and, despite the high doses needed for its efficacy in rescuing ECM mice, it did not further decrease blood pressure in hypotensive mice. Importantly, transdermal GTN reversed cerebrovascular constriction soon after administration and the effect lasted for the time the patch was applied (24 h), whereas this effect was not observed in mice receiving artemether only (Orjuela-Sánchez et al. 2013). Similarly, inhaled NO in combination with artesunate improved survival of PbA-infected mice compared to artesunate alone (Serghides et al. 2011). These data show that reversing hypoperfusion by tackling vasoconstriction is a life-saving intervention in ECM and therefore holds potential for translation to the clinical setting.

While directly providing exogenous NO or other vasodilators such as calcium channel blockers constitute a potentially translatable strategy to restore cerebral perfusion during CM, a better understanding of the mechanisms of vascular dysfunction may reveal improved targets for intervention. We have developed a new cranial window procedure allowing implantation of a superfusion chamber to study cerebrovascular functionality in mice with ECM (Ong et al. 2013a). Using this system, we found that eNOS and nNOS dysfunction are at least partially responsible for the impaired cerebrovascular responses to endothelium-dependent and neuron-dependent vasodilation stimuli (Ong et al. 2013b). Contrary to normal animals, pial arterioles of mice with ECM did not respond (dilate) to acetylcholine (ACh) (which dilates arterioles via NO production by eNOS) or to N-methyl-D-aspartate (NMDA) (which dilates arterioles via NO production by nNOS). ACh and NMDA induced generation of NO (measured as nitrite/nitrate metabolites) during pial superfusion in normal animals, which was abolished by the NOS inhibitor NG-methyl-L-Arg, but no nitrite/nitrate production was observed upon ACh or NMDA superfusion in mice with ECM. Arteriolar responses were partially restored by adding BH4, an essential cofactor for NOS, to the superfusate, suggesting that BH4 shortage may be implicated in NOS dysfunction. C57BL/6 mice infected with *P. berghei* NK65, which does not induce ECM, showed preserved pial arteriolar responses to ACh and NMDA, indicating that eNOS and nNOS dysfunction is specific for the ECM syndrome. Another important aspect of endothelial dysfunction in ECM is related to the vessel wall shear stress. Mice with ECM show increased expression of iNOS and eNOS. However, total NOS activity is decreased and vascular reactivity to vasodilators is impaired, as described above. Although eNOS expression is increased, eNOS phosphorylation is decreased, indicating deficient stimulation of the endothelium by mechanical forces related to blood flow. Indeed, wall shear stress is decreased in ECM (Ong et al. 2013b). In addition to resulting in deficient eNOS activation, decreased shear stress favours pRBC and leukocyte adherence to the endothelium and prevents their washout. In patients that die of CM, persistent adherence of pRBC containing dead parasites or pRBC ghosts contributes to persistent vascular occlusion and pathology (Pongponratn et al. 2003).

Cerebral hypoperfusion in CM is mainly a consequence of two factors: vascular occlusion and vasoconstriction. These two factors greatly decrease the amount of blood that flows to the brain during CM. Interestingly, although artemether rapidly decreases parasitaemia and vascular occlusion in the first 24 h of treatment of mice with late-stage ECM (Clemmer et al. 2011), it has no effect on arteriolar constriction (Orjuela-Sánchez et al. 2013). Being more persistent than vascular obstruction, vasoconstriction represents a fundamental obstacle to restore perfusion in ECM. Reversing constriction is therefore a key intervention to preserve life in moribund individuals. By reversing constriction, blood flow is increased and oxygen delivery is improved, reversing hypoxia and its consequences such as acidosis. With normal vascular tonus restored, resistance to blood flow decreases and normal shear stress helps washing out adhered cells.

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