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Whole blood transfusion as adjunctive therapy for experimental cerebral malaria

SABA GUL

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Whole blood transfusion as adjunctive therapy for experimental cerebral malaria

Thesis presented to Instituto
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for obtaining the title of Doctor of Science

Supervisor: Prof. Dr. Leonardo José de Moura Carvalho

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Whole blood transfusion as adjunctive therapy for experimental cerebral malaria

ABSTRACT

PHD THESIS IN BIOLOGIA PARASITÁRIA

Saba Gul

Some of the main pathological features observed in both human and experimental cerebral malaria (ECM) are endothelial dysfunction and changes in blood components. Blood transfusion has been routinely used in patients with severe malarial anemia and can also benefit comatose and acidotic malaria patients. In the present study, *Plasmodium berghei*-infected mice were first transfused intraperitoneally with 200 µL of whole blood along with 20 mg/kg of artemether. ECM mice showed severe thrombocytopenia and decreases in hematocrit. Artemether treatment markedly aggravated anemia within 24 h. Whole blood administration significantly prevented further drop in hematocrit and partially restored the platelet count. Increased levels of plasma angiopoietin-2 (Ang-2) remained high 24 h after artemether treatment but returned to normal levels 24 h after blood transfusion, indicating reversal to quiescence. Ang-1 was depleted in ECM mice and levels were not restored by any treatment. Blood transfusion prevented the aggravation of the breakdown of the blood-brain barrier after artemether treatment and decreased spleen congestion without affecting splenic lymphocyte populations. Critically, intraperitoneal blood transfusion resulted in markedly improved survival of mice with ECM (75.9% compared to 50.9% receiving artemether only). More significantly, changing the route of transfusion from intraperitoneally to intravenously led to further improvement of all mentioned parameters: survival rate increased to 90% and recoveries of hematocrit, platelet counts, angiopoietins levels (Ang-1, Ang-2 and Ang-2/ Ang-1) and blood-brain barrier integrity were all more pronounced. These findings indicate that whole blood transfusion can be an effective adjuvant therapy for cerebral malaria.

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Transfusão de sangue total como terapia adjuvante para malária cerebral experimental

RESUMO

TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Saba Gul

Algumas das principais características patológicas observadas na malária cerebral (MC) humana e experimental (MCE) são disfunção endotelial e alterações nos componentes do sangue. A transfusão de sangue tem sido usada rotineiramente em pacientes com anemia malárica grave e também pode beneficiar pacientes com malária acidótica e em coma. No presente estudo, camundongos infectados com *Plasmodium berghei* foram primeiramente transfundidos por via intraperitoneal com 200 µL de sangue total junto com 20 mg/kg de artemeter. Os camundongos com MCE apresentaram trombocitopenia grave e diminuição do hematócrito. O tratamento com artemether agravou acentuadamente a anemia em 24 horas. A administração de sangue total preveniu significativamente a queda do hematócrito e restaurou parcialmente a contagem de plaquetas. Níveis aumentados de angiopoietina-2 (Ang-2) plasmática permaneceram elevados 24 horas após o tratamento com artemeter, mas retornaram aos níveis normais 24 horas após a transfusão de sangue, indicando reversão para quiescência. Ang-1 foi depletada em camundongos com MCE e os níveis não foram restaurados por nenhum tratamento. A transfusão de sangue evitou o agravamento da quebra da barreira hematoencefálica após o tratamento com artemether e diminuiu a congestão do baço sem afetar as populações de linfócitos esplênicos. Criticamente, a transfusão de sangue intraperitoneal resultou em uma sobrevida acentuadamente melhorada de camundongos com ECM (75,9% em comparação com 50,9% recebendo apenas artemeter). De maneira muito significativa, a mudança da via de transfusão de intraperitoneal para intravenosa levou a melhorias adicionais em todos os parâmetros analisados: a taxa de sobrevivência aumentou para 90% e as recuperações do hematócrito, contagem de plaquetas, níveis de angiopoietinas (Ang-1, Ang-2 and Ang-2/ Ang-1) e integridade da barreira hematoencefálica foram todas mais marcantes. Essas descobertas indicam que a transfusão de sangue total pode ser uma terapia adjuvante eficaz para a malária cerebral.

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LIST OF ACRONYMS AND ABBREVIATIONS

Ang-1	Angiopoeitin-1
Ang-2	Angiopoeitin-2
ARM	Artemether
BBB	Blood-brain barrier
Blood	Blood
CM	Cerebral malaria
CSF	Cerebrospinal fluid
CSP	Circumsporozoite protein
CT	Computational tomography
EC	Endothelial cell
ECM	Experimental cerebral malaria
EEG	Electroencephalographic
eNOS	Endothelial Nitric Oxide Synthase
EPCR	Endothelial protein C receptor
FDG	Fluorodeoxyglucose
GPI	Glycosylphosphatidylinositols
Hb	Hemoglobin
HBECs	Human brain microvasculature endothelial cells
Hp	Haptoglobin
ICAM-1	Intercellular adhesion molecule-1
IL-6	Interleukin-6
KAHRP	Knob-associated histidine-rich protein
LFA-1	Lymphocyte function-associated antigen 1
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
P	*Plasmodium*
PAMPs	Pathogen-associated molecular patterns
PbA	*Plasmodium berghei* ANKA
PET	Positron emission tomography
PF4	Platelet factor 4
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
pRBCs	Parasitized red blood cells
RBCs	Red blood cells
RNI	Reactive nitrogen intermediates
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion protein 1
VWF	Von Willebrand factor
WHO	World Health Organization
WPB	Weibel-Palade bodies

LIST OF FIGURES

1 Background

1.1 Malaria epidemiology

Malaria is still a disease of public health concern, with high morbidity and mortality predominantly in children under the age of five. In 2019, the World Health Organization (WHO) estimated that there were 229 million cases and 409,000 deaths due to malaria globally. The WHO African region accounts for a disproportionately high share of the global burden, 94% of malaria cases in 2019 alone [1,2] . Nearly all malaria cases in the WHO African region are caused by *Plasmodium falciparum*. In Brazil, malaria cases have fallen more than 75% since a peak in 2005, with 135,000 cases reported in 2020. The vast majority of malaria cases (99.7%) occurs in the Amazon region, and 80% of those occur in only 41 municipalities (Fig. 1) [3].

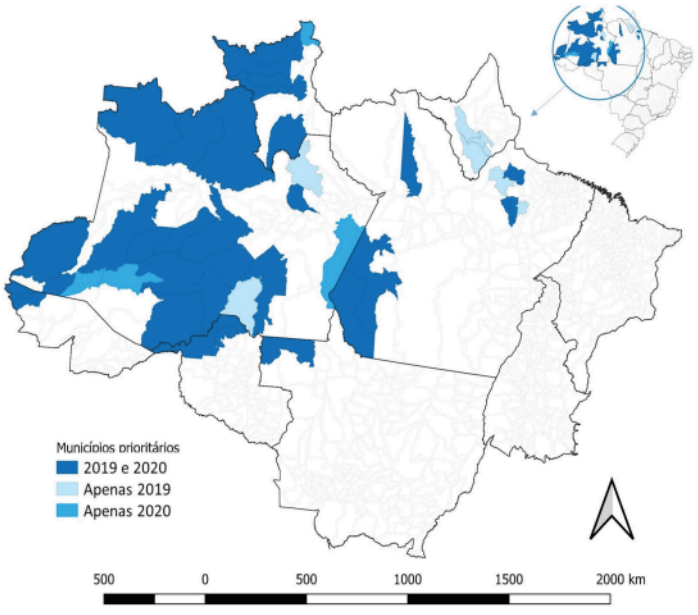

Figure 1 - Map of malaria priority municipalities in Brazil in 2019 and January to June 2020. Nearly 90% of malaria cases in Brazil are caused by *Plasmodium vivax*, which is much less lethal than *P. falciparum*. Due to this fact and also because in Brazil malaria diagnosis and treatment are easily and widely available, with most of cases being detected and treated in the first 48 hours of symptoms, very few deaths by malaria are reported every year in the country. Indeed, in 2020, Brazil registered 30 deaths by malaria.

1.2 Malaria: general aspects

Long before the identification of a causative agent, the periodic symptoms of malaria had already been recognized. The low-lying marshlands and swamps were first suggested as an agent of causing malaria by exuding “bad air”, and the theory led to derived the word “malaria” [4]. Based on microscopic observation of infected blood samples, Alphonse Laveran in 1880 was the first to demonstrate that malaria is caused by a parasite. Over the course of the 1880’s and 1890’s, the biology and transmission malaria parasite were steadily uncovered. Transmission by mosquito vectors among avian and human hosts were independently suggested by Ross and Grassi, respectively [5,6].

The parasites of malaria belong to phylum Apicomplexa, family *Plasmodiidae* and genus *Plasmodium*. Species that cause malaria in humans are *P. falciparum, P. vivax, P. ovale, P. malariae* and most recent addition to the list is primate malaria parasite *P. knowlesi and P. simium* [7–9]. Malaria is transmitted by female *Anopheles* mosquitoes during the blood meal. This mosquito comes from genus *Anopheles*, order Diptera, family Culicidae. More than 400 species of anopheline has been known and, of these, 70 are vectors of malaria [10]. The most prevalent species in the African continent is *An. gambiae*, whereas in Brazil *An. darlingi*, *An. albitarsis* and *An. aquasalis* are most prevalent [10,11].

Transmission begins when an infected mosquito inoculates sporozoites into subcutaneous tissue of the vertebrate host, the migration and penetration of sporozoites into the blood vessels to enter circulation start from this point. Circulating sporozoites make their way to liver, and the circumsporozoite protein (CSP) expressed on the surface of sporozoites facilitates them to recognize heparan sulfate proteoglycans expressed on the surface of hepatocytes [12]. During liver-stage (exoerythrocytic phase), sporozoites replicate and differentiate into pre-erythrocytic trophozoites that multiply giving rise to tissue schizonts, subsequently originating tissue merozoites. This asexual phase is called schizogony [13,14]. While the human host remains

asymptomatic for at least 1-2 weeks, at this stage, infections caused by *P. vivax* and *P. ovale* rest in the liver in a latent form known as hypnozoites and are eventually responsible for some of the late relapses [13,15].

Later, the hepatocytes rupture and release asexual merozoites (10,000-30,000/infected hepatocyte) into the blood streams, and the blood stage begins when these merozoites invade erythrocytes. Maturation of blood-stage parasites is described by a progression of intraerythrocytic forms identified as rings, trophozoites and schizonts. Forty-eight hours post-invasion, daughter merozoites are released when schizont ruptures the Red blood cells (RBCs) and continue to infect further RBCs. Infected individuals during blood-stage infection experience episodes of fever along with headache, fatigue and nausea, and eventually severe cases, which may progress to life-threatening complications. If a person acquired sufficient immunity like in malaria endemic areas, symptoms may be mild or even absent despite significant amount of blood-stage parasitemia. A small number of asexual parasites differentiate into gametocytes, which are taken up in blood meal of a feeding *Anopheles* mosquito [12]. Another stage of *Plasmodium* life cycle begins when gametocytes traverse epithelial lining of mosquito midgut. Male and female gametocytes recombine to form ookinete. The ookinete then breaches the intestinal epithelium of the anopheline and transforms itself into an oocyst. After the sporogonic phase of 8–15 days, the oocyst bursts and releases sporozoites into the body cavity of the mosquito. The sporozoites migrate to the mosquito's salivary glands via mosquito's hemolymph, completing the life cycle [16]. A summary of the biological cycle of *Plasmodium falciparum* is shown in figure 1.

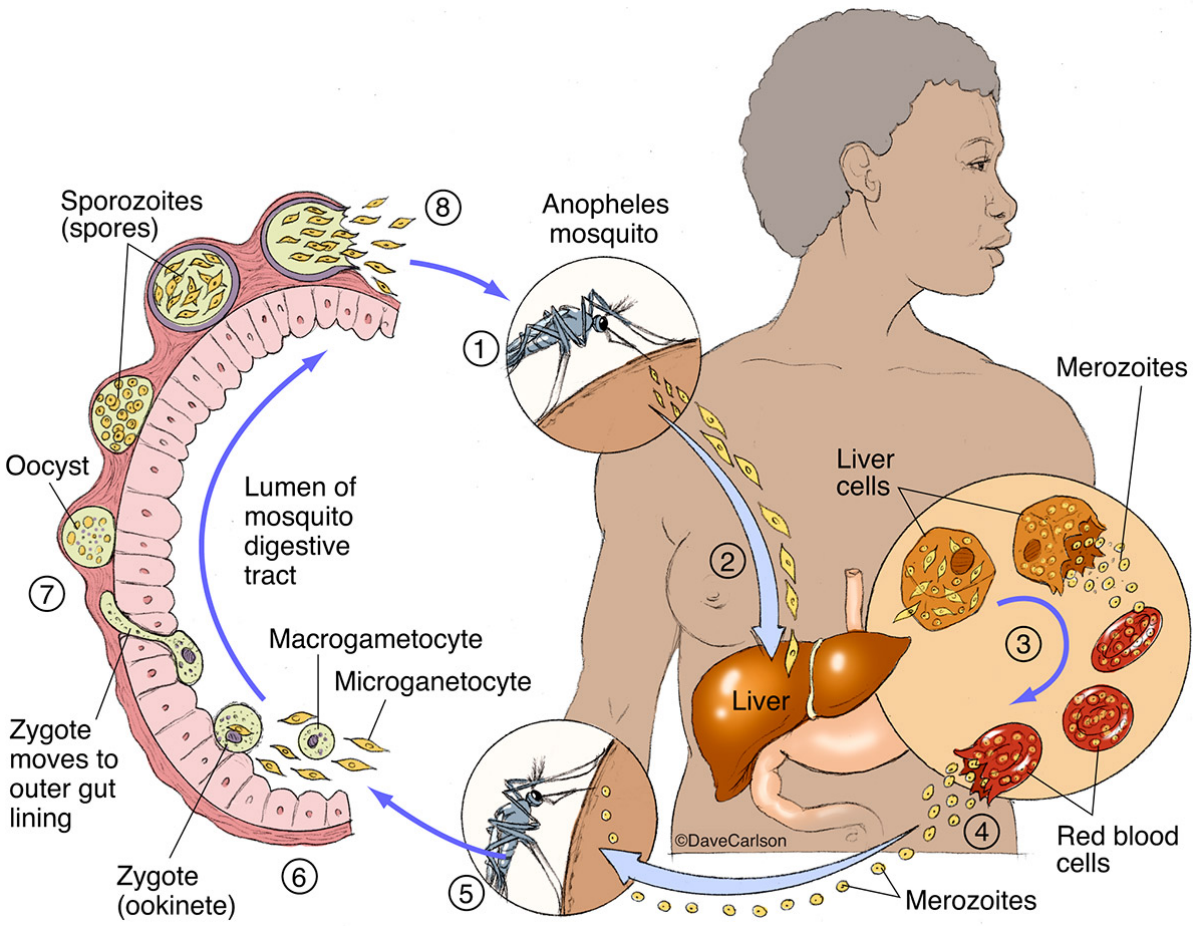

Figure 2 - Biological life cycle of *Plasmodium falciparum*. (1) During blood feed on human host, infected female mosquitoes of the genus *Anopheles* inject sporozoite in the dermis. **(2)** Upon entering to vasculature, sporozoite are transported to the liver to invade hepatocytes, sporozoites replicates and differentiate into trophozoites, schizonts and end up releasing thousands of merozoites into the bloodstream. **(3)** In the bloodstream, merozoites encounter erythrocytes and asexual cycle of schizogony begins. The infected erythrocytes rupture and release new merozoites into the bloodstream. **(4)** Blood stage continues since merozoites infect new red blood cells. Some of the trophozoites differentiate into female or male gametocytes. **(5)** Vector phase start when mosquitoes ingest these gametocytes while performing blood meal on an infected individual. **(6)** The gametocytes reach the vector midgut and give rise to the female gametes (macrogametes) and male gametes (microgametes). Macro and microgametes mate to form a zygote, which then transforms into ookinete and moves to midgut epithelium lining. **(7)** The ookinetes invade the midgut wall of the mosquito where they develop into oocysts, which then ruptures and releases the sporozoites in the vector's hemocele. Motile sporozoites pass to the salivary glands and remain there until the mosquito makes a new blood meal on the human host [17].

Microscopic observation of stained blood smear is the standard diagnosis method of malaria infection. For the detection of *P. falciparum* histidine rich protein 2 and lactate dehydrogenase, rapid diagnostic tests are now available. To achieve higher sensitivity polymerase chain reaction are used for testing parasite DNA or RNA.

1.3 Cerebral malaria

Approximately one to two percent of *P. falciparum* infected individuals develop severe malaria. But because malaria is so prevalent, this low percentage translates in a large absolute number, between 1-2 million severe malaria cases every year. Cerebral malaria (CM) is one of the most severe and frequently overlapping complication, causing majority of malarial morbidity and mortality [18–21]. Development of CM is more pronounced in children under 5 years of age. WHO defined CM as an acute, diffuse encephalopathy, characterized by a persistent coma after seizure, in individuals infected with *Plasmodium*, excluding other causes of encephalopathy [19].

The clinical features of CM include ataxia, seizures, hemiplegia, coma and, eventually, death. The treatment recommended by the WHO is intravenous artesunate, but mortality still exceeds 15-25% even after treatment with antimalarial drugs [20,22]. Most of deaths ensue just hours following admission to a hospital or clinic [23–25]. In addition, it is estimated that 5-30% of children who survive cerebral malaria have neurological sequelae [26,27]. It has been described that clinical features of cerebral malaria notably differ among children and adults. Reasons pertaining to these differences are not entirely clear but may be associated with the immunity of the patients [28].

Cerebral Malaria is a multifactorial neurological syndrome; however, its defined pathogenesis remains poorly understood. In a large cohort study, children (47.6%) under age 14 with CM presented different levels of gross neurological complications such as impaired consciousness, seizures, agitation, retinopathy, epilepticus and coma [29]. An autopsy study described that 24% of patients exhibiting clinical symptoms of CM died of other reasons [30]. Seizures are often followed by the onset of coma in children. With neurological involvement, 80% of children present seizures [31]. Seizures did not seem to be associated with fever, electrolyte disorders and consumption of anti-malarial drugs. The cause behind occurrence of seizures is still unclear but it is suggested that parasite sequestration in the brain, inflammatory mediators or metabolic factors might be responsible. An electroencephalographic (EEG) identified focal origins but seizures appear generalized in adult and pediatric patients [32]. Increased brain volume has been noticed in roughly half of CM affected children [33]. An elevated intracranial pressure was observed in 75–80 % of African children with

cerebral malaria [34]. Abnormal posturing is more common in young children and is associated with raised intracranial pressure or hypoglycemia [35].

Neurological deficits are higher in children than in adults, and ranges from 6 to 29 % at the time of discharge [36]. Children surviving CM episodes are also at the risk of persistent neurological sequelae [37,38]. Such patients may acquire several adverse neurological impairments including ataxia, paresis (hemiparesis and quadriparesis), persistent cortical blindness, epilepsy, deafness, disruptive behavior, cognitive impairments and language deficits. Most of the forementioned deficits resolve within 6 months after discharge. However, some persistent neurologic and cognitive impairments in survivors continues up to 9 years [39].

Plasmodium falciparum malaria is characterized by sequestration of late blood-stage parasites (trophozoites and schizonts) in cerebral vasculature [30,40,41]. Parasitized red blood cells (pRBCs) also adhere to the walls of heart, liver and skin but the brain typically displays the highest density of cytoadherence [42]. Cytoadherence is unique to *P. falciparum*, allowing parasite to reach higher parasitic density. Among human malarias, *P. falciparum* causes higher rates of severe disease than other *Plasmodium* species.

A class of parasite expressed protein ligands collectively referred as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) mediates the cytoadherence of pRBCs. Between 18 to 24 hours post invasion, at trophozoite stage, PfEMP1 proteins are displayed on the surface of host erythrocyte where they form dense knob-like structures [43,44]. PfEMP1 binds to host receptors of which intercellular adhesion molecule-1 (ICAM-1) is the most important and whose expression is upregulated in areas next to sequestered parasites. The sequestered parasite mass is further increased when adherent erythrocytes clump with other pRBCs, form rosettes with non-parasitized erythrocytes. In severe disease, pRBCs receptor adhesion leads to occlusion of the vessels[45,46]. When this occurs in the brain, it can lead to sudden, rapid swelling of the brain, and death can result from respiratory failure due to brain stem herniation. Platelet-mediated clumping is also well-known phenomenon in cytoadherence. Furthermore, pRBCs deform and confront difficulties to pass through the microvasculature [47]. Studies reported endothelial protein C receptor (EPCR) as a

key ligand facilitating cytoadherence in severe malaria. Parasites that express “var” genes encoding PfEMP1 containing EPCR-binding domains have shown a solid association with the progress of severe malaria, including Cerebral Malaria [22,48–50].

Autopsy studies of patients that died with CM revealed the sequestration of parasitized red blood cells in the vascular endothelium [51,52]. Sequestration impairs perfusion and may aggravate coma through hypoxia. Therefore, hypoxia and inadequate tissue perfusion may be major pathophysiological events. Although a critical reduction in metabolite supply (oxygen and glucose) may occur, in most children significant neural tissue necrosis is unlikely since, with specific antimalarial treatment, coma is rapidly reversible. However, in the presence of increased metabolic demand such as during seizures and fever, the risk of neural injury is higher and may be worse if the patient is hypoglycemic [53], or if blood flow is further compromised by intracranial hypertension [34].

Acute endothelial activation is followed by sequestration of pRBCs, leukocytes and platelets in capillaries and postcapillary venules of the brain [54]. Activated endothelium is well illustrated by surface expression of various adhesion molecules (e.g., ICAM-1, Vascular cell adhesion protein 1 (VCAM-1), P-selectin, E- selectin) [54], exocytosis of Weibel-Palade bodies (WPB) [55], breakdown of tight junctions [56] and by several other biomarkers such as endothelial microparticles [57], angiopoeitin-1 and 2 (Ang-1 and Ang-2), vascular endothelial growth factor (VEGF). An in vitro laminar flow model detected pRBCs adhesion to activated endothelium via platelet-decorated ultra large von Willebrand factor (VWF) strings. It was proposed as one of the mechanisms responsible for pRBCs sequestration and endothelium activation during cerebral malaria [58].

Ang-1 and Ang-2 are ligands of the Tie-2 receptor, expressed on endothelial cells. During normal physiological conditions, Ang-1 promotes endothelial cell quiescence and survival. Ang-1 is constitutively produced and excreted into the blood by pericytes and smooth muscle cells and it is also stored in platelets. Ang-1 binds to the Tie-2 receptor, thereby acting as an agonist whereas Ang-2 acts as a functional antagonist [59]. Ang-2 is a vessel destabilizing molecule, Ang-2-Tie-2 interaction results in the blocking of the protective, anti-inflammatory and anti-apoptotic effect of Ang-1.

Therefore, Ang-2 binding to Tie-2 receptors further facilitates endothelial activation and increases vascular permeability [60].

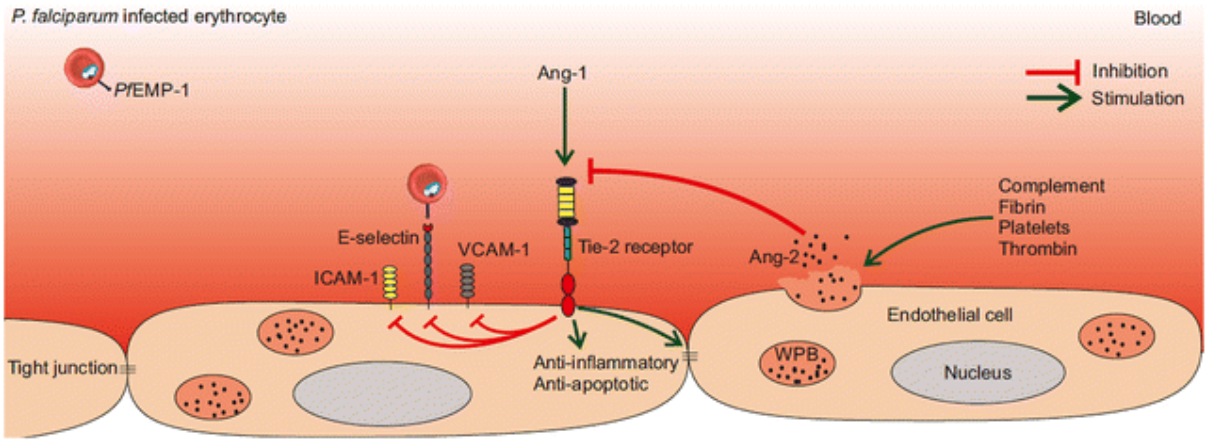

Figure 3 - Schematic overview of function and localisation of Ang-1, Ang-2 and the Tie-2 receptor. Ang-2 is pre-stored in the Weibel–Palade bodies in endothelial cells and is released upon endothelial cell activation. Ang-2 replaces Ang-1 by binding the Tie-2 receptor, preventing its activation and thereby blocking the anti-inflammatory, anti-apoptotic and tight-junction supporting effects of Ang-1. Ang-1, Ang-2, ICAM-1, E-selectin, VCAM-1, adhesion molecules, PfEMP-1, WPB. [61]

Increased levels of pro and anti-inflammatory cytokines have been noticed in patients with CM [54,62]. Malaria pathogen-associated molecular patterns (PAMPs) are *Plasmodium*-derived molecules with ability to induce cytokine responses in vitro. One of the candidate malaria PAMPs are glycosylphosphatidylinositols (GPI). GPI serve as membrane anchors for certain cell surface proteins and are present in all eukaryotic cells, although Plasmodium GPI are structurally different from human GPI [63]. In early pre symptomatic stages of plasmodial infection, innate immunity genes such as Toll-like receptors (TLRs), and many proinflammatory cytokines are already upregulated [64]. The increase of proinflammatory cytokine release is responsible for the overexpression of adhesion molecules on brain endothelial cells causing the development of cerebral malaria [65]. While a more recent study reported that neither plasma nor cerebrospinal fluid (CSF), Tumor necrosis factor (TNF) concentration were indicative of CM-associated mortality, yet elevated levels of TNF in CSF of pediatric CM cases were associated with long-term neurologic and cognitive deficits [66].

Nitric oxide (NO) is a neutral gaseous molecule biosynthesized from L-arginine, oxygen and Nicotinamide adenine dinucleotide phosphate (NADPH) by members of

the nitric oxide synthase (NOS) family. It is a key regulator of cardiovascular and endothelial cell function with anti-inflammatory and anti-adherent properties. In vitro studies exhibited the inhibitory effect of reactive nitrogen intermediates (RNI) on growth and survival of *P. falciparum* [67]. Reduced NO bioavailability has been significantly associated with increased levels of plasma angiopoietin-2, an autocrine regulator of endothelial activation [68]. These findings together suggest a protective role of NO during CM. However, inhalation of NO by CM affected children in a small phase II clinical trial had no significant effect on mortality and neurological defects [69].

Patients with severe malaria show low nitric oxide (NO) bioavailability resulting in impaired microvascular function [70,71]. L-arginine, the substrate for endogenous NO synthesis, is depleted in patients with severe falciparum malaria [72,73]. Plasma concentrations of L-arginine were also found to be low in Tanzanian children with CM as compared to healthy controls [73]. In recent years studies have been conducted, promoting the proposal of L-arginine supplementation as an adjunctive therapy to improve vascular function in severe malaria [72,74,75].

Integrity of the BBB (Blood-brain barrier) is compromised during CM due to sequestration of pRBCs in the brain microvasculature [76]. In the majority of Malawian children, ring hemorrhages and extravascular fibrinogen leakage was particularly referred as the reason behind their death [30,77]. Breakdown of the blood-brain barrier and hemorrhages could allow plasma proteins and other toxic metabolites into brain parenchyma, and lead to adverse neurological complications. Supporting this phenomenon some patients exhibited edema [78,79] and downstream axonal injury [77,80]. An impairment of the blood-brain barrier is responsible for the brain swelling in CM [81]. A study on Malawian patients depicted focal reduction in staining of cell junction proteins in the vessels containing sequestered RBCs [82]. Activation of microglial cells and astrocytes can be stimulated with the leakage of blood-brain barrier which may promote further neuronal injury [83].

Blood-brain barrier integrity is maintained by tight junctions between endothelial cells [84]. Post-mortem report of patients that died with cerebral malaria revealed disruption in the expression pattern of junction proteins. In these studies, the presence of cytoadherent parasitized red cells was associated with aberrant expression of tight

junctions [52,85]. Under normal conditions, activation of endothelial Tie 2 receptors by angiopoietin-1 inhibits RhoA to maintain tight junction expression [86,87]. During severe and cerebral malaria Ang-1/Tie-2 antagonist angiopoietin-2 elevates, resulting in vascular leakage in addition making vascular endothelium sensitive to inflammatory signaling [88–90].

Activation and increased apoptosis of platelets are associated with CM. In human malaria, cell rosetting and clumping phenotypes have been detected as platelet-erythrocyte adhesion involve both infected and uninfected cells [91,92]. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) has been identified as a ligand for platelet CD36 [93]. Thrombocytopenia induced by the pathogenesis of malaria is poorly understood. Various mechanisms have been proposed to be accountable for thrombocytopenia including antibody mediated platelet destruction, platelet activation, splenomegaly, platelet sequestration, bone marrow alterations and reduced platelet lifespan [94].

In an autopsy study, increased platelet accumulation was observed at the site of pRBCs sequestration [95]. In a separate *in vitro* study, addition of platelets to pRBCs on human brain microvasculature endothelial cells (HBECs) resulted in increased endothelial permeability [96]. However, a recent study identified a novel role of platelets, mentioning that platelets can directly kill parasites in the periphery by binding to pRBCs and thereby contribute to the host's ability to control the infection. Platelet binding to infected red cells releases an antimicrobial protein, platelet factor 4 (PF4), which accumulates inside the cell and kills the parasite by lysing the food vacuole. This knowledge may provide novel interventions and therapeutic tools, which are so desperately needed [97].

Vascular flow may be further impaired by malaria-induced RBCs deformability. Squeezing through microcapillaries (diameter range = 3-7 μm) may become difficult for deformed red blood cells with average diameter ~ 7.5μm [98]. During severe malaria, the space provided for passage may be further abridged due to sequestration of pRBCs, intravascular coagulation [99,100] or platelet- facilitated clumping of parasitized red cells [91,101,102]. Increased cytosolic viscosity by the parasite and the insertion of

parasite-derived proteins into erythrocyte membrane could reduce deformability. Knob-associated histidine-rich protein (KAHRP) playing the dominant role, accounting for 51% of overall increase in rigidity in pRBCs [103]. Oxidant stress and related changes in membrane chemistry could also be the reason behind less deformability [98].

Intravascular hemolysis of parasitized red blood cells, results in the release of cell-free hemoglobin (Hb). Cell-free haemoglobin is readily oxidized to methemoglobin which releases plasma haem [104]. Accumulation of free haem in the blood cause tissue damage, inflammation, cytotoxicity [105], and host cell death [106]. The glycoprotein haptoglobin (Hp) is the body's main tool for removing circulating, toxic free Hb [107]. Under normal physiological states, cell-free hemoglobin and haem are promptly bound by circulating haptoglobin and hemopexin, respectively, leading to their inactivation via various pathways, including by haem-oxygenase 1 [108,109]. However, in conditions of intravascular hemolysis these protective pathways may be overwhelmed resulting in oxidative stress, reduced nitric oxide bioavailability, inflammation, endothelial activation, and platelet/fibrin microthrombi that together culminate in vascular dysfunction and multi-organ injury [110,111]. Haptoglobin possesses anti-inflammatory and immunomodulatory properties and functions as an antioxidant by preventing iron-driven oxidative tissue damage [107,112]. The plasma concentration of Hp increases several folds in the event of an inflammatory stimulus such as infection, injury or malignancy, whether local (vascular) or systemic (extravascular) [113–116].

The spleen is a secondary lymphoid organ with multiple functions in physiology and immunity, including removal of senescent red blood cells from circulation, recycling of iron and coordination of innate and adaptive immune responses against blood-borne pathogens [117,118]. One of the main clinical events of malaria in humans and mice is splenomegaly. The spleen turns a dark reddish-brown color together with increase in size and weight due to the accumulation of hemozoin and cellular expansion [119,120]. Insight into the spleen’s importance in malaria control suggests that upon primary infection with *P. falciparum*, the severity of disease (i.e., increased cerebral malaria), death, and parasite burden were augmented in splenectomized patients [121,122]. Spleen is also a primary site for the initiation of the adaptive immune response against blood-stage infection. CD4+ and CD8+ T cell responses are mounted against blood-stage infection [123]. Antibody-dependent cytotoxicity of parasitized RBCs blocks parasite

invasion of red blood cells, together antibody-mediated immunity blocks the activity of parasite toxins. Secondly, during *Plasmodium* infection dendritic cells uptake parasite material, process and present to CD4+ T cell. Blood parasitemia in murine malaria models had shown to be reduced via CD4+ T cell-mediated immunity, while in a human study upon low-dose parasite exposure activated parasite-specific T cells leads to death of parasite. Thirdly, in murine hepatocytes, exoerythrocytic stages can be controlled by CD8+ T cells in *Plasmodium berghei* infection. Blood flow in the spleen is complex with distinct circulation patterns adapted to enable filtration and immune clearance. A subset of T cells carrying T cell receptors (TCRs) has been shown to associate with protection induced by irradiated sporozoites [124]. The open circulation provides two checkpoints where macrophages and other immune cells can survey red blood cells and blood-borne particles. Hence, mechanisms exist to regulate mass and velocity of cell passage through these compartments [125]. Dendritic cells play a primary role of bridging innate and acquired immunity [126]. In the blood stage splenic Dendritic Cells efficiently phagocytize pRBCs, once internalized, the schizonts are destroyed and parasite components are released, which activate both CD8, $CD11b^{+}$and $B220^{+}$ etc Dendritic Cells. Those cells then migrate from the marginal zone to the T cell area and enhance expression of MHC II [127–129].

1.4 Experimental models of cerebral malaria

The complex inter-related events in the beginning and during cerebral malaria makes it difficult to completely understand the progressions and pathogenesis of CM. Most of the information about type and distribution of brain pathology during CM are usually collected through post-mortem examination of brains from patients that succumbed to CM, which are not always accessible. The limitations in the human studies, created the information gaps about fatal cases of CM. For obvious reasons, it is impossible to define the series of incidents promoting onset of CM symptoms or to compare the effect of treatment between lethal cases with those who successfully responded to treatment. Due to the lack of detailed comparative studies of adult and pediatric CM cases, it is hard to resolve whether the pathology of CM fluctuates between children and non-immune adults or not [25].

With the advancement in current imaging technologies, neuroimaging provided a way forward for clinicians to follow the progression of the disease and allow comparisons between fatal and surviving cases. With the use of magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS) and computational tomography (CT), cerebral edema was discovered as a factor of cerebral malaria pathology [79,130]. However, availability of the expensive specialized equipment in malaria-endemic clinical settings is limited because of high cost and ethical constraints [131,132]. Ophthalmoscopy is comparatively a less expensive diagnostic method used to detect malarial retinopathy in patients [133]. The severity of brain involvement and fatality can also be assessed with retinopathy [134]. Moreover, peripheral blood studies in non-fatal as well as fatal cases may provide limited information on the immunological and parasitological conditions in the brain. Again, firstly, patients usually show up at the hospital once the syndrome is well-established; secondly, total parasite biomass cannot be accurately predicted through peripheral blood examination [41]. Severity of malaria infection is strongly correlated to total parasite biomass than to only peripheral parasitaemia [135].

Due to limited access to clinical samples for ethical reasons as well as lack of access to relevant organs and inability to direct the immune response for mechanistic studies, snags are created in human studies to evaluate the pathological events of CM. Thus, experimental models have been timely used to supplement human data and to fully understand the pathogenesis of CM. Commonly used animal models for CM are non-human primates and rodents.

Among non-human primate CM models, Rhesus monkeys (*Mulatta Macaque)* infected with *Plasmodium knowlesi* or *Plasmodium coatneyi* [136,137], or *Plasmodium falciparum* infection in squirrel monkeys [138] have been used. These non-human primate model allowed to comprehend characteristic complications of CM such as the sequestration of parasitized red cells in the cerebral microvasculature and the expression of cell adhesion molecules such as ICAM-1, CD36, etc [136]. A more recent review on brain analysis of Japanese macaques (*Macaca fuscata*) infected with *Plasmodium coatneyi* using 18F-fluorodeoxyglucose (FDG)-positron emission tomography (PET) (FDG-PET) scanning and magnetic resonance imaging of the brain has been published. The authors concluded that neither parenchymal injury nor neuronal necrosis was found in

the tissues, although the monkeys exhibited severe clinical signs, and suggested that lack of abnormalities on MRI may be due to any avoidance mechanisms from ischemia caused by sequestration. This may be one reason why more than half of CM patients have no neurological sequelae following recovery [139]. Despite of being a productive model, non-human primates are prohibitively expensive, harder to study and for ethical reasons restricted to use of low numbers of animals, and these limitations make them not preferred models of experimental cerebral malaria [140,141].

Mouse models are the most commonly used in vivo experimental model of cerebral malaria [142]. *Plasmodium*-infected RBCs induce infection in several mouse models including *P. yoelii* 17XL [143], *P. berghei* ANKA [144–146], *P. berghei* NK65 [147], and *P. berghei* K173 [148]. *Plasmodium berghei* ANKA (PbA) is accepted as the best available experimental model of cerebral malaria, with the ability to reproduce clinically evident neurological events within a precise time course. Genetically CM-susceptible mouse strains are available for PbA infection including CBA and C57BL/6. Infection of these strains of mouse with PbA results in reproducible fatal CM, 6 to 10 days post-infection, with clinical signs including ataxia, paralysis, seizures and coma [149,150]. Like in humans, there is a speedy descent in the condition of infected animals after the onset of neurological signs, with death often occurring within 6 to 24h.

PbA infection of CM-resistant mice, such as the DBA/2 and BALB/c strains, results in non-cerebral malaria, with mice dying of hyperparasitaemia and severe anaemia 3 weeks after infection [151].

Experimental cerebral malaria (ECM) shares specific histopathological events with human cerebral malaria. As for human CM, PbA-infected mice develop neurological signs including limb paralysis, ataxia, and convulsions [149]. The ability of pRBCs and normal RBC to squeeze through capillaries is impaired during *P. falciparum* infection. Mainly sequestration of parasitized red cells due to their interaction with human and murine microvascular endothelium decreases the luminal diameter (although in humans the extent of sequestration of parasitized red blood cells in brain microvessels is greater). Leukocyte sequestration in brain vessels occurs in both mice as well as in man (but murine model presents higher number of sequestered leukocytes) [152,153]. Along with reduced blood flow, increased lactate production and inflammatory

cytokines are upregulated in both ECM and human CM brains [154,155]. *P. falciparum* and PbA induce endothelial activation by dysregulating angiopoietins, lactate, platelet activation and proinflammatory cytokines [62,96,156–158]. Breakdown of the blood-brain barrier, endothelial activation also leads to pronounced expression of adhesion molecules such as ICAM1, VCAM-1 [159].

Hypoargininemia and NO scavenging by cell free hemoglobin and superoxides in plasma may results in low NO bioavailability [110]. Treatment with asymmetric dimethylarginine (an endogenous nitric oxide synthase inhibitor) was sufficient to increase permeability of endothelial cell layer [160]. Administration of NO as a therapy to PbA-infected mice resulted in reduction in cerebral hemorrhages and improvement in endothelial barrier integrity [161]. Another animal model's study revealed activated platelet caspases and circulating platelet-derived microparticles in the plasma of *P. berghei* ANKA infected mice. Also, the reason behind thrombocytopenia is the loss of the majority of platelets by fragmentation in the plasma [162]. Blocking Lymphocyte function-associated antigen 1 (LFA-1, a ligand expressed on platelets) by monoclonal antibodies was found to be protective against experimental cerebral malaria. LFA-1 blockade selectively abolished cerebral sequestration of platelets [163].

Indeed, ECM and human CM are characterized by severe vasculopathy i.e., coagulopathy, vascular leakage, edema, and microhemorrhages. Although no experimental model can wholly reproduce any human disease. The principal objection on this model is its little or no intracerebral sequestration of parasitized RBCs, whereas HCM is associated with intense intracerebral sequestration [164]. Even though the predominant blood cell type sequestered in human or murine CM may differ, the causes and consequences of sequestration seem to be very similar in both syndromes [165].

It has been a decade that our group has focused its research using C57BL/6 mice experimental model infected with PbA to unveil the mechanisms of vasoconstriction and vascular dysfunction in ECM and the development of adjuvant therapies to reverse these processes.

Cabrales et al. (2010) demonstrated that ECM is associated with marked decrease (mean: 60%) of pial arteriolar blood flow attributable to vasoconstriction and decreased blood velocity. Leukocyte sequestration further decreased perfusion by narrowing luminal diameters in the affected vessels and blocking capillaries [166]. Clemmer et al. (2011) concluded that artemether and artesunate are effective over quinine in rescuing mice with late-stage ECM and decreased brain inflammation [167]. The administration of exogenous NO helped in reduction of inflammation, endothelial activation, preserved vascular integrity and decreased leukocyte and platelet accumulation in the brain [168,169]. Using cranial window method for intravital microscopy of the pial microcirculation helped to understand that the pathological impairment of endothelial Nitric Oxide Synthase (eNOS) and neuronal NOS (nNOS) functions contribute importantly to cerebrovascular dysfunction in ECM [170]. Supplementation of L-arginine at various doses were shown to partially reverse aggravation of cerebral vasoconstriction in ECM disease when given as adjuvant therapy with traditional antimalarial drug [171]. More recent work exhibited that systemic administration of L-arginine supplement and inhibition of thromboxane synthase immediately and substantially increases cerebral blood flow hence partially reversing cerebral ischemia [172].

1.5 Antimalarial chemotherapies

Quick reduction of parasite burden with antimalarial drugs is a priority, when treating cerebral malaria patients. Currently the most effective class of antimalarial drugs is Artemisinin derivatives, including artemether, artesunate and dihydroartemisinin. These compounds open wide therapeutic window for elimination of parasites and prevention of transmission, by acting on intraerythrocytic parasite [173,174]. Artemisinin derivatives apparently involves the cleavage of peroxide bridge by free ferrous iron or ferrous haem, thus generating highly reactive radicals to kill parasite by destroying the digestive vacuole or interfering with parasite's ability to detoxify haem by converting it to hemozoin [175,176]. The efficacy of artemether, artesunate and quinine was also evaluated in ECM mice. The results demonstrated that artemether and artesunate are effective in rescuing mice with late-stage ECM and decrease brain inflammation,

hence provided a framework for studies of CM adjunctive therapies using this mouse model [167].

1.6 Blood transfusion in malaria

Whole blood transfusion is a practice already adopted in the adjuvant treatment of patients with severe malarial anemia [177]. In areas of high malaria endemicity, the World Health Organization (WHO) recommends blood transfusion when the hemoglobin concentration is less than 4 g/dL, this threshold is increased to 6 g/dL in case of anemia accompanied by acidosis, impaired consciousness, shock, or parasitaemia greater than 20% [178,179]. The beneficial effects of blood transfusion is improvement in red blood cell deformability [180,181], and maintenance of the hematocrit, that strengthens the oxygen-carrying capacity of the blood, which in a setting of cerebral ischemia may be a critical advantage for the patient. Lower hematocrit also results in decreased vascular wall shear stress [182], with decreased eNOS activity, which together with the nitric oxide-scavenging action of free hemoglobin leads to worsened endothelial dysfunction. Under conditions of decreased oxygen saturation, fresh red blood cells may induce vasodilation of vessel strips by exporting NO bioactivity [183], release of ATP from RBCs also results in RBC-mediated hypoxic vasodilation [184,185]. Therefore, increasing hematocrit through whole blood transfusion should help restore endothelial function and improve tissue perfusion.

2 Justification

Changes in blood and blood vessels of the host are a pathological hallmark of *Plasmodium falciparum* infections, and are particularly intense in CM. These changes in blood cell structure and components are one of the leading causes of neurological complication. The need of appropriate adjuvant treatment along with traditional drugs is crucial to increase survival rate and decrease the incidence of sequelae in patient with CM.

Recently, Ackerman and colleagues have shown that whole blood transfusion was associated with improved survival in children with severe *falciparum* malaria, and

patients with impaired consciousness and hyperlactatemia benefited from transfusion even at moderate levels of anemia [186,187].Therefore, we decided to investigate the effects of whole blood transfusion as an adjuvant therapy in experimental cerebral malaria, in combination with artemether on blood components, survival, and blood-brain barrier permeability. Because of the difficulties in transfusing relatively large amounts of a viscous fluid in mice by means of intravenous infusion, especially in mice with ECM (showing vasoconstriction, vascular clogging by adherent cells and coagulation problems), our first strategy was to perform whole blood transfusion by the intraperitoneal route, as previously described [188]. Given the success of the intervention, we later implemented a method of intravenous transfusion by dissection of the jugular vein in anesthetized mice. Thus, in the present study we investigated the effects of whole blood transfusion in late stages of ECM, to answer the question: whether blood transfusion as an adjunctive therapy would help to restore vascular function and increase survival in this neurological syndrome. Simply to provide a cheap and easily available treatment to save hundreds of thousands of lives every year.

3 Objectives

3.1 General objective

To investigate the effect of whole blood administration (intraperitoneal and intravascular) on cerebral vascular function and as an adjunct therapy to artemether in *Plasmodium berghei* ANKA experimental cerebral malaria.

3.2 Specific Objectives

- Evaluate total blood transfusion associated with the treatment of antimalarial drug artemether in the cerebral ischemic process in ECM.

- Analyze the effect of blood transfusion on hematologic parameters and plasmatic factors in ECM.

- Evaluate the blood transfusion effects on splenocyte population.

- Analyze the effect this new combination on the survival of animals with ECM.

- Analyze the effect of two different routes of blood administration on vascular functions.

- Analyze how the different routes of transfusion affect the efficacy of treatment.

4 Results

4.1 Article 1

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Whole blood transfusion improves vascular integrity and increases survival in artemether-treated experimental cerebral malaria

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Pathological features observed in both human and experimental cerebral malaria (ECM) are endothelial dysfunction and changes in blood components. Blood transfusion has been routinely used in patients with severe malarial anemia and can also benefit comatose and acidotic malaria patients. In the present study *Plasmodium berghei*-infected mice were transfused intraperitoneally with 200 µL of whole blood along with 20 mg/kg of artemether. ECM mice showed severe thrombocytopenia and decreases in hematocrit. Artemether treatment markedly aggravated anemia within 24 h. Whole blood administration significantly prevented further drop in hematocrit and partially restored the platelet count. Increased levels of plasma angiopoietin-2 (Ang-2) remained high 24 h after artemether treatment but returned to normal levels 24 h after blood transfusion, indicating reversal to quiescence. Ang-1 was depleted in ECM mice and levels were not restored by any treatment. Blood transfusion prevented the aggravation of the breakdown of blood brain barrier after artemether treatment and decreased spleen congestion without affecting splenic lymphocyte populations. Critically, blood transfusion resulted in markedly improved survival of mice with ECM (75.9% compared to 50.9% receiving artemether only). These findings indicate that whole blood transfusion can be an effective adjuvant therapy for cerebral malaria.

Changes in blood and blood vessels are a pathological hallmark of *Plasmodium falciparum* infection, and are particularly intense in its deadly complication, cerebral malaria (CM). Mechanical obstruction of cerebral blood vessels by sequestration of parasitized red blood cells (pRBCs) reduces cerebral blood flow and oxygen consumption[1–3]. Anemia and loss of RBC deformability impair the perfusion of various organs[4]. Severe malaria also leads to alterations of biochemical characteristics of the plasma, with depletion of plasmatic factors related with vascular health such as L-arginine[5], haptoglobin[6] and angiopoietin-1 (Ang-1), a critical regulator of endothelial integrity[7]. Ang-1 maintains vascular quiescence by signaling through the Tie-2 receptor[8], whereas Ang-2, stored in Weibel-Palade bodies, can be rapidly released upon endothelial activation and displace Ang-1, sensitizing the endothelium to low concentrations of inflammatory cytokines such as TNF[8]. Indeed, Ang-2 levels are elevated in severe malaria[9] and have been associated with CM retinopathy[10], and blood-retinal breakdown associated with death or sequelae in pediatric CM[11]. Together with coagulation disorders like platelet activation and thrombocytopenia[12,13], these changes disturb endothelial quiescence, leading to vascular dysfunction, impaired cerebral perfusion, acidosis[14] and breakdown of the blood–brain barrier (BBB)[15].

Whole blood transfusion is a practice already adopted in the adjuvant treatment of patients with severe malarial anemia[16]. In areas of high malaria endemicity, the World Health Organization (WHO) recommends blood transfusion when the hemoglobin concentration is less than 4 g/dL, this threshold is increased to 6 g/dL in case anemia is accompanied by acidosis, impaired consciousness, shock, or parasitaemia greater than 20%[17,18]. Recently, Ackerman and colleagues have shown that whole blood transfusion was associated with improved survival in children with severe falciparum malaria, and patients with impaired consciousness and hyperlactatemia benefited from transfusion even at moderate levels of anemia[19,20].

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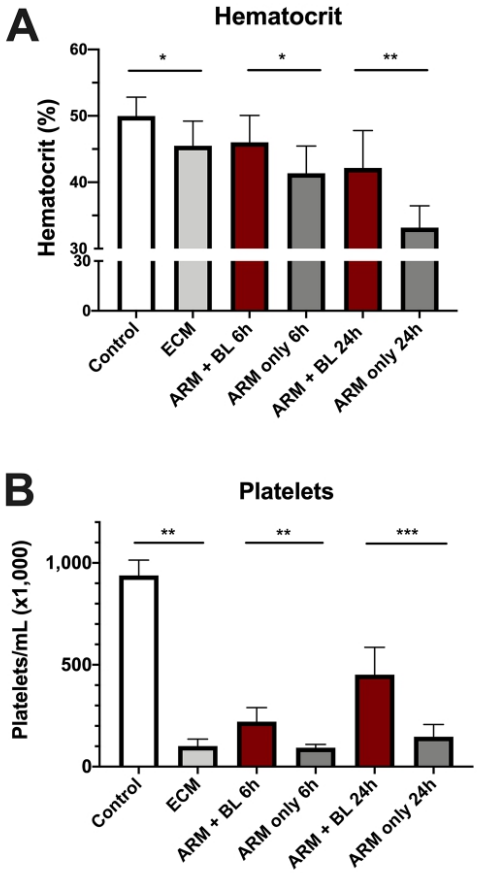


Figure 1. Effect of artemether treatment with and without 200 µL of whole blood on hematocrit level and platelet counts. *Plasmodium-berghei* ANKA-infected mice showing signs of ECM on day 6 of infection (n = 6–11 per group) received artemether (ARM) 20 mg/kg (20 µL) given IP, and mice in one of the groups also received 200 µL of whole blood (BL) also given IP. (**A**) Hematocrit: mice with ECM before treatment showed a mean 9% decrease in hematocrit in relation to uninfected controls (45.5 ± 3.72% versus 50.0 ± 2.83%, P = 0.0345). Treatment with ARM led to further decreases in hematocrit after 6 and 24 h (41.4 ± 4.10% and 33.2 ± 3.72%). Whole blood transfusion given together with ARM prevented the decrease in hematocrit at 6 h (ARM + BL: 46.0 ± 4.06%; ARM only: 41.4 ± 4.10%, P = 0.0352) and provided substantial protection for the strong decrease in hematocrit at 24 h (ARM-BL: 42.2 ± 5.63%; ARM only: 33.2 ± 3.72%, P = 0.0015, versus). (**B**) Platelet count: platelet count was drastically reduced by nearly 90% in mice with ECM compared to uninfected controls (101 ± 33.9 versus 938 ± 75.2, P = 0.0012). Treatment with ARM only did not change platelet levels within 6 h and 24 h. Whole blood transfusion given together with ARM led to significant recoveries in platelet counts compared to ARM only-treated mice at 6 h (221 ± 68.8 versus 93 ± 16.5, P = 0.0061) and 24 h (451 ± 134.5 versus 147 ± 59.9, P = 0.0004). Data are shown as mean ± standard deviation and Student t-test was performed for statistical analyses comparing two groups.

In the present study, a well-characterized and commonly used experimental model for cerebral malaria (ECM), C57BL/6 mice infected with *P. berghei* ANKA (PbA)[21,22], was used to investigate the effects of whole blood transfusion as adjunctive therapy to artemether in the late stages of the disease. This experimental model shows a number of similarities with human CM as well as some differences. The pros and cons of this model have been discussed[21–24]. A major difference is that a hallmark of human CM is sequestration of *Plasmodium falciparum*-infected erythrocytes in the brain post-capillary venules, resulting in vascular blockage and impaired perfusion[25,26]. In ECM, leukocyte adhesion with vascular plugging of cerebral vessels is the common pathological

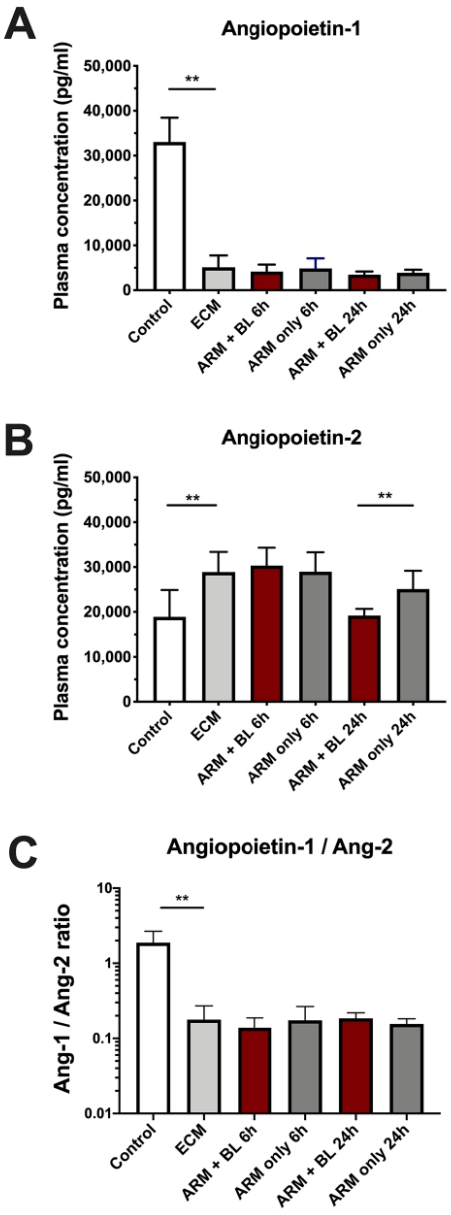


Figure 2. Plasma levels of Angiopoietin-1 and Ang-2 in mice with ECM treated with artemether with or without blood transfusion. Angiopoietins were assessed using ELISA ($n = 8$ mice per group). Levels of Ang-1 (**A**) were decreased by 85% in mice with ECM in relation to uninfected controls ($P < 0.0001$). Treatment with ARM only or ARM + BL did not change Ang-1 levels within 24 h. Levels of Ang-2 (**B**) were elevated by a mean 52% in mice with ECM in relation to uninfected controls ($P = 0.0038$). With blood transfusion after 24 h, concentration of Ang-2 decreased back to normal levels, whereas in ARM only treated mice levels remained high ($P = 0.0090$). The ratio of Ang-1 to Ang-2 (**C**) was decreased tenfold in mice with ECM compared to uninfected controls ($P = 0.0002$) and was not changed by ARM only or ARM + BL treatments within 24 h. Data are shown as mean ± standard deviation and Student t-test was performed for statistical analyses comparing two groups.

Figure 3. Plasma haptoglobin levels, blood–brain barrier permeability and spleen weight in mice with ECM treated with artemether with or without blood transfusion. (**A**) Plasma haptoglobin was evaluated using ELISA ($n = 8$ mice per group). Mice with ECM showed a twofold increase in plasma haptoglobin compared to uninfected control ($P = 0.0074$). The levels of haptoglobin were not affected by either treatment at 6 h. At 24 h, levels of haptoglobin had decreased in treated animals of both groups, being significantly different from ECM mice before treatment ($P = 0.0480$) and showing no significant differences in relation to uninfected control mice. (**B**) Permeability of blood brain barrier (BBB) was quantified by Evans blue assay. Mice with ECM showed increased permeability of the BBB in relation to uninfected controls ($P = 0.0005$). ECM mice treated with ARM only showed further increased permeability 6 h after treatment, but this increase was prevented in mice treated with ARM + BL. Indeed, permeability was significantly higher in ARM only-treated mice compared with mice treated with ARM + BL at 6 h ($P = 0.0263$). At 24 h, permeability decreased in both groups and was lower than in ECM mice before treatment (ARM only: $P = 0.0065$; ARM + BL: $P = 0.0006$), but was still higher than in uninfected controls (ARM only: $P = 0.0032$; ARM + BL: $P = 0.0371$). (**C**) Spleen weight: mice with ECM showed splenomegaly, with spleen weight increased more than threefold in relation to uninfected controls ($P < 0.0001$). Treatment with ARM only did not change spleen weight within 6 or 24 h. However, treatment with ARM + BL resulted in significant decreases in spleen weight at 6 h ($P = 0.0019$ and $P = 0.0005$ in relation to ECM mice before treatment and ARM only-treated mice, respectively) and 24 h ($P = 0.0003$ and $P < 0.0001$ in relation to ECM mice before treatment and ARM only-treated mice, respectively). However, spleen weight of ARM + BL-treated mice remained higher than uninfected controls 6 and 24 h ($P < 0.0001$) after treatment. Data are shown as mean ± standard deviation and Student t-test was performed for statistical analyses comparing two groups.

feature of the neurological syndrome, but *P. berghei*-infected erythrocyte accumulation in the brain has been documented and a recent study showed that *P. berghei*-infected erythrocytes are trapped in brain capillaries and contribute to impaired cerebral blood flow[27–29]. In both human and experimental CM, cerebrovascular blockage and severe vasculopathy occur, making this model appropriate to investigate interventions intended to restore vascular function, cerebral blood flow and cerebral oxygenation. Indeed, in this study whole blood transfusion showed a marked benefit on survival and on parameters associated with vascular integrity in ECM.

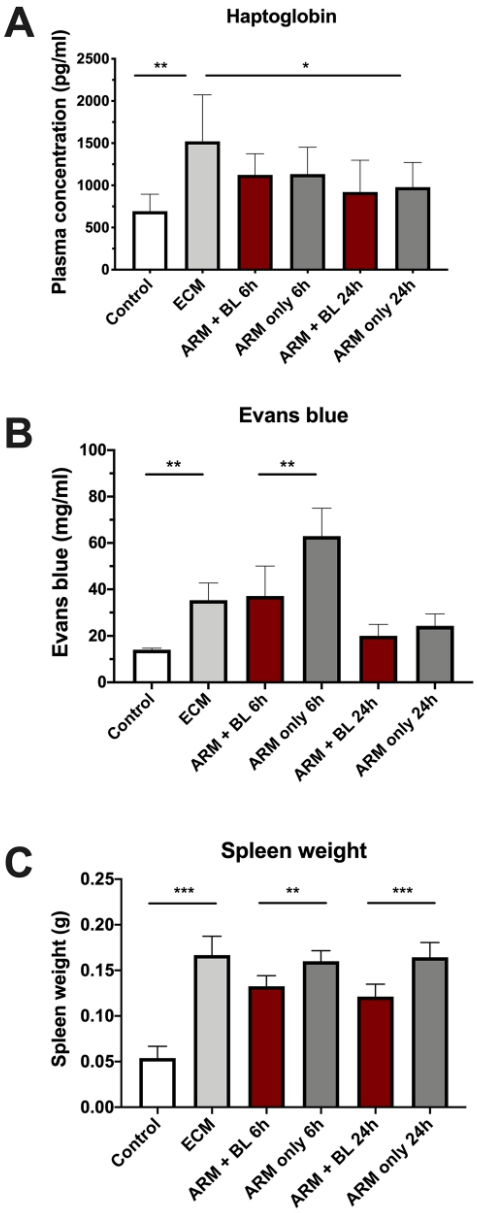
Material and methods

Animals, parasites and infection. Eight-to-ten-week-old female C57BL/6 mice (16-20 g, Fiocruz's Institute of Science and Technology in Biomodels—ICTB-Fiocruz) were infected intraperitoneally (IP) with 1×10^6 PbA (MR4 number: MRA-865) pRBCs and parasitemia checked by microscopy or flow cytometry. Hypothermia (rectal temperature between 31 and 36 °C) was used for defining late-stage ECM and as the objective criterion for treatment on day 6 post-infection[30]. Thermocouple probe (Oakton® Acorn TM; Oakton Instruments, IL, USA) was used to measure rectal temperature of mice on day 6 post-infection. All methods were performed in accordance with the relevant guidelines and regulations and the Fiocruz Animal Welfare Committee approved the experiments (license number L-037/21). The ARRIVE guidelines were taken in consideration while designing and performing experiments.

Treatments. Two preliminary experiments were performed to determine the conditions for the main experiments. First, an experiment was performed to establish the effect of artemether and artemether plus whole blood transfusion on hematocrit in mice with late-stage ECM. Transfusion of whole blood in mice poses a substantial challenge. The typical whole blood transfusion in humans is made with an amount of 20 mL/kg, which in our mice would translate approximately to a volume of 400 µL. Transferring this amount of blood to mice, in particular mice with late-stage ECM, which present with vasoconstriction and vascular plugging by leukocyte, is even more challenging. Therefore, we followed a protocol for whole blood transfusion by means of IP injection, as previously described[31]. Following this experiment, a decision was made to use half the volume (200 µL) of whole blood for the main experiments (see "Results"). A second (survival) preliminary experiment was then conducted with three arms to define a suitable control for whole blood (200 µL) transfusion: (*i*) artemether only; (*ii*) artemether plus 200 µL of saline and; (*iii*) artemether plus 200 µL of plasma (obtained from healthy C57BL/6 mice). Artemether only showed the best outcome and was used therefore for the main experiments (see Supplementary Data).

For the main experiments, on day 6 post infection, hypothermic mice (31–36 °C) were equally and randomly distributed in two groups (ECM treated with artemether only and ECM treated with artemether plus 200 µL of whole blood transfusion). Mice received artemether (Artesiane, a kind gift of Dafra Pharma, Turnhout, Belgium) IP at 20 mg/kg as previously defined[30]. Blood was collected by cardiac puncture from a number of healthy C57BL/6 mice in sodium heparin, pooled and intraperitoneally administered (200 µL). The time elapsed between blood collection, pooling and administration to sick mice was kept below 30 min. Uninfected mice and mice with ECM, untreated, were used as controls. In the survival experiments, mice with late-stage ECM were treated with artemether with or without 200 µL of whole blood and, in the subsequent days, they received artemether only daily for another 4 days. After last artemether dose, mice were followed for 7 days before being euthanized with pentobarbital.

Blood sample collection and analysis. Mice with ECM treated with artemether only or artemether + whole blood had their blood collected after 6 h and 24 h post treatment for hematological and biochemical analysis. Blood from uninfected, healthy mice and from mice with ECM, untreated, were used as


A
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ARM only 6h
ARM + BL 24h
ARM only 24h

Figure 4. Effect of artemether treatment with and without blood transfusion on splenocyte populations. (**A**) Mice with ECM showed a mean 36% increase in the total number of splenocytes in relation to uninfected controls ($P = 0.0323$), which was not significantly affected by treatments after 24 h. (**B**) The number of myeloid (CD11b + cells) were not changed in mice with ECM compared to uninfected controls, but treatment with artemether, with or without blood transfusion, led to a marked increase in this population after 24 h ($P < 0.0001$ and $P = 0.0038$, respectively, compared to mice with ECM untreated). (**C**) Mice with ECM showed a marked (90%) increase in B cells (B220+) in relation to uninfected controls ($P = 0.0002$); 24 h after treatment, the number of B cells returned to normal levels with either treatment. (**D**) The number of T cells (TCRβ + cells) in mice with ECM was not different from that observed in uninfected controls ($P = 0.5853$), but numbers increased 60–78% after ARM ($P = 0.0023$) or ARM + BL ($P = 0.0077$) treatments, compared to mice with ECM untreated. (**E**) and (**F**) The effect on CD4+ and CD8+ populations was similar to that observed in TCRβ+ cells. (**G**) and (**H**) When CD4+ and CD8+ cells were analyzed in relation to their relative proportions in relation to TCRβ+ cells, an increase in CD4+ and a decrease in CD8+ cells was observed in mice with ECM, compared to uninfected controls or with ECM mice treated with artemether or artemether plus whole blood ($P = 0.0004$ for CD4+ cells and $P = 0.0005$ for CD8+ cells); these changes led to an increase in the CD4/CD8 ratio in mice with ECM untreated. Data are shown as mean ± standard deviation and Student t-test was performed for statistical analyses comparing two groups.

controls. Blood was drawn by cardiac puncture and 300 µL were transferred to EDTA-coated microtubes and analyzed for hematological components, including hematocrit and platelet counts, using a pocH-100i automated hematology analyzer (Sysmex) at the Institute of Science and Technology in Biomodels (ICTB-Fiocruz). For plasma components analysis, blood was collected in heparinized tubes and centrifuged for 6 min at 6000 rpm. Plasma was collected and aliquots were made and stored at − 20 °C until needed. Spleen tissues were removed, weighed, and then processed for further analysis.

Determination of concentrations of plasma components by enzyme-linked immunosorbent assays (ELISA). Plasma samples were thawed and diluted to measure concentration of Ang-1 and Ang-2, using mouse Ang-1 and Ang-2 Picokine ELISA kits (Boster), or to measure mouse haptoglobin (Duoset). All ELISAs were performed according to the manufacturer's instructions.

Evans blue dye for blood–brain barrier permeability assay. The procedure was performed as previously described[32]. Six hours after treatment, mice were anesthetized with urethane (2 mg/g ip) with final volume 100 µL per animal. 2% solution of Evans blue dye (Sigma) in PBS 1X with final volume 150 µL was intravenously injected through orbital sinus. After 1 h of dye circulation animals were euthanized and perfused transcardially with 10 mL of ice-cold saline. Later, the brain was harvested and incubated for 48 h at 37 °C in 3 mL of 99.5% formamide (Sigma). The same procedure was done with uninfected controls, and 100 µL of formamide from each brain was then collected and absorbance measured at 620 nm. The amount of Evans blue extracted was calculated using a standard curve ranging from 1285 to 1.25 µg/mL.

Spleen processing and immunophenotyping. Animals were submitted to cardiac perfusion with 10 mL of cold PBS. Spleens were removed, weighed and mechanically dissociated, single cell suspensions were treated with lysis buffer (Sigma) and splenocytes counted with a hematocytometer. Approximately 1×10^6 spleen cells in PBS containing 5% FCS were incubated with an anti-Fc-γ III/II (CD16/32) receptor Ab (2,4G2, BD Biosciences) and pool of fluorochrome-conjugated antibodies. The following antibodies were used: PE anti-mouse TCRβ chain (H57-597, BD); APC anti-mouse CD45R/B220 (RA3-6B2, BD); APC-H7 anti-mouse CD4 (GK1.5, BD); PE-Cy7 anti-mouse CD8a (53–6.7, BD) and Percp-Cy5.5 anti-mouse CD11b (M1/70, eBioscience). Cells were incubated for 30 min at 4 °C and protected from light, according the manufacturers' instructions. Samples were collected using a FACS CANTO II flow cytometer (BD Biosciences). Data analysis was performed using the FlowJo 10.0 program (BD Biosciences).

Statistical analysis. All experiments were repeated at least once. Data were analyzed using a statistical software package (GraphPad Prism 7.0, La Jolla, CA). Shapiro–Wilk test was used to check distribution among the tested groups. Data are reported as mean ± standard deviation, where values of $p < 0.05$ were considered significant. Comparisons between 2 groups were performed using Student t-test and Mann–Whitney test, and multiple groups were compared using One-way ANOVA. For survival analysis, the Mantel-Cox log-rank test was used.

Results

Preliminary evaluation of whole blood transfusion on hematocrit and of saline or plasma infusion on survival in ECM. Preliminary experiments were performed in order to define the feasibility of blood transfusion via intraperitoneal injection, as described[31], and to define a suitable treatment to compare with the performance of artemether plus whole blood transfusion in late-stage ECM. As shown in Supplemental Fig. 1A, intraperitoneal injection of 400 µL of whole blood restored hematocrit levels. However, 400 µL (20 mg/kg) is suitable for transfusion in scenarios of severe anemia, and since mice with late-stage ECM showed only mild to moderate decreases in hematocrit at the time of treatment, all the experiments were henceforth performed with 200 µL of whole blood to avoid potential deleterious effects of overtransfusion. Overtransfusion occurs when hematocrit/hemoglobin levels after transfusion of a given volume of blood exceeds the target levels, and this is known to increase the risk of death[33]. Supplemental Fig. 1B shows that artemether only was an

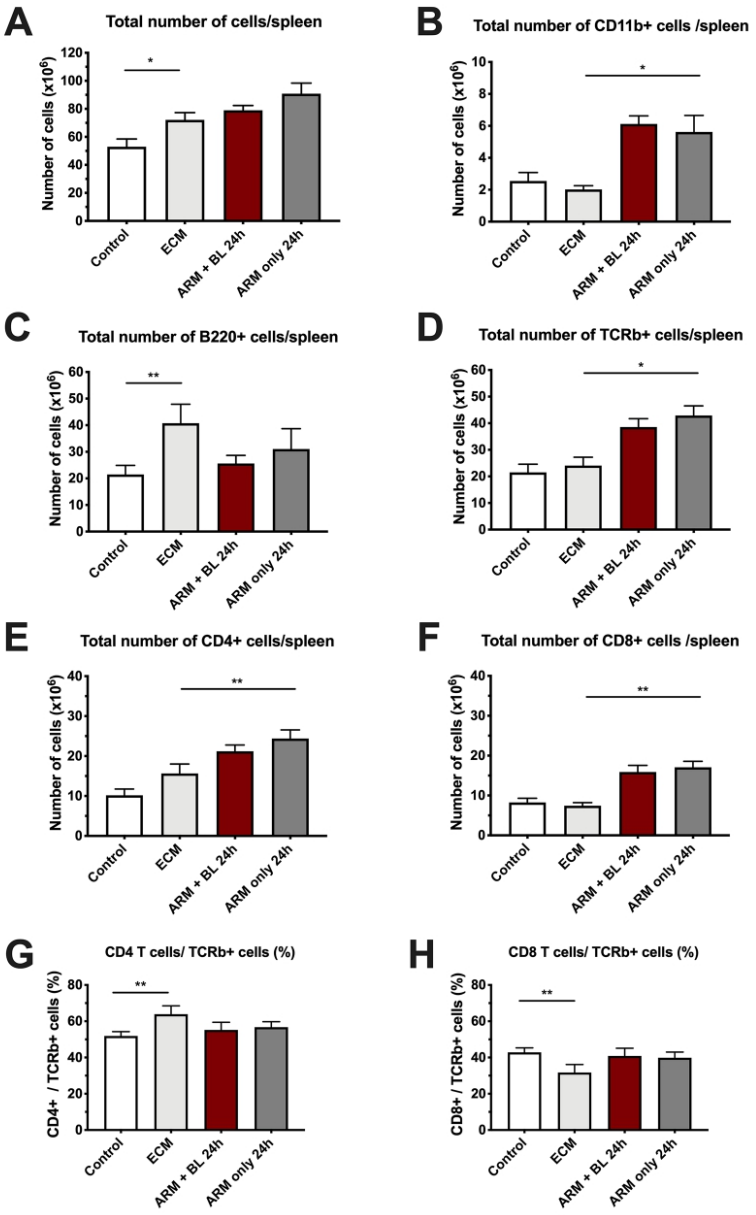

A
Total number of cells/spleen
Number of cells (x10^6)
Control
ECM
ARM + BL 24h
ARM only 24h
B
Total number of CD11b+ cells /spleen
C
Total number of B220+ cells/spleen
D
Total number of TCRb+ cells/spleen
E
Total number of CD4+ cells/spleen
F
Total number of CD8+ cells /spleen
G
CD4 T cells/ TCRb+ cells (%)
CD4+ / TCRb+ cells (%)
H
CD8 T cells/ TCRb+ cells (%)
CD8+ / TCRb+ cells (%)

Figure 5. Effect of artemether treatment with and without blood transfusion on survival, parasitemia and body weight of mice with ECM. (**A**) Survival: mice with ECM treated with ARM only showed a survival rate of 50.9% (n = 53). Whole blood given as adjunctive therapy to ARM resulted in significant increase in survival to 75.9% (n = 54; P = 0.0085). Four survival experiments where conducted and results pooled. (**B**) Parasitemia: treatment with artemether, with or without whole blood transfusion, led to a marked decrease in parasitemia in 24 h. At the 24-h timepoint, parasitemia was higher in mice treated with ARM only compared to ARM + BL (3.10 ± 0.57% versus 1.94 ± 0.25%; P = 0.0046). (**C**) Body weight: mice with ECM showed a mean 24% decrease in body weight compared to uninfected controls (13.7 ± 0.79 g versus 18.1 ± 1.05 g, P < 0.0001). After 5 days of treatment, mice that received ARM + BL in the first day showed body weight (17.4 ± 0.91 g) higher than mice that received ARM only (16.2 ± 0.75 g, P = 0.0040) and not different from uninfected controls (P = 0.1391). For survival, four separate studies were performed, the results combined and log-rank test was performed for statistical analysis. For parasitemia and body weight, data are shown as mean ± standard deviation and Student t-test was performed for statistical analyses comparing two groups.

adequate control for the experiments compared to artemether plus plasma or saline, with a better performance in survival. Indeed, addition of saline actually led to a worse outcome, with all mice dying in 24 h.

Whole blood transfusion prevents the post-artemether decrease in hematocrit in ECM. Mice with ECM showed a drop in hematocrit (45.5 ± 3.72%) compared to uninfected controls (50.0 ± 2.83%) (Fig. 1A). ECM mice treated with artemether alone showed a post-treatment decrease in hematocrit, reaching 41.4 ± 4.1% at 6 h and 33.2 ± 3.27% at 24 h after treatment. In contrast, ECM mice that received artemether plus 200µL of whole blood showed a preservation of hematocrit levels at 6 h (46.0 ± 4.06%) and at 24 h (42.2 ± 5.63%) (Fig. 1A).

Whole blood transfusion partially corrects thrombocytopenia in ECM. During ECM, platelet counts (per µL of blood) fell by nearly 90% (101 ± 33.9 compared to 938 ± 75.2 of uninfected controls). Artemether treatment did not improve the platelet count at 6 h (92.8 ± 16.5) nor at 24 h (147.0 ± 60.0) post-treatment (Fig. 1B). The combination, however, of artemether treatment plus whole blood raised platelet counts by twofold at 6 h (221.0 ± 68.9) and threefold at 24 h (451.4 ± 134.5) compared to artemether-only treated mice (Fig. 1B).

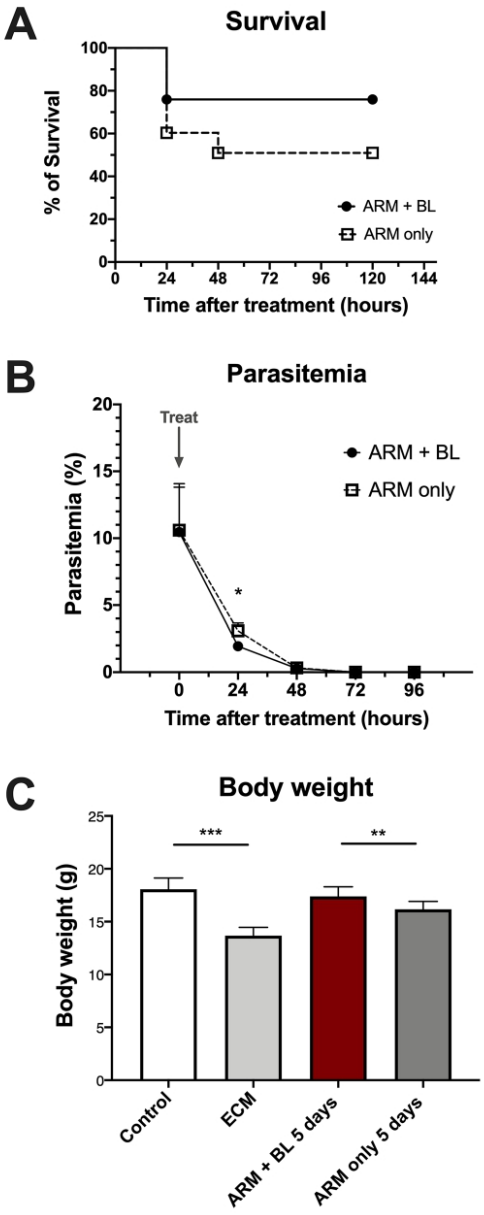
Blood transfusion decreases plasma Ang-2 levels at 24 h in ECM. Mice with ECM showed very low levels of Ang-1 (5106 ± 2647 pg/mL; uninfected: 33,043 ± 5410 pg/mL) (Fig. 2A). Ang-1 levels did not recover after artemether treatment whether combined with blood transfusion or not. In contrast, Ang-2 increased to higher levels than normal from 18,887 ± 5982 to 28,882 ± 4489 pg/mL and remained high at 25,085 ± 4085 pg/mL 24 h after artemether-only treatment (Fig. 2B). Blood transfusion reversed Ang-2 levels back to normal, to 19,169 ± 1507 pg/mL after 24 h. Despite resolution of elevated Ang-2 levels after blood transfusion, the Ang-1 to Ang-2 ratios remained low (Fig. 2C).

Haptoglobin levels, blood–brain barrier integrity and spleen weight in ECM. Figure 3A depicts the rise in plasma haptoglobin in mice with ECM (1,521 ± 525 pg/mL) compared to healthy controls (693 ± 202 pg/mL). Artemether treatment was followed by a substantial decrease in plasma haptoglobin levels (979 ± 292 pg/mL) after 24 h, and blood transfusion did not alter this decrease (921 ± 376 pg/mL).

Mice with ECM showed increased Evans blue dye leakage (35.3 ± 7.44 mg/mL; uninfected: 14.1 ± 0.66 mg/mL) (Fig. 3B). BBB integrity worsened 6 h after treatment with artemether alone (63.0 ± 12.0 mg/mL), which was prevented by whole blood transfusion (37.1 ± 13.0 mg/mL). Twenty-four hours after treatment, BBB integrity recovered in both treatment groups compared to untreated ECM mice or at 6 h post-treatment. Mice with ECM showed increased spleen weight, which did not improve 6 or 24 h after artemether-only treatment, but mice treated with artemether plus whole blood had decreased spleen weight at 6 and 24 h after treatment (Fig. 3C).

Splenic leukocyte populations in ECM before and after treatment. Data regarding splenocyte populations are shown in Fig. 4A–H. The increase in spleen weight in mice with ECM was paralleled by an increase in total number of splenocytes, which was not affected by the antimalarial treatment. The number of splenic myeloid cells was not changed in ECM mice compared to uninfected controls, but artemether treatment induced an increase in CD11b + cells. Mice with ECM showed increased numbers of splenic B cells, which return to normal 24 h after artemether treatment. The numbers of T cells and subsets (CD4+ and CD8+) did not differ in mice with ECM compared with uninfected mice. However, the relative balance was changed, with an increase in CD4+ and a decrease in CD8+ T cells. Upon treatment with artemether, the numbers and percentage of T cells and subsets increased compared to ECM mice before treatment, and the balance between CD4+ and CD8+ cells was restored. In all cases, blood transfusion had no effect on the spleen cell populations compared to artemether alone.

Blood transfusion increases survival in artemether-treated ECM. Groups of mice presenting ECM were treated with artemether (20 mg/kg) alone or combined with 200 µL of whole blood administered IP. Adjuvant therapy with blood transfusion resulted in a marked improvement of survival (75.9% versus 50.9% in the group of mice treated with artemether-only) (Fig. 5A). There was a sharp decrease of parasitemia 24 h


A
Survival
% of Survival
100
80
60
40
20
0
0
24
48
72
96
120
144
Time after treatment (hours)
ARM + BL
ARM only
B
Parasitemia
Parasitemia (%)
20
15
10
5
0
Treat
*
0
24
48
72
96
Time after treatment (hours)
ARM + BL
ARM only
C
Body weight
Body weight (g)
25
20
15
10
5
0
***
**
Control
ECM
ARM + BL 5 days
ARM only 5 days

after treatment, and at this timepoint parasitemia was lower in mice treated with artemether plus whole blood (1.94 ± 0.25%) than in mice treated with artemether only (3.10 ± 0.57%) (Fig. 5B). This difference was not observed in the subsequent timepoints. Mice with ECM showed decreased body weight (13.7 ± 0.79 g) compared to uninfected controls (18.1 ± 1.05 g) (Fig. 5C). Even after 5 days of artemether-only treatment the body weight did not fully recover (16.2 ± 0.75 g), but transfusion of whole blood in the first day of treatment with artemether led to a faster recovery of the body weight (17.4 ± 0.91 g), which was not different from uninfected controls.

Discussion

The high lethality and post treatment neurological sequalae in patients with cerebral malaria demand new adjuvant therapies. The main finding of the present study is that whole blood transfusion resulted in substantial improvement of survival of mice with late-stage ECM treated with artemether.

Mice with ECM showed only mild to moderate (~ 10%) decrease in hematocrit. However, artemether treatment resulted in further marked drops in hematocrit after 6 and 24 h, a phenomenon also reported in human malaria[34], especially in non-immune travelers with hyperparasitemia[35]. The fact that mice with ECM show parasitemia over 10% and parasites are rapidly killed by artemether may help to explain the rapid decrease in hematocrit following treatment.

Transfusion of 200 µL of whole blood to mice with ECM resulted in improved hematocrit at both 6 and 24 h, whereas saline had the opposite effect, and all mice died in 24 h. This worse outcome may reflect similar findings in human severe malaria, where fluid resuscitation did not improve and actually worsened patient outcomes[55–57]. Although the findings in the experimental CM model cannot be directly extrapolated for the human disease, it is expected that maintenance of the hematocrit strengthens the oxygen-carrying capacity of the blood, which in a setting of cerebral ischemia may be a critical advantage for the patient. Fresh RBCs also improve the hemorheological properties, which is known to be deteriorated in severe malaria infections[36,37]. And finally, lower hematocrit results in decreased vascular wall shear stress[38], with decreased eNOS activity, which leads to worsened endothelial function[39]. Therefore, increasing hematocrit through whole blood transfusion should help restore endothelial function[40,41], help clear vessels blocked by parasitized erythrocytes and inflammatory cells, increasing tissue perfusion and decreasing acidosis[19,42,43] as well as immune cell-mediated endothelial damage. The effects on endothelial function are supported by the observation that blood transfusion prevented worsening of BBB breakdown and restored Ang-2 levels. Indeed, decreased levels of Ang-1 and increased levels of Ang-2, disturbing endothelial quiescence with loss of vascular health[7,8], have been associated with pediatric severe malaria[10,44,45]. Since Ang-2 has been proposed as a risk factor for cognitive injury in pediatric CM, this finding is of critical importance, indicating that fresh blood counteracts the ECM-related inflammation and vascular insult. These findings are in line with data showing that interventions that counteract vascular dysfunction and inflammation are beneficial in ECM[32,40,41,46–48]. It is noteworthy, however, that the improvement in Ang-2 levels by transfusion was observed at 24 but not at 6 h, indicating that the intervention takes time to show benefit on this parameter. Since in human CM most deaths occur in the first 24–48 h after hospitalization, the relevance of this finding for clinical disease still needs to be established.

Thrombocytopenia is also one of the risk factors for mortality in African children with falciparum malaria[49]. Whole blood transfusion induced significant upturn in circulating platelets in mice with ECM. The availability of fresh, quiescent platelets, could help restoring the normality of the coagulation system without the deleterious inflammatory actions of activated platelets[50]. This effect of partially restoring the platelet counts in 24 h cannot be ascribed only to a passive, repository effect due to the platelets present in the limited amount of transfused blood. Therefore, it is apparent that blood transfusion stimulates the body to actively respond, increasing platelet production. Other effects such as improved BBB response and decreased splenic congestion also support an active modulatory, rather than just repository, effect of blood transfusion.

Acute and severe hemolysis usually leads to a consumption of haptoglobin, as seen in severe malaria[51]. In mice with ECM, anemia was only mild to moderate whereas inflammation is overwhelming, and this might help to explain why haptoglobin levels were high, since haptoglobin is an acute phase protein that increases with conditions such as inflammation and infection[52]. On the other hand, a hemolytic event might also help to explain the decrease in haptoglobin levels following artemether treatment. Blood transfusion did not seem to interfere with haptoglobin levels following artemether treatment.

In this study, 10 mL/kg (200 µL) of blood was administered, which is half the usual amount given for severe anemia, because mice with ECM showed only mild to moderate anemia and also because malaria induces splenic congestion and a sudden increase in hematocrit might exacerbate this condition. Interestingly, blood transfusion actually helped to decrease the weight of enlarged spleen in mice with ECM, suggesting that it helped to decrease congestion. It is possible that fresh red blood cells improved blood flow throughout the body, improving overall hemodynamics and decreasing the burden at the spleen. Indeed, in sickle cell disease acute splenic sequestration is treated by RBC transfusion[53]. The increase in spleen weight in mice with ECM was paralleled by an increase in the total splenocyte population. However, the magnitude of the increase in total splenocyte population was much lower than the increase in spleen weight (20% versus 200%), indicating that congestion with accumulation of blood-circulating cells was the major reason for the increased spleen weight. The vast majority of the increase in splenocyte numbers was in the B cell compartment, in line with previous findings[54]. Although there was a change in the dynamics of different splenocyte populations in mice with ECM and after treatment with artemether, blood transfusion had no effect on the outcomes of each cell population.

The benefit of blood transfusion, however, on hematological and vascular parameters in mice with ECM treated with artemether was associated with a marked improvement in survival. These findings are in line with a recent prospective multicenter observational study showing that blood transfusion improved survival of children hospitalized with severe falciparum malaria[19,20]. Blood transfusion is currently recommended by the WHO in

severe malaria when hemoglobin is below 4 g/dL, or below 6 g/dL when associated with complications such as acidosis and coma. However, the authors showed that when signs of vital organ hypoperfusion are present, blood transfusion can benefit patients even at higher hemoglobin thresholds (7.7 g/dL for all severe malaria, and even higher in case of impaired consciousness or severely elevated lactate concentration)[19]. The effect of whole blood transfusion on survival in pediatric severe malaria must be evaluated in randomized controlled trials that would balance the potential benefits of transfusion against the potential risks such as infection transmission, hemolytic reactions, and circulatory overload. For patients with higher hemoglobin levels, our study in mice indicates that even the transfusion of half the usual blood volume can be of great benefit, an approach that could reduce the risk of circulatory overload.

In conclusion, the transfusion of whole blood as an adjuvant therapy in ECM showed promising results and identified an unexpected interaction between transfusion and vascular inflammation. Future clinical studies of transfusion will be necessary to evaluate the potential of this strategy as a viable, cheap and effective adjunctive therapy for cerebral malaria.

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Author contributions

S.G. conducted the experiments, analyzed the data and wrote the article. F.L.R.G. was responsible for the immunophenotyping experiments. A.S.M., G.S.S. and F.G.C. assisted in performing the experiments. H.C.A. was involved with study conception and, with C.T.D.R., helped with data analysis and interpretation. L.J.M.C. conceived and was overall responsible for planning and supervising the study, data analysis and interpretation, and wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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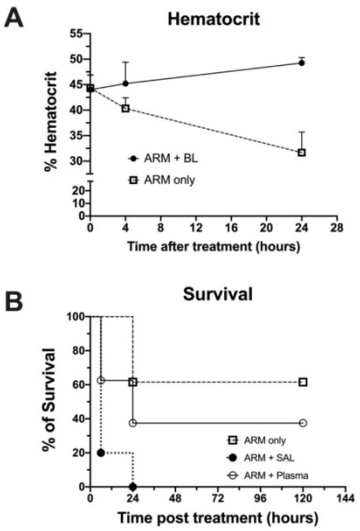
Supplemental Figure 1



Supplemental Figure 1. **Preliminary evaluation of the effect of whole blood, plasma and saline transfusion in mice with ECM. (A)** Effect of whole blood transfusion on hematocrit: *Plasmodium-berghei* ANKA-infected mice showing signs of ECM on day 6 of infection (n = 5 per group) received artemether (ARM) 20 mg/kg (20 µL) given intraperitoneally (IP), and mice in one of the groups also received 400 µL of whole blood (BL) also given IP. At treatment, mice in both groups presented similar hematocrit levels, with mild to moderate anemia (44.0 ± 2.91 % for ARM + BL and 44.3 ± 2.51 % for ARM only; uninfected controls: 50.0 ± 2.83 %). ARM only treated mice showed progressive decreases in hematocrit 4 and 24 hours after treatment, reaching 31.6 ± 4.04 % at 24 hours (n = 3). On the other hand, mice that received 400 µL of blood transfusion together with ARM showed no further decreases in hematocrit, and actually recovered to 49.3 ± 1.06 % at 24 hours (n = 2). **(B)** Effect of saline or plasma infusion on survival: Mice with late-stage ECM received artemether (ARM) 20 mg/kg (20 µL) given IP (n = 6-13 mice per group) and were divided in three groups: *i*) received 200 µL of sterile saline, IP; *ii*) received 200 µL of plasma obtained from healthy C57BL/6 mice, IP; *iii*) received nothing additional. Mice that received artemether only showed 61% survival. In the group that received artemether + saline all mice died within 24 hours. Adding saline or plasma did not improve outcome, on the contrary.

4.2 Article 2

Open Forum Infectious Diseases

Intravenous whole blood transfusion results in faster recovery of vascular integrity and increased survival in experimental cerebral malaria

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Abstract:	Transfusion of 10 mg/kg of whole blood via intraperitoneal route to mice with late-stage experimental cerebral malaria (ECM) along with artemether has been shown to result in markedly increased survival (76%) compared to artemether alone (51%). Intraperitoneal route was used to overcome the restrictions imposed by injection of large volumes of viscous fluid in small and deranged blood vessels of mice with ECM. In the present study, a method of intravenous transfusion was implemented by injecting 200µL of whole blood through the right jugular vein in mice with late-stage ECM, together with artemether given intraperitoneally, leading to even more remarkable increase in survival, from 54% to 90%. On the contrary, mice receiving artemether plus plasma transfusion showed a worse outcome, with only 18% survival. Compared to the intraperitoneal route, intravascular transfusion led to faster and more pronounced recoveries of hematocrit, platelet counts, angiopoietins levels (ANG-1, ANG-2 and ANG-2/ANG-1) and blood brain barrier integrity. These findings indicate that whole blood transfusion when given intravenously show more efficacy over intraperitoneal transfusion, reinforcing evidence for benefit as an adjuvant therapy for cerebral malaria.
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Intravenous whole blood transfusion results in faster recovery of vascular integrity and increased survival in experimental cerebral malaria

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Author contributions

SG conducted the experiments, analyzed the data and wrote the article. HCA was involved with study conception and, with CTDR, helped with data analysis and interpretation. LJMC conceived and was overall responsible for planning and

supervising the study, data analysis and interpretation, and wrote the manuscript. All authors reviewed the manuscript.

Key words: experimental cerebral malaria; blood transfusion; artemether; adjunctive therapy; endothelial dysfunction.

Abstract

Transfusion of 10 mg/kg of whole blood via intraperitoneal route to mice with late-stage experimental cerebral malaria (ECM) along with artemether has been shown to result in markedly increased survival (76%) compared to artemether alone (51%). Intraperitoneal route was used to overcome the restrictions imposed by injection of large volumes of viscous fluid in small and deranged blood vessels of mice with ECM. In the present study, a method of intravenous transfusion was implemented by injecting 200μL of whole blood through the right jugular vein in mice with late-stage ECM, together with artemether given intraperitoneally, leading to even more remarkable increase in survival, from 54% to 90%. On the contrary, mice receiving artemether plus plasma transfusion showed a worse outcome, with only 18% survival. Compared to the intraperitoneal route, intravascular transfusion led to faster and more pronounced recoveries of hematocrit, platelet counts, angiopoietins levels (ANG-1, ANG-2 and ANG-2/ANG-1) and blood brain barrier integrity. These findings indicate that whole blood transfusion when given intravenously show more efficacy over intraperitoneal transfusion, reinforcing evidence for benefit as an adjuvant therapy for cerebral malaria.

Introduction

Despite advances in medical science and global technical strategies for malaria control, there were an estimated 229 million malaria cases with 409,000 deaths in 2019 [1]. Cerebral malaria (CM) is prominently involved in majority of malaria related deaths, and frequently results in long-term cognitive deficits in survivors [2,3]. Majority of deaths befall in the first 24-48 hours of hospital admission, demanding a quick administration of treatments to reduce death [4,5]. In order to address these issues, to increase survival and overcome the occurrence of post treatment neurological sequelae, there is utmost need of adjuvant therapies.

Cerebral malaria is a multifactorial disorder accompanied by hematological and vascular changes, including mild to moderate to severe anemia, altered hemorheologic properties, thrombocytopenia, along with alterations of biochemical characteristics of the plasma such as the levels of angiopoietins, haptoglobin, L-arginine and proinflammatory cytokines, among others [6–12]. Recently, a multicenter study in Africa showed that children hospitalized with severe *P. falciparum* malaria, particularly those with impaired consciousness or severe acidosis, benefited from whole blood transfusion, exhibiting increased survival [13,14]. Accordingly, whole blood transfusion, when administered intraperitoneally to mice with late-stage ECM also receiving artemether, resulted in increased survival and had beneficiary effects in terms of restoring hematocrit and markers of vascular health [15]. These findings with whole blood transfusion in ECM follow prior evidence indicating that interventions that restore vascular function are beneficial as adjunctive therapy in ECM [16–19].

In the study showing the effect of blood transfusion along with artemether in ECM, transfusion was performed by means of intraperitoneal injection. This route was

chosen for its simplicity and due to the complicated task of injecting relatively large amounts (200μL) of a viscous fluid in the commonly used lateral tail vein in very sick mice with ECM, which shows vasoconstriction and vascular plugging by adherent monocytes [16,18–23]. The downside of this strategy is that it does not properly mimic the intervention as it happens in humans and, as a consequence, the benefit of transfusion may not be as immediate as it would be with an intravenous injection. Besides, not 100% of the injected red blood cells reach the circulation [24] and it is expected that losses occur as well for the transfused plasma components. Therefore, in the present study we performed whole blood transfusion as adjunctive therapy to artemether in mice with late-stage ECM by means of intravenous injection of heparinized blood using a technique of right jugular vein injection. The results show that the outcome in terms of survival, which reached 90%, was markedly improved compared to intraperitoneal transfusion as previously described [15], and recovery of hematological and vascular parameters was also more prominent.

Materials and methods

Mice

The Institute of Science and Technology in Biomodels (ICTB) of FIOCRUZ provided specific pathogen-free, female, 8-12-week-old C57BL/6 mice (16-20 g). A state of 12-hour light/dark cycle and a constant temperature of 21–22 °C was maintained where animals had free access to water and chow. In experimental protocols every regulation and guideline were properly followed. All the experimental work has been ratified by Animal Welfare Committee of the FIOCRUZ (CEUA/FIOCRUZ), under license number L-037/21.

Infection with *Plasmodium berghei* ANKA (PbA)

For passage, healthy C57BL/6 mouse were used to cultivate *Plasmodium berghei* ANKA (PbA) parasites (kindly donated by MR4; the Malaria Research and Reference Reagent Resource Center, Manassas, VA, USA). PbA samples were removed from liquid nitrogen and brought to room temperature and injected to passage mice by intraperitoneal (ip) injection. Infection in experimental group began after intraperitoneal inoculation of 1×10^6 infected RBC (day 0), and parasitemia assessed on day 6 by flow cytometry or Giemsa-stained blood smears. The onset of cerebral malaria in mice was assessed through clinical signs and body (rectal) temperature, using a thermocouple probe (Oakton® Acorn TM; Oakton Instruments, IL, USA).

Treatments

In a first set of experiments, on day 6 post inoculation, hypothermic mice (31-36 °C) [25] were randomly allocated in three equal groups to define the effect of treatments on survival rate: *i*) artemether only; *ii*) artemether plus 200 µL of blood and; *iii*) artemether plus 200 µL of plasma. The amount of blood and plasma to be transfused was previously defined [15] (200 µL, approximately equivalent to 10 mL/kg) and were obtained from healthy C57BL/6 mice. Artemether 20mg/ml (Dafra Pharma GmbH, Basel, Switzerland) was injected intraperitoneally at 20 mg/kg [25]. Healthy mice (uninfected) and mice with ECM (untreated) were used as controls. Artemether plus plasma showed the worst outcome in terms of survival, therefore this group was removed from the other experiments (see Results).

Intravenous blood transfusion and sample collection

All mice received a single intraperitoneal injection of artemether (20 mg/kg), while a group of mice additionally received whole blood into the right jugular vein. Briefly, after anesthetizing mice with 75-100 mg/kg ketamine and 10mg/kg xylazine, mice were kept in ventral recumbent position. Jugular vein was made visible by making small incision on the neck. Blood was transfused using 30G-insulin syringe, the needle was entered into lumen of vein after passing through pectoral muscle. All injections were manually performed and mocking was done to mice receiving only artemether.

Sample collection included collection of whole blood and plasma 6hr and 24hr post treatment. For hematological analysis, whole blood was collected in EDTA-coated microtubes through cardiac puncture. For biochemical analysis, plasma samples were withdrawn from whole blood after centrifuging it at 6,000 rpm for 6 minutes. Later on, plasma was distributed in small aliquots and stored at −80 °C. Same procedure was done to uninfected and ECM mice which served as controls in the study.

Survival experiments

Experiments were performed to check whether intravenous blood transfusion as adjunctive therapy has a beneficiary effect on survival of ECM mice over only artemether and artemether plus plasma. ECM mice (day 6 of infection, rectal temperature of 31-36 °C) were randomly assigned to three treatments groups: (1) artemether 20 mg/kg ip, (2) artemether 20 mg/kg ip plus 200 µL of whole blood iv and (3) artemether 20 mg/kg ip plus 200 µL of plasma. On daily basis one dose of 20mg/kg artemether ip was administered for 5 consecutive days. After the last dose of

artemether, mice were followed for another one week and then euthanized with pentobarbital.

Study of blood hematological components

At time 0 hour, 6 hours and 24 hours, whole blood samples were collected from controls and treated animals and sent to Institute of Science and Technology in Biomodels (ICTB) of FIOCRUZ for hematological components analysis, using a pocH-100i automated hematology analyzer (Sysmex).

Enzyme-linked immunosorbent assays (ELISA) for biochemical analysis of plasma components

To determine levels of Angiopoietin 1 & 2, Boster Mouse Angiopoietin 1 & 2 Picokine ELISA kits were used. Prestored plasma samples were brought to room temperature and subjected to 1:5 dilution in PBS and the ELISA performed according to the manufacturer's instructions. For haptoglobin measurement, plasma samples were diluted 1:5,000 and then Douset ELISA kit was used.

Assessment of blood-brain barrier permeability

To assess the permeability of the blood-brain barrier at 6 hours and 24 hours after treatment, the animals were anesthetized with urethane (2 mg/g ip) with final volume 150 µL per animal. Each animal received an intravenous injection (in the eye plexus) of 2% Evans blue stain (Sigma) diluted in 1X PBS. After 1 hour of the dye injection, the animals were euthanized and 10 mL of ice-cold saline has been transcardially

perfused to them, followed by brain removal. Later the harvested brains were incubated in 3 mL of 99.5% formamide (Sigma) for 48 hours at 37°C. healthy controls and ECM untreated mice went through same procedure. To determine the concentration of the dye leaked out of brain in the formamide, 100µl of formamide from each brain was then separated and the absorbance of each sample was evaluated by spectrophotometry, with a wavelength of 630 nm. A standard curve ranging from 1,285 µg/ml to 1.25 µg/ml was used to calculate the amount of Evans blue extracted per brain tissue.

Results

Intravenous blood transfusion prevents artemether-induced anemia

To better mimic a clinically relevant scenario when children present with neurological signs, mice were treated after the development of neurological symptoms on day 6 post infection with artemether (20 mg/kg) alone, artemether plus plasma (200 µL), or artemether plus whole blood (200 µL) IV. Mild to moderate anemia with mean hematocrit value 46.2 % was recorded in ECM mice (uninfected controls: 50.1 %) (**Figure 1A**). The data on hematocrit level per time point showed a continuous decline of anaemia levels (mean: 41.4 % to 33.1 % after 6 hours and 24 hours respectively) in mice treated with artemether alone. On the other hand, treatment with whole blood prevented the further decline in hematocrit (mean: 48.6 % at 6 hours and 46.0 % at 24 hours). Indeed, hematocrit levels in transfused mice at 6 and 24 hours were not different from that observed in ECM mice before treatment.

Intravenous blood transfusion improves ECM-induced thrombocytopenia

The mean platelet count on day 6 post infection in mice with ECM was depleted by 90% (104,000 platelets per mL versus 942,000 of uninfected controls) (**Figure 1B**). Treatment with artemether alone had little effect on platelet counts at 6 hours and 24 hours. Intravenous blood transfusion as an adjuvant to artemether significantly hastened the recovery of platelets at 6 hours and at 24 hours (2.7-fold and 4.4-fold compared to ECM mice treated with artemether only, respectively). Intravenous blood transfusion recovered the platelet count to around 60% of that noticed in healthy controls, whereas artemether alone had no affect at any time point (13% of controls).

Blood transfusion improves endothelial quiescence by restoring balance of angiopoietin-1 and 2

Plasma levels of ANG-1 and ANG-2 among four groups were assessed at different time points before and after treatment. With disease severity on day 6 post infection, plasma levels of endothelial-protective ANG-1 strongly declined (**Figure 2A**). Compared to uninfected healthy control, mean plasma levels of ANG-1 declined 84% in ECM mice. Treatment with artemether alone did not modify ANG-1 levels at 6 and 24 hours post treatment. Comparatively, at 24 hours mice receiving blood transfusion showed ANG-1 levels two times higher than artemether-only treated animals, indicating that transfusion induced a faster recovery of endothelial function in artemether-treated animals. On the other hand, the mean plasma levels of endothelial-inflammatory ANG-2 increased 52% in mice with ECM (**Figure 2B**). The levels of ANG-2 remained high 6 hours after either artemether or artemether plus blood transfusion. However, intravenous blood transfusion helped bring ANG-2 levels back to normal at

24 hours. As an additional measure, the ratio of ANG-2 to ANG-1 of ECM mice was found to be significantly different between artemether-only and artemether plus transfused blood groups at 24 hours (**Figure 2C**). No significant difference was noticed in the ratio of ANG-2 to ANG-1 between uninfected control and blood transfused groups. These data combined indicate that intravenous whole blood transfusion restores angiopoietin balance in artemether-treated ECM mice 24 hours after administration.

Intravenous blood transfusion prevents blood-brain barrier (BBB) breakdown

The improved survival of PbA-infected mice treated with whole blood in combination with artemether can be associated with preservation of the BBB, quantified by Evans blue extravasation into the brain parenchyma. Mice with ECM showed increased cerebrovascular Evans blue leakage, which was aggravated 6 hours post treatment with artemether alone (**Figure 3A**). Mice with ECM that received intravenous blood transfusion along with artemether showed no aggravation of vascular leakage, with Evans blue extravasation being much lower than that observed in mice that received artemether alone. Actually, these mice even showed some recovery of cerebrovascular integrity, as Evans blue leakage was significantly smaller than that observed in ECM mice prior to treatment. Also, after 24 hours of treatment, mice that received blood transfusion showed enhanced BBB integrity, comparable to the level of healthy controls, whereas animals treated with artemether alone still presented increased vascular leakage at this point.

Plasma haptoglobin levels after intravenous blood transfusion

Mice with ECM showed a mean 134% increase in plasma haptoglobin levels (1,539 ± 217 pg/mL) compared to healthy control (655 ± 136 pg/mL) (**Figure 3B**). Treatment with artemether, with or without intravenous blood transfusion, led to decreased haptoglobin levels at 6 hours (1,116 ± 201 pg/mL and 1,145 ± 227 pg/mL, respectively) and further at 24 hours (911 ± 198 pg/mL and 896 ± 248 pg/mL, respectively).

Intravenous whole blood transfusion plus artemether improves survival of mice with ECM compared to artemether plus plasma or artemether alone

When PbA-infected mice present signs of ECM, if left untreated they will die, mostly within 24 hours. Treatment of mice with late-stage ECM with artemether led to a survival rate of 54% (**Figure 4**). Whole blood transfusion given together with artemether resulted in 90% survival, which represents a dramatic increase of 67% in survival compared to mice treated with artemether alone. Plasma transfusion (200 μL), on the other hand, worsened disease severity, resulting in very low survival rate (18%).

Overall, these findings suggest that whole blood, when transfused via intravenous route, improved survival and reduced disease severity by preserving endothelial components.

Discussion

Clinical observational studies have suggested that intravenous whole blood transfusion may improve survival of children with cerebral malaria even in the absence of severe anemia. However, the mechanisms underlying the apparent protection of whole blood remain undefined. Here, we find that in a murine model cerebral malaria,

intravenous whole blood restored the balance of angiopoietin 1 and 2, key factors that regulate endothelial inflammation and barrier integrity. Indeed, intravenous whole blood transfusion prevented the blood brain barrier breakdown that is a cardinal feature of experimental cerebral malaria, and improved survival. Notably, infusion of plasma instead of whole blood did not convey any protection and instead worsened survival. Thus, we must infer that red blood cells are a necessary component of endothelial-preserving property of whole blood.

The beneficial effects of blood transfusion include improvement in red blood cell deformability [26,27], maintenance of hematocrit strengthening the oxygen-carrying capacity of the blood, which in a setting of cerebral ischemia may be a critical advantage for the patient. Lower hematocrit also results in decreased vascular wall shear stress [28], with decreased eNOS activity, which together with the nitric oxide-scavenging action of free hemoglobin leads to worsened endothelial dysfunction. Under conditions of decreased oxygen saturation, fresh red blood cells may induce vasodilation of vessel strips by exporting NO bioactivity [29], release of ATP from RBCs also results in RBC-mediated hypoxic vasodilation [30,31]. Therefore, increasing hematocrit through whole blood transfusion should help restore endothelial function and improve tissue perfusion.

Prior work has demonstrated the therapeutic efficacy of whole blood transfusion in PbA-infected C57BL/6 mice with ECM, using intraperitoneal route for blood administration, resulting in 76% survival (compared to 51% survival in ECM mice receiving artemether only) [15]. The present study demonstrates that a single intrajugular transfusion of whole blood along with artemether resulted in a 90% survival of mice with ECM.

In the previous study, the intraperitoneal route of transfusion was chosen due to the difficulties of performing intravenous injection of large volumes in mice with ECM. However, the natural pathway for blood transfusion is the intravenous route, and a solution was achieved by means of intrajugular injection. This strategy resulted in a stronger increase in survival, and this effect was related to a more effective action on measured parameters of hematological and vascular health. Indeed, the findings in the present study independently confirm the protective effects of intraperitoneal whole blood transfusion, and now extend those findings to the more clinically relevant intravenous route of administration. Interestingly, intravenous transfusion appeared to have a greater effect on hematocrit, platelet counts, ANG-1 and ANG-2 levels, blood-brain barrier integrity, and survival compared to intraperitoneal transfusion.

Endothelial cell system activation and dysfunction are hallmarks of malaria pathogenesis [32–34]. Endothelial protein markers such as angiopoietins have been found to play a critical physiological role in maintenance of vascular integrity. It has been previously shown that the levels of the endothelial cell quiescence promoter ANG-1 are depleted, whereas the levels of endothelial cell activation/inflammation promoter ANG-2 are elevated, in mice with ECM [15]. It is noteworthy that blood transfusion given intraperitoneally had no effect on restoring ANG-1 levels, but when given intravenously a timid but significant increase in ANG-1 levels was observed after 6 hours, and a more robust increase was seen at 24 hours. And while ANG-2 levels were equally restored by either intraperitoneal of intravascular transfusion, the better effect on ANG-1 levels resulted also in a much better ANG-2/ANG-1 ratio by intravascular transfusion. Since ANG-1 and ANG-2 compete for binding to Tie2, with opposite effects, this improved profile achieved by intravascular transfusion likely results in a faster recovery of endothelial function. Indeed, reversing ANG-2/1

imbalance promotes endothelial cell survival, stabilizes endothelial interactions with supporting cells and limits vascular permeability [35–37]. ANG-1 stabilizes the BBB while ANG-2 weakens the pericyte-endothelial cell interaction, resulting in BBB disruption [34,38]. Since severity of cerebral malaria is related to vascular dysfunction [39] and much of the mortality occurs in the first 24 hours after antimalarial treatment, this faster recovery of endothelial function may be a critical factor explaining the substantial increase in survival in these animals.

A faster recovery of endothelial function may help to explain a more rapid reversal of BBB breakdown. The overall profile of Evans blue leakage observed after treatment of ECM mice with artemether or artemether plus intravenous blood transfusion was similar to that observed in the previous study using intraperitoneal transfusion [15]. However, ECM mice receiving intravenous transfusion showed a more profound recovery of BBB integrity, both at 6 and 24 hours after treatment. These findings are in line with a better profile of plasma ANG-1 and ANG-2/ANG-1 levels and with a better survival outcome. In the case of haptoglobin levels, the effects of intravenous transfusion were not different from those observed with intraperitoneal transfusion [15].

Malaria is a true hematological infectious disease, heavy parasite burden and hemolysis of parasitized and non-parasitized red blood cells may result in rapid decline of hematocrit [40,41]. Intravenous transfusion again resulted in a better hematological outcome compared to intraperitoneal transfusion. Whereas the latter only partially prevented the marked fall of hematocrit following artemether treatment observed in non-transfused mice [15], intravenous transfusion resulted in a more consistent maintenance of hematocrit. This can be in part explained by the fact that not all red blood cells transfused by intraperitoneal route actually reach the circulation [24]. But

there is also indication that blood transfusion does not lead only to a passive recovery of circulating red blood cell numbers. The magnitude of hematocrit recovery at 24 hours in transfused compared to non-transfused mice was greater than it would be expected by the amount of red blood cells transfused. Therefore, whole blood transfusion may either induce an active response of the recipient mice, enhancing bone marrow production of new red blood cells, or transfusion has a protective effect on the hemolytic effect of artemether. The actual mechanisms explaining these findings still need to be shown.

In any case, the maintenance of higher hematocrit has beneficial effects such as increasing shear stress [28], managing acid-base balance [13,42], improving red blood cell deformability, reducing microvascular obstruction, all of which help improving brain tissue oxygen delivery [43,44] [45].

Another one of the more well-known hematologic changes observed in patients with malaria is thrombocytopenia [46]. *Plasmodium falciparum* infected patients with a platelet count below 20×10^9/L were five times more likely to die than patients with a higher platelet count [47]. Intraperitoneal blood transfusion partially restored platelet counts in ECM mice [15]. But, again, the effect of intravenous blood transfusion was stronger, leading to a faster recovery of platelet counts and again the magnitude of the recovery was greater than it would be expected by a passive transfer of platelets contained in the transfused blood. Because platelets have been implicated with the pathogenesis of ECM, in processes such as coagulopathy [48], endothelial activation [32], cytoadherence [49] and auto-agglutination [50], restoring fresh platelets in a timely manner may be helpful in preventing mortality.

Finally, this study confirmed that, while whole blood transfusion is an intervention with high efficacy in preventing death by ECM, healthy plasma transfusion has the opposite effect, leading to increased mortality. This is probably related to increased circulating volume without increased oxygen carrying capacity which may worsen cardiovascular performance exacerbate pulmonary edema [51,52].

Collectively our data suggest that intravenous blood transfusion reversed anemia and thrombocytopenia, facilitated restoration of endothelial quiescence, improved vascular stability, leading to improved survival in experimental CM. These findings deserve more scrutiny to better understand the mechanisms behind the benefit achieved and provide additional support for translational studies.

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Figure Legends

Figure 1. Improved survival after blood transfusion plus artemether compared to artemether alone and plasma plus artemether in mice with ECM. *Plasmodium-berghei* ANKA-infected mice showing signs of ECM and rectal temperatures between 31 and 36 °C on day 6 of infection were treated with either: *i*) artemether 20mg/kg given intraperitoneally (IP) alone (n = 13); *ii*) artemether IP plus 200 µL of whole blood given intravenously (IV) (n = 19) or; *iii*) artemether ip plus 200 µL of plasma IV (n = 11). Whole blood or plasma were given only once, together with first artemether dose, whereas artemether was given daily for a total of 5 days. Adjunctive therapy with whole blood resulted in marked improved survival (90%) compared to artemether alone (54%; p = 0.0004). On the other hand, associating plasma therapy with artemether resulted in worsened outcome, with only 18% survival. Three separate experiments were performed, and the results were combined. For statistical analysis, log-rank Mantell-Cox test was performed.

Figure 2. Effect of intravenous whole blood transfusion plus artemether on plasma angiopoietin-1 (ANG-1) and angiopoietin-2 (ANG-2) profiles in mice with ECM. Plasma levels of ANG among studied groups (healthy controls, ECM =

experimental cerebral malaria, blood plus artemether [ARM] and artemether alone) were measured using ELISA (*n* = 10 mice per group). Mice with ECM showed depleted plasma ANG-1 levels (**A**) compared to uninfected controls (P < 0.0001). Treatment of ECM mice with artemether had no effect on ANG-1 levels, which remained very low. However, ANG-1 levels were slightly (mean 43%) but significantly higher in ECM mice that received whole blood transfusion compared to mice that received artemether alone at 6 hours (P = 0.0499) and at 24 hours whole blood transfusion led to a 2-fold increase in ANG-1 levels compared to artemether alone (P < 0.0001). (**B**) Plasma ANG-2 levels increased by 1.5 folds in ECM mice compared to normal controls (P = 0.0001). Treatment with artemether alone or artemether plus whole blood transfusion did not modify the increased ANG-2 levels 6 hours after treatment. At 24 hours, ECM mice that received whole blood transfusion showed plasma ANG-2 levels back to normal, whereas ECM mice that received artemether alone still showed increased levels (p = 0.0462). (**C**) The ratio of ANG-2 to ANG-1 was increased 10-fold in ECM mice compared to healthy controls (P < 0.0001). The levels kept increasing after 6 hours of artemether alone treatment but stabilized with artemether plus whole blood treatment (P = 0.0357). Within 24 hours, ANG-2 levels were still high in ECM mice that received artemether only, but were much lower in ECM mice that received artemether plus whole blood transfusion (P = 0.0002). Data are shown as mean ± standard deviation. For comparing groups at a given timepoint, Student's t-test was performed.

Figure 3: **Blood-brain barrier (BBB) permeability and plasma levels of haptoglobin in mice with ECM after treatment with artemether with or without intravenous blood transfusion. (A)** The BBB permeability (Evans blue assay) in mice with ECM was increased 2.7-fold in relation to healthy controls (P < 0.0001). BBB

permeability kept increasing (to 4.6-fold) 6 hours after treatment with artemether (ARM) alone, but intravenous transfusion of whole blood prevented the further increase. Indeed, Evans blue leakage in the brain of transfused mice was less than half that observed in mice receiving artemether alone ($p < 0.0001$). At 24 hours, BBB leakage decreased but was still evident in mice treated with artemether alone, whereas it returned to normal levels in mice that received artemether plus blood transfusion ($p = 0.0126$). **(B)** Haptoglobin levels in whole plasma was assessed using ELISA. Mice with ECM showed a mean 135% increase in plasma haptoglobin levels compared to uninfected controls ($P < 0.0001$). The levels of haptoglobin showed a decline in both treatment groups at 6 and 24 hours. No difference between the treatments was noticed any time point. Data are shown as mean ± standard deviation. For comparing groups at a given timepoint, Student's t-test was performed.

Figure 4. Effect of adjuvant intravenous blood transfusion at 6 and 24 hours on levels of hematocrit and platelet counts in mice with ECM. (A) Hematocrit: In ECM mice, pretreatment and pretransfusion hematocrit was 8% less than in healthy controls (mean; 46.16 % versus 50.12 %, $P = 0.0390$). Mice treated with artemether (ARM) alone showed further decrease in hematocrit after 6 and 24 hours ($P < 0.0001$). Intravenous blood transfusion plus ARM stabilized hematocrit levels at 6 and 24 hours (mean 48.8% and 46%, respectively). **(B) Platelet count:** ECM mice displayed around 90% reduction in platelet counts in relation to healthy controls ($P < 0.0001$). Intravenous blood transfusion led to partial recovery in platelet counts at 6 hours (2.9-fold; $P < 0.0001$) and at 24 hours (5.4-fold; $P < 0.0001$) compared to mice that received artemether alone. Data are shown as mean ± standard deviation. For comparing groups at a given timepoint, Student's t-test was performed.

Figure 1

Figure 1

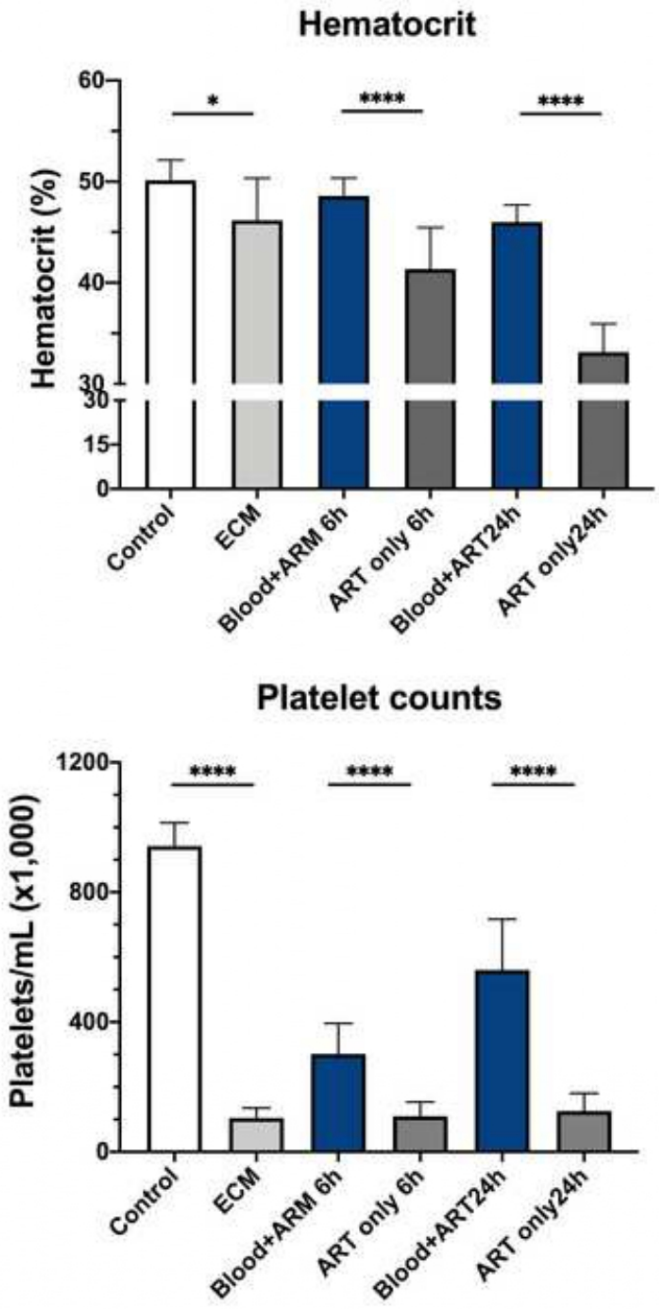

Figure 2

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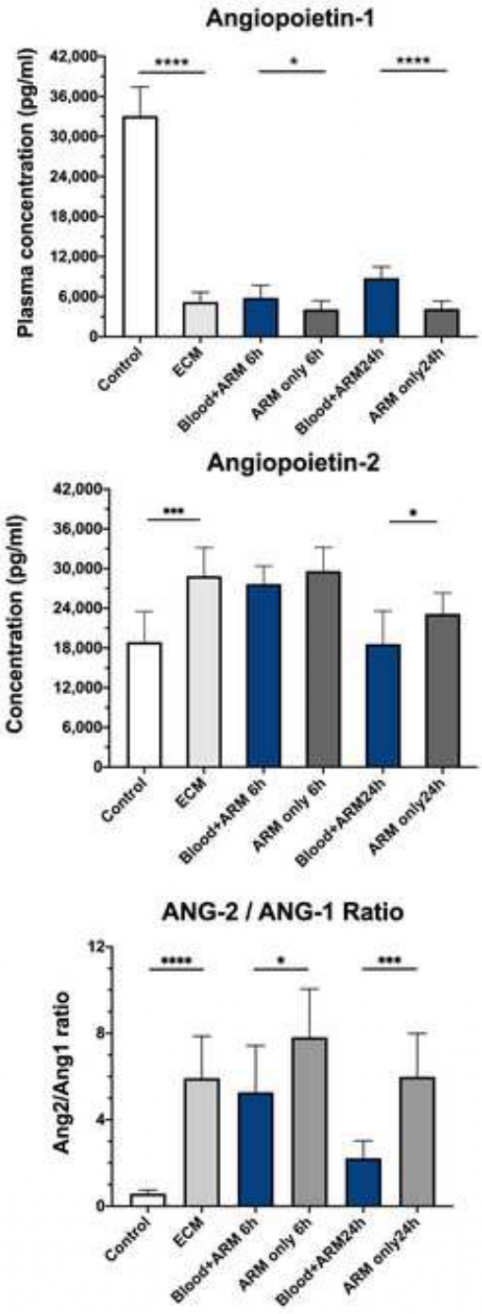

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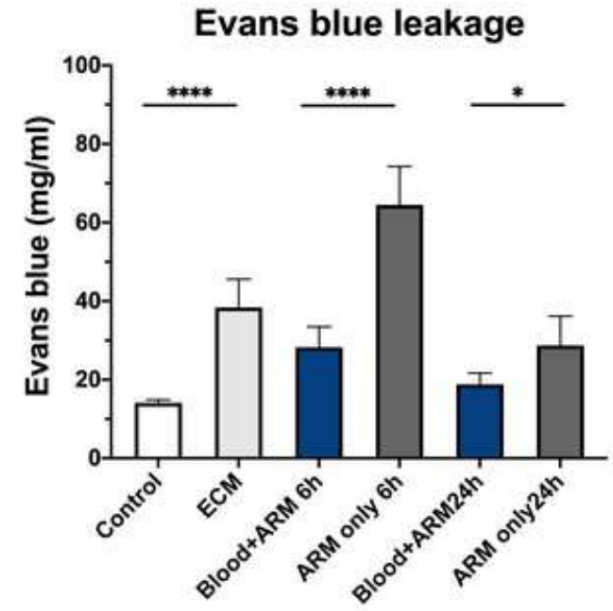

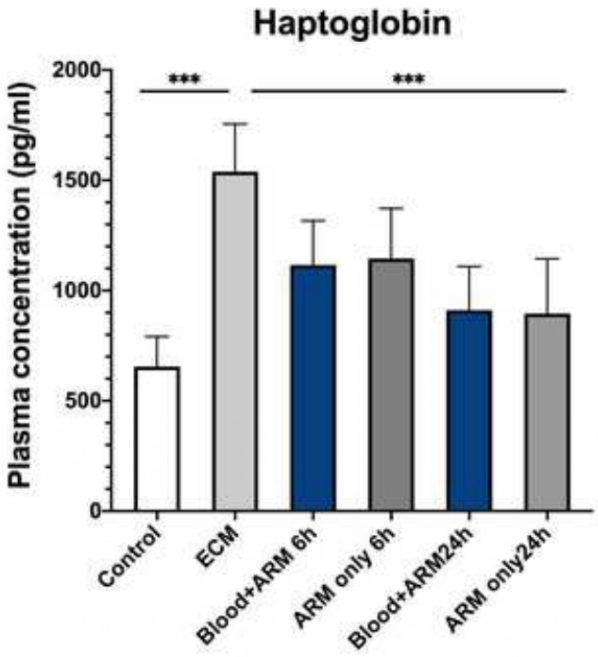

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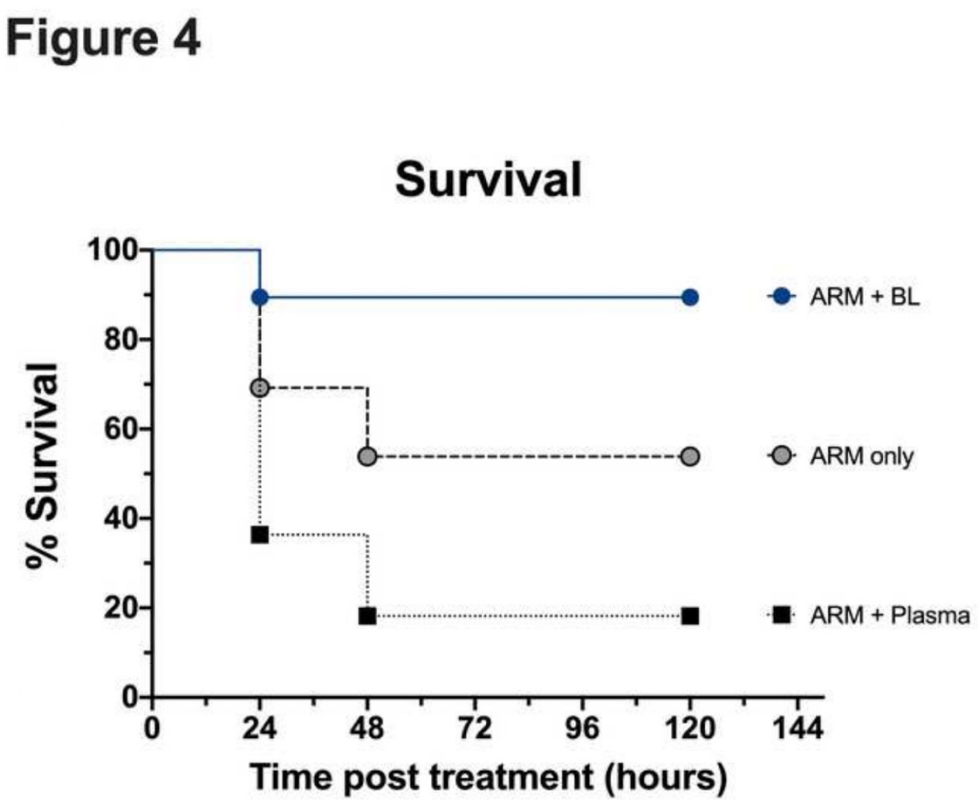

5 Discussion

Due to the high lethality and post treatment neurological sequalae in patients, cerebral malaria demands new adjuvant therapies. Blood transfusion has been extensively used in medical practice since early 20th century to treat anaemia and haemorrhage. Whole blood and exchange blood transfusion is used for severe *falciparum* malaria particularly in patients with severe anemia [177,189,190].

In the present study, whole blood transfusion resulted in substantial improvement in survival of mice with late-stage ECM treated with artemether. Mice with ECM showed only moderate (~10%) decrease in hematocrit at the time of neurological manifestations and treatment. However, artemether treatment resulted in further marked drops in hematocrit after 6 and 24 hours. This is possibly a result of the killing of parasites and consequent removal of infected red blood cells from the circulation,

but the magnitude of hematocrit drop in a short period suggests that other mechanisms may be acting. This phenomenon of red blood cell destruction following treatment with artemisinin derivatives has been reported also in human malaria [191–193]. A study conducted with children with uncomplicated malaria in a hyperendemic area in the Congo showed that patients treated either with intravenous artesunate or quinine had decreases in hemoglobin levels peaking at 48 hours, but only 5% in each group had decreases greater than 10% [194]. Children with severe malaria were not included in the study because intravenous artesunate has been shown to be clearly superior to quinine to treat severe malaria and, therefore, no randomization to a quinine group could be performed. However, more intense hemolysis (median reduction in hemoglobin of 6 g/dL), frequently requiring blood transfusion, has been reported in returned travelers presenting severe malaria treated with artemisinin [195]. In these cases, hemolysis is usually reported to occur several days after initiation of treatment, it is related with delayed lysis of once-infected erythrocytes resulting from splenic pitting and is associated with hyperparasitemia. The fact that mice with ECM show parasitemia over 10% and parasites are rapidly killed by artemether may help to explain the rapid decrease in hematocrit following treatment. Also, for practical purposes, mice with ECM were treated with a dose of artemether (20 mg/kg) that is 5-6 times higher than that used for the loading dose in human malaria (3.2-4.0 mg/kg) [196]. This is because a commercial artemether pediatric formulation (Artesiane 20 mg/mL in coconut oil) was used in this study and a dose equivalent to that of humans would require administration of very small volumes of 3-4 µL. Therefore, to avoid mistakes and variations in dosing, a volume of 20 µL was chosen to be delivered instead. While a higher dose may have potentiated the hemolytic effect of artemether, the findings of a number of studies showing that antimalarial drugs can cause different degrees of hemolysis especially in severe malaria should be taken into account when considering improvements in treatment for this condition.

Transfusion of 200 µL of whole blood to mice with ECM resulted in improved hematocrit at both 6 and 24 hours. Fresh red blood cells improve the hemorheological properties of the blood, which is known to be deteriorated in severe malaria infections [177,181]. And finally, lower hematocrit also results in decreased vascular wall shear stress [182], with decreased eNOS activity, which together with the nitric oxide-scavenging action of free hemoglobin leads to worsened endothelial dysfunction.

Therefore, increasing hematocrit through whole blood transfusion should help restore endothelial function and improve tissue perfusion. The effects on endothelial function are supported by the observation in this study that BBB breakdown worsened after artemether treatment and that this effect was prevented by blood transfusion.

Thrombocytopenia is one of the risk factors for mortality in African children with falciparum malaria. In a study of 75 Senegalese children with cerebral malaria, those with a platelet count of less than 100,000/μL were at greater risk of a fatal outcome than children with higher platelet counts [197]. Based on the platelet's well-known abilities to bind both infected and noninfected red cells as well as the endothelium, they are thought to be a major mediator of cerebral malaria. Activated platelets bind to infected red cells through platelet receptors, CD36 and gC1qR [198,199], and kill the parasites within [200]. Binding of activated platelets to infected erythrocytes and to endothelial cells may explain the sharp decline in the platelet counts in ECM mice.

When whole blood was used in combination with artemether as adjunctive therapy to ECM, it was found to induce significant upturn in circulating platelets in mice with ECM. The availability of fresh, quiescent platelets could help restoring the normality of the coagulation system without the deleterious inflammatory actions. This effect of partially restoring the platelet counts in 24 hours cannot be ascribed only to a passive, repository effect due to the platelets present in the transfused blood. If all platelets in the 200 μL of transfused blood reached the circulation, an 80% increase in platelet counts in artemether only-treated animals would be expected, but the actual increase reached 200%. Therefore, it is apparent that blood transfusion stimulates the body to actively respond, increasing platelet production. Other effects such as improved BBB response and decreased splenic congestion also support an active modulatory, rather than just repository, effect of blood transfusion. A thorough study of the effects of blood transfusion on hematological, cardiovascular, immunological, neurological and other parameters is necessary to unveil the critical events associated with the benefit of blood transfusion in ECM.

Angiogenic factors such as angiopoietins are critical regulators of endothelial integrity. Ang-1 maintains vascular quiescence by constitutively expressing and signaling through the Tie-2 receptor Ang-2 is stored in Weibel-Palade bodies in endothelial cells

and can be rapidly released upon endothelial activation and displace Ang-1, sensitizing the endothelium to low concentrations of inflammatory cytokines such as TNF [201]. Decreased levels of Ang-1 and increased levels of Ang-2 have been associated with pediatric severe, including cerebral, malaria and Ang-2 has been proposed as a marker for disease severity and a risk factor for cognitive injury [202–204]. The time course of the increase of von Willebrand factor in experimental malaria [205] suggests that Ang-2 release has a potential role in endothelial activation early in the course of disease. Altered angiopoietin concentrations were observed in ECM in the present study, with depletion of Ang-1 and increased levels of Ang-2, indicating relevant loss of vascular health [206]. In children with CM, plasma levels of Ang-2 as well as of soluble ICAM1 and C-reactive protein remain elevated for few weeks even after antimalarial treatment, indicating persistent inflammation and endothelial activation [207]. Accordingly, in the present study Ang-2 levels remained elevated in ECM mice treated with artemether only but went back to normal after 24 hours of blood transfusion, indicating that fresh blood rapidly counteracts the ECM-related inflammation and vascular insult.

Haptoglobin is an acute phase alpha2-glycoprotein that increases with conditions of inflammation and trauma [114]**.** The plasma concentration of haptoglobin increases several folds in the event of an inflammatory stimulus such as infection, injury or malignancy [208]. Interleukin-6 (IL-6) is the main inducer of the expression of this protein [209]. Higher haptoglobin levels were observed in individuals with symptomatic malaria than those with symptomless infection [210]. Consistent with the inflammatory response seen during ECM, our results found a positive correlation between the levels of Ang-2 and acute-phase protein such as haptoglobin. The increased levels of plasma haptoglobin in ECM is also supposed to be a response to intravascular hemolysis, leading to the release of free hemoglobin into the plasma [210–212]. The iron moiety of free hemoglobin can be oxidized by nitric oxide leading to oxidative stress [107,213]. To avoid this, free hemoglobin is scavenged by plasma haptoglobin [114,214] forming a complex, which in the case of humans is recognized by the CD163 receptor in monocytes and internalized [215]. Mouse haptoglobin-hemoglobin complex, on the other hand, presents low-affinity binding of hemoglobin to CD163, and CD163-mediated clearance seems to only account for a part of free hemoglobin removal in mice [216]. Acute and severe hemolysis usually leads to a consumption of haptoglobin, reducing

plasma levels, as seen in severe malaria [217]. It is possible that, in the case of ECM, a mild to moderate anemia in the ambient of an overwhelming pro-inflammatory response resulted in increased rather than decreased haptoglobin levels. This finding is of particular importance, because indeed, it had been assumed that acute-phase-driven stimulation of haptoglobin synthesis would counterbalance haptoglobin depletion in hemolysis [218,219]. The decrease in haptoglobin levels 24 hours after artemether treatment may be a result of the decrease in the inflammatory environment following parasite killing as well as consumption due to post-treatment hemolysis. Blood transfusion did not seem to interfere with haptoglobin levels following artemether treatment.

Neurological signs of ECM have been associated with the breakdown of the neuro-immunological BBB [163,220] and the disruption of microvascular permeability in humans has been associated with fatal CM [221–223]. In the present study, mice with ECM showed increased leakage of Evans blue in the brain, which was aggravated 6 hours after artemether treatment. This aggravation probably reflects a deterioration of endothelial function before the benefit of the antimalarial treatment can become apparent. On the other hand, whole blood transfusion prevented this deterioration of vascular health, and this is certainly a critical event to explain the beneficial effect of the intervention on survival. The mechanisms behind the beneficial effect of whole blood transfusion on BBB health are still unclear. Potential factors include increased oxygenation resulting from better oxygen-carrying capacity and also from improved cerebral perfusion as increased hematocrit with better hemorheological properties may help clear vessels blocked by parasitized erythrocytes and inflammatory cells, increasing blood flow and decreasing immune cell-mediated endothelial damage. Indeed, neuroinflammation and ischemia resulting from events such as stroke are known to disrupt the BBB [224]**.** Further studies are necessary to dissect the potential mechanisms involved.

In this study, we chose to administer whole blood at 10 mL/kg, which is half the usual amount in the setting of severe anemia, because mice with ECM showed only mild to moderate anemia at the time of treatment and also because malaria induces splenic congestion and a sudden increase in hematocrit might exacerbate this condition. Interestingly, blood transfusion actually helped to decrease the weight of enlarged

spleen in mice with ECM, suggesting that it helped to decrease congestion. It is possible that fresh red blood cells improved blood flow throughout the body, improving overall hemodynamics and decreasing the burden at the spleen. Indeed, in sickle cell disease acute splenic sequestration is treated by red blood cell transfusion [225]. The increase in spleen weight in mice with ECM was paralleled by an increase in the total splenocyte population. However, the magnitude of the increase in total splenocyte population was much lower than the increase in spleen weight (20 % versus 200 %), indicating that congestion with accumulation of blood-circulating cells was the major reason for the increased spleen weight. The vast majority of the increase in splenocyte numbers was in the B cell compartment. This is in line with previous findings showing that mice with ECM show extraordinary activation and proliferation of B cells, both in the germinal centers and in the marginal zones and intense extrafollicular plasmacytogenesis [226]. The absolute numbers of the T cell population in mice with ECM did not change significantly in relation to uninfected controls, but there was a change in the balance between CD4+ and CD8+ cells, with a marked drop in the percentage of CD8 T cells. CD8 T cells have been shown to play critical roles in the pathogenesis of ECM and also human CM [227,228]. At the time of manifestation of clinical signs, CD8 T cells are found in the brain and mediate endothelial cell damage through cytotoxicity mechanisms. Depletion of CD8 T cells has been shown to prevent development of ECM [229]. Therefore, the decrease in CD8 T cells in the spleen in mice with ECM as shown here may suggest that CD8 T cells are activated and then leave the spleen to migrate to other tissues, particularly the brain. Treatment with artemether, with or without blood transfusion, resulted in the increase in the number and percentage of T cells, with a restoration of the balance between CD4 and CD8 T cells. Also, treatment resulted in increased CD11b+ population, which is a marker of monocytes/macrophages, and this may be due to increased phagocytic activity following killing of parasites and resulting overload of cell debris and hemozoin. Overall, for all the phenomena discussed above involving splenocyte populations, mice treated with artemether that received or not blood transfusion behaved similarly, that is, blood transfusion had no effect on these specific outcomes.

The benefit of blood transfusion, however, on parameters such as hematocrit and platelet count restoration, decrease of plasma Ang-2 levels, prevention of further disruption of the BBB, decrease of spleen congestion, that is, its beneficial effect on

vascular function in mice with ECM treated with artemether was associated with a marked improvement in survival. This is obviously the outcome that really matters. These findings are in line with a recent elegant prospective multicenter observational study showing that blood transfusion improved survival of children hospitalized with severe falciparum malaria [186]. Blood transfusion is currently recommended by the WHO in severe malaria when hemoglobin is below 4.0 g/dL, or below 6.0 g/dL when associated with complications such as acidosis and coma. However, the authors showed that when signs of vital organ hypoperfusion are present, blood transfusion can benefit patients even at higher hemoglobin thresholds, indicating that it should be considered in severe malaria when hemoglobin is below 7.7g/dL. Furthermore, in children with impaired consciousness, transfusion can be considered at hemoglobin lower than 10.5g/dL, and when the patient shows severely elevated lactate concentration, transfusion should be considered regardless of the hemoglobin levels [186]. For patients with higher hemoglobin levels, our study in mice indicates that even the transfusion of half the usual blood volume can be of great benefit.

In the previous experiments, the intraperitoneal route of transfusion was chosen due to the difficulties of performing intravenous injection of large volumes in mice with ECM. However, the natural pathway for blood transfusion is the intravenous route, and a solution was achieved by means of intrajugular injection. This strategy resulted in a 90% survival of mice with ECM, and this effect was related to a more effective action of this route of transfusion on measured parameters of hematological and vascular health. Indeed, the findings in the present study largely reproduced those of the previous study, but intravascular transfusion resulted in greater effects on hematocrit, platelet counts, Ang-1and Ang-2 levels and blood-brain barrier integrity compared to intraperitoneal transfusion.

Endothelial cell (EC) system activation and dysfunction are hallmarks of malaria pathogenesis [68,230,231]. Endothelial protein markers such as Angiopoietins have been found to play a critical physiological role in maintenance of vascular integrity. It has been previously shown that the levels of the EC quiescence promoter Ang-1 are depleted, whereas the levels of EC activation/inflammation promoter Ang-2 are elevated, in mice with ECM [116]. It is noteworthy that blood transfusion given intraperitoneally had no effect on restoring Ang-1 levels, but when given intravenously

a timid but significant increase in Ang-1 levels was observed after 6 hours, and a more robust increase was seen at 24 hours. And while Ang-2 levels were equally restored by either intraperitoneal or intravascular transfusion, the better effect on Ang-1 levels resulted also in a much better Ang-2/ Ang-1ratio by intravascular transfusion. Since Ang-1 and Ang-2 compete for binding to Tie-2, with opposite effects, this improved profile achieved by intravascular transfusion likely results in a faster recovery of endothelial function. Indeed, reversing Ang-1/2 imbalance promotes EC survival, stabilizes endothelial interactions with supporting cells and limits vascular permeability [232–234]. Ang-1 stabilizes the BBB while Ang-2 weakens the pericyte-endothelial cell interaction, resulting in BBB disruption [231,235]. Since severity of cerebral malaria is related to vascular dysfunction [236] and much of the mortality occurs in the first 24 hours after antimalarial treatment, this faster recovery of endothelial function may be a critical factor explaining the substantial increase in survival in these animals.

A faster recovery of endothelial function may help to explain a more rapid reversal of the BBB breakdown. The overall profile of Evans blue leakage observed after treatment of ECM mice with artemether or artemether plus intravenous blood transfusion was similar to that observed in the previous study using intraperitoneal transfusion [116]. However, ECM mice receiving intravenous transfusion showed a more profound recovery of BBB integrity, both at 6 and 24 hours after treatment. These findings are in line with a better profile of plasma Ang-1 and Ang-2/Ang-1 levels and with a better survival outcome.

In the case of haptoglobin levels, the effects of intravenous transfusion were not different from those observed with intraperitoneal transfusion [116].

Malaria is a true hematological infectious disease, heavy parasite burden and hemolysis of parasitized and non-parasitized red blood cells may result in rapid decline in hematocrit [237,238]. Intravenous transfusion again resulted in a better hematological outcome compared to intraperitoneal transfusion. Whereas the latter only partially prevented the marked fall of hematocrit following artemether treatment observed in non-transfused mice [116], intravenous transfusion resulted in a more consistent maintenance of hematocrit. This can be in part explained by the fact that not all red blood cells transfused by intraperitoneal route actually reach the circulation [239]. But

there is also indication that blood transfusion does not lead only to a passive recovery of circulating red blood cell numbers. The magnitude of hematocrit recovery at 24 hours in transfused compared to non-transfused mice was greater than it would be expected by the amount of red blood cells transfused. Therefore, whole blood transfusion may either induce an active response of the recipient mice, enhancing bone marrow production of new red blood cells, or transfusion has a protective effect on the hemolytic effect of artemether. The actual mechanisms explaining these findings still need to be shown.

In any case, the maintenance of higher hematocrit has beneficial effects such as increasing shear stress [182], managing acid-base balance [186,240], improving red blood cell deformability, reducing microvascular obstruction, all of which help improving brain tissue oxygen delivery [241,242 243].

Another one of the more well-known hematologic changes observed in patients with malaria is thrombocytopenia [244]. *Plasmodium falciparum* infected patients with a platelet count below 20×10^9/L were 5 times more likely to die than patients with a higher platelet count [245]. Intraperitoneal blood transfusion partially restored platelet counts in ECM mice [116]. But, again, the effect of intravenous blood transfusion was stronger, leading to a faster recovery of platelet counts and again the magnitude of the recovery was greater than it would be expected by a passive transfer of platelets contained in the transfused blood. Because platelets have been implicated with the pathogenesis of ECM, in processes such as coagulopathy [246], endothelial activation [230], cytoadherence [247] and auto-agglutination [92], restoring fresh platelets in a timely manner may be helpful in preventing mortality.

Finally, this study confirmed that, while whole blood transfusion is an intervention with high efficacy in preventing death by ECM, healthy plasma transfusion has the opposite effect, leading to increased mortality. This is probably related to increased fluid volume which may exacerbate lung water [248,249]. Collectively our data suggest that intravenous blood transfusion facilitated reversal of endothelial quiescence, improved vascular stability and reversed anemia and thrombocytopenia, resulting in improved survival in experimental CM.

In conculsion, the transfusion of whole blood as an adjuvant therapy had a dynamic outcome both on physiological parameters of blood and on endothelium activation. Shortcomings of blood transfusion especially in transmission of other blood related diseases can be addressed, and even in resource-limited settings the availability of tests for HIV, hepatitis C virus and other blood-borne pathogens makes blood and blood products very safe for transfusion completed [250]. Younger children will benefit more as they are the most affected and because only a small amount of blood will be required, which can be easily obtained from parents and relatives. Moreover, it is important to mention that in highly endemic areas such as Africa virtually the entire adult population is hyperimmune to malaria and almost all blood donors have high levels of antibodies against *P. falciparum* adhesins, which would be of great potential benefit to eliminate sequestered parasites and reverse vessel blockage. Also, the expense is much lower than that of keeping patients in ICUs while antimalarial treatment is completed [250]. Conduction of additional studies to prove the potential of this strategy as a viable, cheap and effective adjunctive therapy for cerebral malaria is therefore worth pursuing. These findings deserve more scrutiny to better understand the mechanisms behind the benefit achieved and provide additional support for translational studies.

6 Conclusions

- Animals with experimental cerebral malaria (ECM) show mild to moderate anemia, severe thrombocytopenia and deranged vascular function revealed by increased angiopoietin-2 levels, depleted angiopoietin-1 levels and breakdown of the blood-brain barrier, as well as potent inflammatory responses revealed by increased haptoglobin levels and intense proliferation of splenocytes.
- In animals with ECM, artemether treatment resulted in continuous decline of hematocrit.
- Blood transfusion in combination with artemether preserved the hematocrit levels of ECM animals.
- The treatment of animals with ECM with blood transfusion as adjuvant treatment helped to partially restore platelets counts.

- Animals that developed experimental cerebral malaria showed a significant increase in haptoglobin levels on the sixth day after infection when compared to uninfected control animals. Haptoglobin levels returned to normal level after 24 hours post treatments.
- Blood transfusion combined with artemether had reversed Ang-2 levels back to normal. Intravenous blood transfusion has additional beneficial outcomes by improving Ang-1 levels. The ratio of Ang-2/ Ang-1 showed no significant difference to healthy control after 24 hours post treatment.
- Cerebrovascular Evans blue leakage, showed aggravation at 6 hours post treatment with artemether alone. Treatment of animals with artemether plus blood transfusion stopped further aggravation of breakdown of the blood-brain barrier.
- Animals with ECM showed increased spleen weight, treatment with artemether plus whole blood helped to reverse splenic congestion.
- Blood transfusion had no additional effect on the spleen cell populations compared to artemether alone.
- Transfusion of whole blood on the first day of treatment with artemether led to a faster recovery of the body weight.
- Treatment of ECM animals with intraperitoneal blood transfusion resulted in 75.9% survival rate.
- Intravenous blood transfusion evaluated survival rate to 90%, whereas plasma transfusion worsened the disease severity.

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