Invited Review

Severe malaria: what's new on the pathogenesis front?

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ABSTRACT

Plasmodium falciparum causes the most severe and fatal form of malaria in humans with over half a million deaths each year. Cerebral malaria (CM), a complex neurological syndrome of severe falciparum malaria, is often fatal and represents a major public health burden. Despite vigorous efforts, the pathophysiology of CM remains to be elucidated, thereby hindering the development of adjunctive therapies. In recent years, multidisciplinary and collaborative approaches have led to groundbreaking progress both in the laboratory and in the field. Here we review the latest breakthroughs in severe malaria pathogenesis, with a specific focus on new pathogenetic mechanisms leading to CM. The most recent findings point towards specific parasite phenotypes targeting brain microvasculature, endothelial dysfunction and subsequent oedema-induced brain swelling.

1. Introduction

Malaria is still a leading cause of morbidity and mortality in the developing world. The virulence of *Plasmodium falciparum* is caused by several factors including parasite proteins on the surface of infected erythrocytes (IE). These allow the binding of these cells to the microvascular endothelium of various organs and tissues during infection. Proteins of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family mediate this adhesion through specific binding to multiple cell receptors. These include intercellular adhesion molecule-1 (ICAM-1), CD36, E-selectin, neural cell adhesion molecule (NCAM) and CD31 (PECAM-1) for endothelial beds, as well as chondroitin sulfate A (CSA) for placental syncytiotrophoblasts. Binding to endothelium results in widespread sequestration of IE, which can lead to lead to endothelial activation as well as pro-inflammatory and pro-coagulant responses.

Severe falciparum malaria encompasses a broad range of diseases, the development of which may be influenced by age, exposure and immune status (Wassmer et al., 2015). It includes complications that affect specific organs such as the brain in cerebral malaria (CM) or the placenta in malaria in pregnancy (MiP). Histopathology and laboratory studies allowed investigators to establish a causal link between placenta-specific sequestration of *P. falciparum* and MiP. Indeed, the ability of PfEMP1 variants to target different receptors, the expression of which varies depending on the organ, could explain why some patients with malaria develop organ-specific syndromes. Researchers have speculated that a specific PfEMP1 variant could bind receptors that are preferentially expressed in cerebral microvasculature, and could account for the focal manifestations observed in CM, the most lethal complication of *P. falciparum* infection. Two recent reports simultaneously shed new light on the pathogenetic mechanisms leading to CM. First, endothelial protein C receptor (EPCR) was identified as a binding partner for PfEMP1. Second, normally low levels of EPCR in brain microvessels were shown to be further down-regulated in CM, with a loss of EPCR and thrombomodulin at sites of IE sequestration. These studies provided new clues towards parasite and host cell interactions leading to CM, and connected for the first time brain-specific sequestration of EPCR-binding parasites to the loss of the protein C anti-coagulant function and endothelial cytoprotective pathways (Aird et al., 2014).

While the relative frequency of severe malaria is low, its reported case fatality rate has not substantially changed over decades, especially for CM (Manning et al., 2014). Due to the lack of specific neuro- and vasculoprotective therapies, treatments for CM are currently still precariously limited to antimalarial drugs and

emergency supportive care. The former are quickly dwindling, as the resistance of *P. falciparum* malaria against artemisinin combination treatments, the recommended first-line therapy for infected patients, is on the rise in southeastern Asia. Multi-drug-resistant falciparum malaria is increasingly difficult to treat and new antimalarials are not expected to become available within the next few years. This underlines the necessity for molecular markers for surveillance of partner drug resistance, in conjunction with the implementation of new biomarkers for early diagnosis and outcome prediction, as well as effective adjunct therapies.

Here we review some recent data with a focus on newly developed research approaches aimed at a better understanding of the pathogenetic mechanisms of severe malaria in general and CM in particular.

2. Parasite-brain microvasculature specificity in CM: a virulence factor?

The severity of *P. falciparum* is linked to sequestration of IEs within the microvasculature of various organs including the brain. This sequestration is driven both by the expressed *var* gene in the parasite, leading to the expression of a specific variant of PfEMP-1, and the presence of its associated receptors on microvascular walls (Hviid and Jensen, 2015). Since there are considerable variations in both adhesion molecule expression and functional properties of endothelial cells depending on their position within the vascular bed of a particular tissue, it has been hypothesised that CM may result from a brain endothelial-specific adhesive type of parasite (Moxon et al., 2014). Indeed, IE expressing the domain cassettes (DC) 8 and 13 of the cytoadherent ligand PfEMP-1 adhere to EPCR (Turner et al., 2013). By interfering with EPCR anti-coagulant and pro-endothelial barrier functions, IE adhesion could promote coagulation and vascular permeability that contribute to the pathogenesis of CM (Moxon et al., 2014). To better understand parasite factors that contribute to disease severity, Gillrie et al. (2015) developed in vitro binding models for different microvascular beds to examine the adhesion of DC8- and DC13-expressing parasite lines to endothelial cells from different microvasculature, and the consequences of EPCR engagement on endothelial cell function. They reported that IE from IT4var19 (DC8) and IT4var07 (DC13) parasite lines adhere to human brain, lung and dermal endothelial cells under shear stress. However, the relative contribution of EPCR to parasite cytoadherence on different types of endothelial cell varied.

Divergent functional outcomes for DC8 cysteine-rich interdomain region (CIDR) α1.1 and DC13 CIDRα1.4 domains were also observed. IT4var07 CIDRα1.4 inhibited generation of activated protein C (APC) on lung

89   and dermal endothelial cells and blocked the APC-EPCR binding interaction on brain endothelial cells.

90   IT4var19 CIDRα1.1 inhibited thrombin-induced endothelial barrier dysfunction in lung endothelial cells,

91   whereas IT4var07 CIDRα1.4 inhibited the protective effect of APC on thrombin-induced permeability. Overall,

92   these findings reveal a much greater complexity of how CIDRα1-expressing parasites may

93   modulate malaria pathogenesis through EPCR adhesion (Gillrie et al., 2015). DC8 PfEMP1 encode multiple

94   endothelial binding domains, including binding activity for EPCR. These results show that PfEMP1 domains

95   compete with protein C for EPCR binding but the extent of competition differs between domains.

96   Bernabeu et al. (2016) recently investigated these parasite virulence factors in adult patients in India and

97   demonstrated that specific EPCR-binding parasites lead to severe malaria in that population. In addition,

98   parasite phenotype and biomass are associated with patient hospitalization and disease severity. The authors

99   show a broad range of EPCR binding activity from severe malaria isolates and even parasite domains that

100   partially obstructed the interaction between EPCR and APC were sufficient to interfere with the cytoprotective

101   functions of APC (Bernabeu et al., 2016). Taken together, their findings suggest that parasites may be under

102   selection for phenotypic variation in a key host pathway that regulates coagulation and endothelial barrier

103   properties, and has important implications for pathogenic mechanisms in severe malaria. Full- length

104   sequences of PfEMP1 encoding transcripts were characterized in clinical isolates from children with severe

105   malaria admitted to hospital in Tanzania, and EPCR-binding CIDRα1 domains dominated PfEMP1 transcript

106   profiles of children suffering from CM and/or severe malarial anaemia, further strengthening the evidence for a

107   crucial pathogenic role of the PfEMP1–EPCR interaction in severe malaria (Jespersen et al., 2016).

108   EPCR is not the only receptor suspected to play an important role in the development of severe malaria.

109   Studies of parasite isolates have demonstrated high rates of in vitro ICAM-1 binding among wild strains but

110   reported correlations between ICAM-1 binding and disease severity have been inconsistent. Recent results

111   suggest that ICAM-1 is a co-receptor for a subset of EPCR-binding parasites (Avril et al., 2016). Indeed, Avril

112   et al. (2016) showed that parasites expressing DC13 have dual binding specificity for EPCR and ICAM-1,

113   suggesting for the first time that ICAM-1-binding variants can be grouped into CD36 and EPCR co-receptor-

114   binding traits. This leads to different cytoadherence abilities on TNF-stimulated endothelial cells, which has

115   important implications for understanding parasite organ-specific microvascular bed tropism in pro-inflammatory

116   conditions.

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3. Microvascular endothelial dysfunction: new causes and repercussions

In the recent years, convincing evidence has been presented to support the role of both endothelial cell activation and platelets in modulating the pathogenesis of severe *P. falciparum* malaria. Thrombin, a common factor in both processes, is now thought to be a driver of pathology in CM. The relative contribution of EPCR-binding parasites versus loss of EPCR from the endothelial surface in mediating CM is not known, although both are associated with disease in clinical studies (Turner et al., 2013; Moxon et al., 2014). The key mediator of inflammation in both cases appears to be thrombin, which is a potent mediator of both pro-and anti-inflammatory pathways, depending on the context of endothelial signalling. Thrombin engages thrombomodulin on the plasma membrane of intact endothelium, where it promotes activation of protein C. The latter is accelerated by the presence of EPCR, which binds to protein C and presents it for optimal activation by the thrombin-thrombomodulin complex. Further to its anticoagulant activity, generated APC can also trigger numerous cell-signaling pathways initiating protective cellular responses upon exposure to pro-inflammatory, pro-apoptotic, or toxic insult. IEs expressing PfEMP1 compete with protein C and APC for EPCR, thereby down-regulating protein C activation by the thrombin-thrombomodulin complex. The resulting loss of EPCR-APC–induced cytoprotective signalling is consistent with a decrease in blood-brain barrier (BBB) properties, potentially leading to vasogenic oedema in CM.

Direct protein C pathway alteration by binding of EPCR-specific IEs is not the only cause of endothelial dysfunction in CM. Indeed, *P. falciparum* infection also initiates early endothelial and platelet activation, leading to coagulation dysregulation and microvascular lesions locally (Wassmer et al., 2011a). Furthermore, IEs elicit a myriad of signalling pathways leading to aberrant pro-coagulant effects, ultimately resulting in enhanced endothelial activation, damage and apoptosis (O'Sullivan et al., 2016). Lastly, severe falciparum malaria has been associated with a down-regulation of normal endogenous anticoagulant pathways. EC surface expression of thrombomodulin and EPCR are both reduced, likely through cytokine-enhanced shedding, leading to an increase of their soluble levels in plasma (Moxon et al., 2013). Together, these effects combine and lead to a significant reduction in generation of anti-inflammatory and cytoprotective APC on the endothelial surface. These findings suggest new avenues for acute therapeutic intervention and match well with post-mortem observations and magnetic resonance imaging (MRI) findings linked to mortality in CM.

145   Such an endothelial dysfunction is not only observed in severe falciparum malaria, but was also reported in

146   severe *Plasmodium vivax* (Barber et al., 2015) and *Plasmodium knowlesi* (Yeo et al., 2007) cases, which

147   raises the question of possible common pathogenic pathways in these various types of infection. All plasmodia

148   species can cause severe and fatal malaria. In falciparum malaria, the most common cause of severe malaria,

149   impaired tissue perfusion has long been recognised to arise from microvascular obstruction by IE adherent to

150   endothelial cells. More recently recognised is the contribution of concurrent impairment of nitric oxide (NO)

151   bioavailability, endothelial activation and microvascular dysfunction to impaired tissue perfusion and severe

152   disease (Yeo et al., 2014). Angiopoietin-2, released from endothelial cell Weibel-Palade bodies and an NO-

153   inhibited autocrine mediator of endothelial activation, is markedly elevated in severe falciparum malaria and

154   consistently associated with impaired tissue perfusion and fatal outcome in both adult and paediatric severe

155   malaria. This is independent of both total and sequestered parasite biomass, suggesting that microvascular

156   obstruction and microvascular dysfunction make separate contributions to pathogenesis. Microvascular

157   function, the capacity to increase flow and oxygen delivery in response to ischaemia, is decreased in severe

158   falciparum malaria and associated with an increased risk of death. Endothelial activation, decreased

159   endothelial NO bioavailability and microvascular dysfunction are also associated with impaired tissue perfusion

160   in vivax and knowlesi malaria, a factor likely to contribute to organ dysfunction and severe malaria caused by

161   infection with these species. Initial clinical trials of agents aimed at restoring NO bioavailability in severe

162   falciparum malaria have recently been completed (Hawkes et al., 2011; Serghides et al., 2011), and

163   microvascular function can be improved by L-arginine infusion in severe falciparum malaria (Yeo et al., 2013).

164   These results suggest that new compounds aimed at increasing microvascular NO and microvascular

165   functions have potential clinical benefits as adjunctive treatments in severe malaria from all species.

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167   **4. Rosetting and clumping: consequences for sequestration and microvascular obstruction**

168   Parasite adhesion interactions in severe falciparum malaria are not restricted to the endothelium. Indeed,

169   IEs can bind uninfected erythrocytes to form rosettes (Handunnetti et al., 1989), or platelets to form clumps

170   (Pain et al., 2001). Both processes have been associated with severe malaria (Rowe et al., 1995; Pain et al.,

171   2001) and CM (Carlson et al., 1990; Wassmer et al., 2008).

In this context, recent advances have been made in elucidating the molecular mechanisms underlying rosetting, with a view to development of rosette-reversing therapies. Rosetting generally requires the presence of soluble serum factors such as IgM, and the ability of IE to form rosettes and to bind non-immune IgM is correlated (Rowe et al., 2002). While the role of IgM binding in rosetting remains unclear, it appears to strengthen the bond of the central IE with the surrounding erythrocytes. Stevenson et al. (2015) recently reported that the serum protein α_2-macroglobulin (α_2M) is able to induce rosetting in vitro and ex vivo, using several parasite isolates. In contrast to IgM, α_2M elicits rosetting alone, while the presence of IgM significantly lowers the concentration of α_2M required. The authors of the study postulate that α_2M allows the crosslinking of several individual PfEMP1 molecules, thereby increasing their combined avidity for carbohydrate receptors on surrounding erythrocytes and promoting the formation of rosettes. These results suggest that *P. falciparum* hijacks soluble host proteins for its own benefit, and avoids immune destruction by manipulating those to facilitate adhesion of IEs to low-affinity carbohydrate receptors. In addition, another report recently showed that PfEMP1 is not the only parasite ligand used in rosetting, potentially opening new rosette-disrupting approaches. Goel et al. (2015) showed in a very elegant study that the repetitive interspersed family of proteins (RIFINs) mediates binding of IEs with a preference for blood group A, leading to the formation of large rosettes of 10 or more IEs. This was not observed with IEs from group O. Indeed, blood group O is common in malaria endemic areas and IEs of that blood group were shown to form small, weak rosettes (Rowe et al., 2007). The role of RIFINs was confirmed by the disruption of rosettes in the presence of anti-RIFIN antibodies. These results suggest that RIFINs not only play a fundamental role in the development of severe malaria but also contribute to the virulence of *P. falciparum*.

Puzzlingly, formed rosettes have never been described in the peripheral bloodstream, suggesting that they sequester in the microvasculature and aggravate microvascular obstructions during severe malaria in general, and CM in particular. Understanding the mechanisms by which rosettes sequester (i.e., by direct binding of rosetting IE to endothelial cells, to platelets on endothelial cells or to non-rosetting cytoadherent IE is fundamental, as it may open new adjunct therapies to reduce the biomass of sequestered IE, a parameter associated with disease severity (Hendriksen et al., 2012). IE of the IT/R29 strain expressing a rosette-mediating PfEMP1 variant (IT4var09) were recently shown to cytoadhere to human brain microvascular endothelial cells using heparan sulfate proteoglycans as ligands (Adams et al., 2014). This process is distinct

from rosetting, which is primarily mediated by interactions with between the NTS-DBL1α domain of PfEMP1 and complement receptor 1. This study shows for the first time that IT4var09-expressing parasites are capable of dual interactions with both endothelial cells and uninfected erythrocytes via distinct receptor-ligand interactions, and therefore could aggravate microvascular obstruction in severe malaria by facilitating the sequestration of platelet-mediated clumps.

One of the most recently described *P. falciparum* cytoadherence phenotypes is the ability of IEs to bind to platelets in suspension in vitro to form platelet-mediated clumps. Similar to rosetting, the formation of clumps is a common phenotype and has been reported in a variety of endemic settings (Rowe et al., 2009). This is mediated by the binding of IEs to platelet receptors which include CD36 (Pain et al., 2001), P-selectin (Wassmer et al., 2008) and globular C1q receptor (gC1qR) (Biswas et al., 2007). In all cases, the parasite ligands are unknown, although PfEMP1 is a likely candidate molecule. A recent study performed using clinical isolates from Mozambican patients to evaluate cytoadherence properties such as platelet-mediated clumping, rosetting and adhesion to purified receptors (CD36, ICAM1 and gC1qR) revealed that, compared with matched controls, prevalence of both rosetting and platelet-mediated clumping and adhesion to gC1qR was higher in severe cases (Mayor et al., 2011). Inhibition of these cytoadherence phenotypes may therefore reduce the occurrence or improve the prognosis of severe malaria outcomes. Similar to rosettes, platelet-mediated clumps have not been observed in the bloodstream and it is likely that they sequester in the microvasculature using IE or platelet receptors (or both) to cytoadhere on endothelial cells (Wassmer et al., 2011a). Since this may potentially aggravate microvascular obstruction in CM, further studies aimed at understanding the molecular process involved in their sequestration are warranted.

5. Clinical consequences of BBB opening in CM: vasogenic oedema and brain swelling

Following the report by Seydel et al. (2015), a study was initiated to investigate the different mechanisms potentially responsible for brain swelling in both pediatric and adult CM patients in India (Wassmer et al., 2015). Brain swelling was identified by MRI in over 50% of patients enrolled in the ongoing study, irrespective of their age group. The frequent occurrence of brain swelling in CM has been previously reported in separate studies on Indian adults using computed tomography (CT) (Mohanty et al., 2011), as well as in Bangladeshi adults (Maude et al., 2014) and Malawian children using MRI (Potchen et al., 2012). The cause of the swelling,

228   which can lead to brain herniation in fatal cases (Seydel et al., 2015), remains to be identified and may be due

229   to cytotoxic or vasogenic causes. Recent results show that patients who develop CM exhibit an over-reaction

230   of their endothelial cells to systemic inflammation, which is not observed in uncomplicated malaria (Wassmer

231   et al., 2011b). Such vulnerability, coupled to a loss of cytoprotective EPCR in the brain, could lead to a

232   disruption of the BBB and the leakage of fluids and proteins from the vascular system into

233   the extracellular space. Also called vasogenic oedema, this process is consistent with the description of ring

234   haemorrhages, a characteristic sign of BBB disruption, in pediatric CM (Taylor et al., 2004; Dorovini-Zis et al.,

235   2011). In addition, these ring hemorrhages correlate positively between retinal and cerebral tissues, owing to

236   their common embryological origin from the neuroectoderm (Barrera et al., 2015; Greiner et al., 2015). Malaria

237   retinopathies seen in pediatric CM are therefore a direct reflection of the neurovascular disease process, and

238   the high frequency of ring haemorrhages both in the brain and in the retina in fatal disease point towards the

239   occurrence of vasogenic oedema. However, evidence for a generalised increase in BBB permeability leading

240   to vasogenic oedema is still debated. A study performed in Thailand showed no evidence of BBB impairment,

241   as radioactively-labelled albumin given intravenously was not found in the cerebrospinal fluid (CSF) of CM

242   patients during coma (Warrell et al., 1986). Additionally, post-mortem analyses of adult Vietnamese patients

243   who died of CM showed that its pathophysiology only involved subtle functional changes in BBB integrity

244   (Brown et al., 2000), and evidence from a different cohort demonstrated that localised loss of vascular integrity

245   did not correlate with the occurrence of pre-mortem coma (Medana et al., 2011). Brain swelling may also occur

246   as a result of cytotoxic oedema, which can result from cells in the cerebral tissue being unable to maintain

247   membrane potential after the failure of Na^+/K^+ ATP-dependent pumps, due to hypoxia or nutrient deprivation.

248   This may cause a redistribution of water from the extra-cellular to the intracellular compartments, ultimately

249   leading to cell swelling, cell death and tissue damage. Ischaemic or hypoxic insults due to mechanical effects

250   of microvascular obstruction by IEs, rosettes and platelet-mediated clumps, as well as nutrient "steal" by local

251   metabolically active parasites could account for cytotoxic oedema in CM. This mechanism is consistent with

252   abundant sequestration in the cerebral microvasculature during the neurologic syndrome (White et al., 2013),

253   and the first in vivo magnetic resonance study of experimental CM revealed a preponderant role for cytotoxic

254   oedema in fatal outcome (Penet et al., 2005).

255   An increase in intravascular fluid volume within the brain due to sludging of blood flow with sequestration of

256 IEs, rosettes and platelet-mediated clumps could also cause brain swelling and would explain the diffuse mild

257 brain swelling reported in severe malaria patients from Bangladesh (Maude et al., 2014), without evidence of

258 either cytotoxic or vasogenic oedema. However, cytoadherence itself may not be directly or solely responsible

259 for this clinical syndrome (Storm and Craig, 2014).

260 Lastly, it is entirely plausible that both mechanisms are not mutually exclusive. A study in live mice using

261 high-field MRI with whole-brain coverage showed that vasogenic oedema occurs first in infected animals, and

262 starts in the olfactory bulb before spreading deeper into the brain along a specific path called the rostral

263 migratory stream, eventually reaching the brain stem. Microvascular pathology and ischemic brain injury

264 develop only secondarily, after vasogenic oedema formation (Hoffmann et al., 2016). Additional MRI studies

265 are currently underway in CM patients from Asia and Africa, and are aiming to elucidate these pathogenetic

266 processes (Wassmer et al., 2015).

267

268 **6. New adjunct therapies and critical care approaches in severe malaria**

269 Even under optimal conditions, the case–fatality rate in severe malaria treated with either artemisinin

270 derivatives or quinine remains high. In addition, multi-drug-resistant falciparum malaria is increasingly difficult

271 to treat and new effective antimalarial agents are not expected to become available within the next few years.

272 In an effort to reduce malaria-related mortality, numerous adjunctive therapies that may alter severe malaria-

273 induced physiological abnormalities are being evaluated, some of which have been described in other sections

274 of this review. Such therapies are, in nature, highly specific to distinct syndromes of severe malaria, as their

275 aim is to target precise pathophysiological processes. For example, protein C system defects inflicted by

276 the malaria parasite protein PfEMP1 in CM can be overcome by a soluble EPCR variant (Petersen et al.,

277 2015), and could therefore represent a revolutionary approach to dampen the pathogenetic mechanisms and

278 decrease mortality in affected patients. Promising advances in adjunct therapies for other severe malaria

279 syndromes such as acute respiratory distress, acute kidney injury, severe anaemia and metabolic acidosis are

280 still lacking.

281 The potential clinical benefit of fluid resuscitation was recently investigated and liberal fluid loading in adults

282 with severe malaria showed no improvement in the acidosis and acute kidney injury, but increased the

283 incidence of acute pulmonary oedema (Hanson et al., 2014). However, a more conservative fluid strategy,

284 using a simple weight-based algorithm, led to a low incidence of acute respiratory distress without significant

285 deterioration in acid-base status, renal function, electrolyte profile or systemic haemodynamics, and was

286 associated with increased survival (Aung et al., 2015).

287   In addition to *P. falciparum*, severe cases of *P. vivax* and *P. knowlesi* have also been reported, although

288 sequestration is not a specific feature of either infection and the relative contribution of co-morbidities to clinical

289 manifestations, particularly in vivax malaria, remains to be investigated (Wassmer et al., 2015). Both parasitic

290 infections can cause acute pulmonary oedema with a clinical phenotype similar to that seen in severe

291 falciparum malaria. No studies of fluid resuscitation have been performed in these patients, but those are

292 warranted.

293

294 **7. Novel parasite factors involved in malarial pathogenesis and possible therapeutic targets**

295   In parallel to the development of adjunct therapies, a growing effort in the search for new and effective

296 pharmacotherapies has been triggered by the emergence of multi-drug resistance in *P. falciparum*. While still

297 in their infancy, these approaches are promising and provide a wide range of new therapeutic targets. Several

298 new parasite factors were recently identified and have emerged as potential drug target candidates. Among

299 those, G-quadruplex (G4) DNA motifs and RecQ helicases are newly described players in virulence gene

300 control in *P. falciparum*. G4s are four-stranded structures formed by the stacking of quartets of guanines, and

301 recent work has shown that they can form in vivo as well as in vitro, affecting DNA replication, transcription,

302 translation and telomere maintenance. Harris and Merrick (2015) first demonstrated that DNA helicases, as

303 well as histone-modifying proteins, can influence *var* gene dynamics in *P. falciparum*. Understanding the G4-

304 mediated regulation of the parasite virulence may open the door to novel therapeutic interventions.

305   *Plasmodium falciparum* exports parasite-encoded proteins involved in structural and functional remodelling

306 of the host cell. This process is essential for the development of the parasite and is also associated with its

307 virulence (Maier et al., 2009). Molecular chaperones of the heat shock protein (Hsp) family are prominent

308 members of the exportome, including various Hsp40s and one Hsp70. The first biochemical evidence for a

309 specific functional co-chaperone interaction between the exported malarial PFA0660w and PfHsp70-x was

310 recently reported (Daniyan et al., 2016). PFA0660w can stimulate the ATPase activity of PfHsp70-x and work

311 additively with it in a co-chaperone/chaperone interaction, resulting in protein aggregation suppression. The

authors also showed that PFA0660w could potentially act independently as a chaperone. These findings support the proposed role of PfHsp70-x and PFA0660w in parasite protein trafficking and folding in IEs. Further studies are underway to determine the molecular basis for the specificity of this interaction, and to identify inhibitors capable of disrupting it. All of the modifications to the IEs are induced by parasite proteins, most of which initially traffic from the parasite via the secretory pathway to the parasitophorous vacuole. There, proteins interact with a translocon complex called PTEX (*Plasmodium* translocon of exported proteins) that transports them across the vacuole membrane into the IE. Blocking protein export through blocking PTEX function leads to the arrest of parasite growth and loss of virulence (Kalanon et al., 2016), making PTEX an excellent drug target (Gilson et al., 2016).

Post-translational modification of histones is one of the key gene regulation mechanisms during the intra-erythrocyte development cycle of *P. falciparum*. Studies of proteins, which recognise and interact with histone post-translational modifications, are pivotal for understanding *P. falciparum* pathogenesis. Bromodomain proteins bind to acetylated lysines, often on histones, and frequently play a role in regulation of gene expression. *Plasmodium falciparum*-specific bromodomain protein 1 (PfBDP1) binds to chromatin at transcriptional start sites of invasion-related genes and directly controls their expression. Conditional PfBDP1 knockdown causes a significant defect in parasite invasion and growth (Josling et al., 2015). In parallel to these studies, several small molecule inhibitors have recently been reported to have a high affinity and specificity to bromodomains, and could represent a new therapeutic avenue in *P. falciparum* infection (Padmanabhan et al., 2016).

The unique plasticity of the epigenetic regulation in *P. falciparum* has also emerged as a pivotal virulence and pathogenicity factor in recent years. Karmodyia et al. (2015) performed genome-wide mapping of multiple histone modifications of *P. falciparum* and reported H3K36me2 as a global repressive mark, with gene regulation being fine-tuned by the ratio of activation marks to H3K36me2 (Karmodiya et al., 2015). Moreover, *var* genes are mostly poised and marked by a unique set of activation (H4ac) and repression (H3K9me3) marks, which are mutually exclusive to other *P. falciparum* housekeeping genes. A better characterization of epigenetic regulation in *P. falciparum* will lead to the identification of potential therapeutic targets (Ay et al., 2015). Indeed, disrupting the function of proteins responsible for maintaining heterochromatin, such as HP1 (Brancucci et al., 2014), could be an effective strategy to block parasite

340 replication during the asexual cycle. PfAP2-G, a transcription factor shown to drive gametocytogenesis, also

341 represents a promising target to disrupt malaria transmission (Coleman et al., 2014). Extensive

342 characterisations of the parasite epigenetic factors, as well as its post-transcriptional and translational control

343 processes, are likely to open new avenues for drug development against *P. falciparum*.

344

345 **8. Pathologies other than CM: MiP**

346 *Plasmodium falciparum* infection during pregnancy can result in ~~MiP,~~ a pathology ~~resulting from~~caused by

347 the accumulation of IEs in the placental intervillous space and the infiltration of maternal

348 monocytes/macrophages (Rogerson et al., 2003), with detrimental outcomes for both the mother and the

349 foetus. Expression of PfEMP1-var2csa at the surface of IEs mediates their adhesion to the placenta. Adaptive

350 immunity is progressively acquired during sequential malaria infections in pregnancy and is mediated by the

351 production of anti-VAR2CSA antibodies, which promote IE adhesion blocking and opsonisation (Desai et al.,

352 2007). This naturally acquired immunity is the key basis for development of a vaccine to protect women during

353 pregnancy, and VAR2CSA is currently the leading candidate (Fried and Duffy, 2015). However, recent findings

354 indicate that broadly neutralising antibodies of multigravidae are not depleted on VAR2CSA recombinant

355 antigens. Using a new approach to assess VAR2CSA domains for functional epitopes recognized by naturally

356 acquired antibodies, Doritchamou et al. (2016) recently demonstrated that different Duffy binding-like (DBL)

357 domain-specific IgG could react to both homologous as well as heterologous antigens and parasites,

358 suggesting that conserved epitopes are shared between allelic variants. In addition, IE binding was blocked by

359 ID1-DBL2-ID2a, DBL4 and DBL5-specific IgG, while partial cross-inhibition activity was observed with purified

360 IgG specific to ID1-DBL2-ID2a and DBL4 antigens. Interestingly, plasma from patients still showed broadly

361 adherence-blocking activity after complete depletion of these VAR2CSA specificities. These results suggest

362 that VAR2CSA vaccines based on a single construct and variant might induce antibodies with limited broad

363 blocking activity, and confirm that a multivalent vaccine comprised of up to five different alleles or with the

364 addition of multiple placental malaria vaccine candidates may be needed to elicit the broad blocking activity

365 observed in African multigravidae (Avril et al., 2010; Hommel et al., 2010).

366 The generation of protective vaccines is becoming a priority, especially in areas where the prevalence of

367 malaria has decreased due to control and elimination campaigns. Indeed, a recent study conducted in

368 Mozambique showed a close relationship between antibody levels and the intensity of malaria transmission.

369 Mayor et al. (2015) showed convincing evidence that a decline in the prevalence of malaria documented in the

370 study area was accompanied by reductions in levels of IgG antibodies not only against VAR2CSA, but also

371 against non-pregnancy-specific malaria antigens. In pregnant women with MiP, this was associated with an

372 increase in parasite densities and a higher adverse effect of *P. falciparum* infection on maternal haemoglobin

373 levels and newborn weights (Mayor et al., 2015). Although they also suggest that immunity may be regained as

374 exposure increases, the findings of this study indicate that malaria control and elimination programmes could

375 precede a resurgence of pregnancy-associated malaria pathologies.

376 Additionally, MiP has also recently been linked to placental pathology in a low malaria transmission area in

377 Brazil, where *P. vivax* is predominant (Souza et al., 2013). While MiP was not associated with severe outcome

378 in a second study performed by the same team in this region, an increased ratio of peripheral receptor tyrosine

379 kinase Tie-2 to angiopoetin (Ang-1) was associated with the occurrence of MiP. Both Ang-1 and Ang-2 had

380 similar magnitudes but inverse associations with placental barrier thickness. MiP is an effect modifier of the

381 association between Ang-1 and placental barrier thickness (Ataide et al., 2015). These findings provide a

382 possible pathway through which placental pathological changes occur during MiP.

383 Lastly, further to imbalances in cytokine cascades, IE cytoadhesion and angiogenic dysregulation,

384 excessive or dysregulated complement activation as part of the host innate immune response to malaria

385 infection can also exacerbate the severity of MiP, leading to poor pregnancy outcomes (McDonald et al.,

386 2015b). Using an experimental model of MiP in conjunction with micro-CT and HPLC analysis of

387 neurotransmitter levels, McDonald et al. (2015a) showed that complement activation, in particular C5a,

388 contributes to foetal neuropathologic outcomes during MiP. The offspring of infected animals showed

389 persistent neurocognitive deficits in memory and affective-like behaviour compared with unexposed controls

390 (McDonald et al., 2015a). These impairments were linked with decreased tissue levels of neurotransmitters in

391 regions of the brain associated with the deficits. The inhibition of maternal C5a complement receptor signaling

392 restored the levels of neurotransmitters and reversed the associated phenotype, suggesting new targets for

393 intervention in MiP aimed at decreasing foetal neuropathologic outcomes.

394

395 **9. New investigative tools and experimental models**

396   A vast array of new tools and models has recently become available to facilitate the investigation of severe

397   malaria pathogenesis, with a particular focus on CM. These are detailed elsewhere (Sahu et al., 2015). Further

398   to this, the rise of the 'omic' era during the past decade has provided the malaria research community with

399   unprecedented approaches and technologies to better understand the biology, evolution and pathogenesis of

400   different *Plasmodium* spp. The Malaria Host-Pathogen Interaction Centre (MaHPIC) is a large international

401   systems biology consortium developed in 2012 and based in the USA

402   (http://www.systemsbiology.emory.edu/index.html), which allowed the development of a variety of distinctive

403   hypothesis-generating and hypothesis-driven collaborations with scientific teams in malaria-endemic regions.

404   These collaborations involve investigators from several countries in South America, southeastern Asia and

405   sub-Saharan Africa, with a focus on infections caused by the predominant, less predominant, or mixed species

406   of *Plasmodium* (Anderson et al., 2015; Lapp et al., 2015), to study disease states, pathogenesis and

407   physiological or immunobiological questions through the use of untargeted high-resolution metabolomics, as

408   well as clinical and demographic metadata (Salinas et al., 2014). In addition, the MaHPIC team is intensively

409   studying malaria using non-human primate model systems (macaque and New World monkey species, (Joyner

410   et al., 2015)). An overarching goal, beyond the team's specific research quests, is to develop and make

411   available unique large 'omic' datasets (e.g., transcripts, proteins, lipids, metabolites and immune responses),

412   integrated models and tools for the use and benefit of the research community at large. This unique and

413   integrated combination of 'omic' approaches represents a revolutionary platform to identify, develop and

414   evaluate new diagnostic tools, antimalarial drugs and vaccines for different types of malaria parasites.

415   In parallel, live imaging, including multi-photon approaches, has contributed to the understanding of CM

416   pathogenesis, notably its haemodynamics (Nacer et al., 2014) and its immune cell mobilisation (Pai et al.,

417   2014) components. Recently, in experimental CM, vibrational spectroscopies provided evidence of

418   peroxidative stress and protein oxidation within cerebellar gray matter, which were co-localised with elevated

419   non-haeme iron at the site of microhaemorrhages. A novel combination of chemical probe-free, multimodal

420   imaging to quantify molecular markers of disturbed energy metabolism and peroxidative stress thus provides

421   new insights into understanding CM pathogenesis (Hackett et al., 2015).

422

423   **10. Conclusions and future perspectives**

424   Despite the recent leap in our understanding of pathogenetic mechanisms leading to severe malaria, the

425   translational outputs to improve the clinical outcome of patients remain meager. Collaborative and

426   multidisciplinary approaches using clinical samples from field sites in endemic areas, in vitro and ex vivo

427   models, as well as animal models of the disease, are crucial to allow global advances in the fight not only

428   against severe falciparum malaria, but also emerging public health issues such as severe malaria caused by *P.*

429   *vivax* and *P. knowlesi*.

430

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References

439

440   Adams, Y., Kuhnrae, P., Higgins, M.K., Ghumra, A., Rowe, J.A., 2014. Rosetting *Plasmodium falciparum*-

441   infected erythrocytes bind to human brain microvascular endothelial cells in vitro, demonstrating a dual

442   adhesion phenotype mediated by distinct *P. falciparum* erythrocyte membrane protein 1 domains. Infect

443   Immun 82, 949-959.

444   Aird, W.C., Mosnier, L.O., Fairhurst, R.M., 2014. *Plasmodium falciparum* picks (on) EPCR. Blood 123, 163-

445   167.

446   Anderson, D.C., Lapp, S.A., Akinyi, S., Meyer, E.V., Barnwell, J.W., Korir-Morrison, C., Galinski, M.R., 2015.

447   *Plasmodium vivax* trophozoite-stage proteomes. J Proteomics 115, 157-176.

448   Ataide, R., Murillo, O., Dombrowski, J.G., Souza, R.M., Lima, F.A., Lima, G.F., Hristov, A.D., Valle, S.C., Di

449   Santi, S.M., Epiphanio, S., Marinho, C.R., 2015. Malaria in Pregnancy Interacts with and Alters the

450   Angiogenic Profiles of the Placenta. PLoS Negl Trop Dis 9, e0003824.

451   Aung, N.M., Kaung, M., Kyi, T.T., Kyaw, M.P., Min, M., Htet, Z.W., Anstey, N.M., Kyi, M.M., Hanson, J., 2015.

452   The Safety of a Conservative Fluid Replacement Strategy in Adults Hospitalised with Malaria. PloS One

453   10, e0143062.

454   Avril, M., Bernabeu, M., Benjamin, M., Brazier, A.J., Smith, J.D., 2016. Interaction between Endothelial Protein

455   C Receptor and Intercellular Adhesion Molecule 1 to Mediate Binding of *Plasmodium falciparum*-Infected

456   Erythrocytes to Endothelial Cells. mBio 7, e00615.

457   Avril, M., Cartwright, M.M., Hathaway, M.J., Hommel, M., Elliott, S.R., Williamson, K., Narum, D.L., Duffy, P.E.,

458   Fried, M., Beeson, J.G., Smith, J.D., 2010. Immunization with VAR2CSA-DBL5 recombinant protein elicits

459   broadly cross-reactive antibodies to placental *Plasmodium falciparum*-infected erythrocytes. Infect Immun

460   78, 2248-2256.

461   Ay, F., Bunnik, E.M., Varoquaux, N., Vert, J.P., Noble, W.S., Le Roch, K.G., 2015. Multiple dimensions of

462   epigenetic gene regulation in the malaria parasite *Plasmodium falciparum*: gene regulation via histone

463   modifications, nucleosome positioning and nuclear architecture in *P. falciparum*. BioEssays : News Rev

464   Mol Cell Dev Biol 37, 182-194.

Barber, B.E., William, T., Grigg, M.J., Parameswaran, U., Piera, K.A., Price, R.N., Yeo, T.W., Anstey, N.M., 2015. Parasite biomass-related inflammation, endothelial activation, microvascular dysfunction and disease severity in vivax malaria. PLoS Pathog 11, e1004558.

Barrera, V., Hiscott, P.S., Craig, A.G., White, V.A., Milner, D.A., Beare, N.A., MacCormick, I.J., Kamiza, S., Taylor, T.E., Molyneux, M.E., Harding, S.P., 2015. Severity of retinopathy parallels the degree of parasite sequestration in the eyes and brains of malawian children with fatal cerebral malaria. J Infect Dis 211, 1977-1986.

Bernabeu, M., Danziger, S.A., Avril, M., Vaz, M., Babar, P.H., Brazier, A.J., Herricks, T., Maki, J.N., Pereira, L., Mascarenhas, A., Gomes, E., Chery, L., Aitchison, J.D., Rathod, P.K., Smith, J.D., 2016. Severe adult malaria is associated with specific PfEMP1 adhesion types and high parasite biomass. Proc Natl Acad Sci (USA), 113, E3270-E3279.

Biswas, A.K., Hafiz, A., Banerjee, B., Kim, K.S., Datta, K., Chitnis, C.E., 2007. *Plasmodium falciparum* uses gC1qR/HABP1/p32 as a receptor to bind to vascular endothelium and for platelet-mediated clumping. PLoS Pathog 3, 1271-1280.

Brancucci, N.M., Bertschi, N.L., Zhu, L., Niederwieser, I., Chin, W.H., Wampfler, R., Freymond, C., Rottmann, M., Felger, I., Bozdech, Z., Voss, T.S., 2014. Heterochromatin protein 1 secures survival and transmission of malaria parasites. Cell Host Microbe 16, 165-176.

Brown, H.C., Chau, T.T., Mai, N.T., Day, N.P., Sinh, D.X., White, N.J., Hien, T.T., Farrar, J., Turner, G.D., 2000. Blood-brain barrier function in cerebral malaria and CNS infections in Vietnam. Neurology 55, 104-111.

Carlson, J., Helmby, H., Hill, A.V., Brewster, D., Greenwood, B.M., Wahlgren, M., 1990. Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. Lancet 336, 1457-1460.

Coleman, B.I., Skillman, K.M., Jiang, R.H., Childs, L.M., Altenhofen, L.M., Ganter, M., Leung, Y., Goldowitz, I., Kafsack, B.F., Marti, M., Llinas, M., Buckee, C.O., Duraisingh, M.T., 2014. A *Plasmodium falciparum* histone deacetylase regulates antigenic variation and gametocyte conversion. Cell Host Microbe 16, 177-186.

492   Daniyan, M.O., Boshoff, A., Prinsloo, E., Pesce, E.R., Blatch, G.L., 2016. The Malarial Exported PFA0660w Is

493   an Hsp40 Co-Chaperone of PfHsp70-x. PloS One 11, e0148517.

494   Desai, M., ter Kuile, F.O., Nosten, F., McGready, R., Asamoa, K., Brabin, B., Newman, R.D., 2007.

495   Epidemiology and burden of malaria in pregnancy. Lancet. Infect Dis 7, 93-104.

496   Doritchamou, J.Y., Herrera, R., Aebig, J.A., Morrison, R., Nguyen, V., Reiter, K., Shimp, R., MacDonald, N.J.,

497   Narum, D.L., Fried, M., Duffy, P.E., 2016. VAR2CSA domain-specific analysis of naturally acquired

498   functional antibodies to *P. falciparum* placental malaria. J Infect Dis, 214. 577-586.

499   Dorovini-Zis, K., Schmidt, K., Huynh, H., Fu, W., Whitten, R.O., Milner, D., Kamiza, S., Molyneux, M., Taylor,

500   T.E., 2011. The neuropathology of fatal cerebral malaria in malawian children. Am J Pathol 178, 2146-

501   2158.

502   Fried, M., Duffy, P.E., 2015. Designing a VAR2CSA-based vaccine to prevent placental malaria. Vaccine 33,

503   7483-7488.

504   Gillrie, M.R., Avril, M., Brazier, A.J., Davis, S.P., Stins, M.F., Smith, J.D., Ho, M., 2015. Diverse functional

505   outcomes of *Plasmodium falciparum* ligation of EPCR: potential implications for malarial pathogenesis.

506   Cell Micro 17, 1883-1899.

507   Gilson, P.R., Chisholm, S.A., Crabb, B.S., de Koning-Ward, T.F., 2016. Host cell remodelling in malaria

508   parasites: a new pool of potential drug targets. Int J Parasitol. [This issue].

509   Goel, S., Palmkvist, M., Moll, K., Joannin, N., Lara, P., Akhouri, R.R., Moradi, N., Ojemalm, K., Westman, M.,

510   Angeletti, D., Kjellin, H., Lehtio, J., Blixt, O., Idestrom, L., Gahmberg, C.G., Storry, J.R., Hult, A.K., Olsson,

511   M.L., von Heijne, G., Nilsson, I., Wahlgren, M., 2015. RIFINs are adhesins implicated in severe

512   *Plasmodium falciparum* malaria. Nat Med 21, 314-317.

513   Greiner, J., Dorovini-Zis, K., Taylor, T.E., Molyneux, M.E., Beare, N.A., Kamiza, S., White, V.A., 2015.

514   Correlation of hemorrhage, axonal damage, and blood-tissue barrier disruption in brain and retina of

515   Malawian children with fatal cerebral malaria. Front Cell Infect Microbiol 5, 18.

516   Hackett, M.J., Aitken, J.B., El-Assaad, F., McQuillan, J.A., Carter, E.A., Ball, H.J., Tobin, M.J., Paterson, D., de

517   Jonge, M.D., Siegele, R., Cohen, D.D., Vogt, S., Grau, G.E., Hunt, N.H., Lay, P.A., 2015. Mechanisms of

518   murine cerebral malaria: Multimodal imaging of altered cerebral metabolism and protein oxidation at

519   hemorrhage sites. Sci Adv 1, e1500911.

Handunnetti, S.M., David, P.H., Perera, K.L., Mendis, K.N., 1989. Uninfected erythrocytes form "rosettes" around *Plasmodium falciparum* infected erythrocytes. Am J Trop Med Hyg 40, 115-118.

Hanson, J., Anstey, N.M., Bihari, D., White, N.J., Day, N.P., Dondorp, A.M., 2014. The fluid management of adults with severe malaria. Crit Care 18, 642.

Harris, L.M., Merrick, C.J., 2015. G-quadruplexes in pathogens: a common route to virulence control? PLoS Pathog 11, e1004562.

Hawkes, M., Opoka, R.O., Namasopo, S., Miller, C., Conroy, A.L., Serghides, L., Kim, H., Thampi, N., Liles, W.C., John, C.C., Kain, K.C., 2011. Nitric oxide for the adjunctive treatment of severe malaria: hypothesis and rationale. Med Hypotheses 77, 437-444.

Hendriksen, I.C., Mwanga-Amumpaire, J., von Seidlein, L., Mtove, G., White, L.J., Olaosebikan, R., Lee, S.J., Tshefu, A.K., Woodrow, C., Amos, B., Karema, C., Saiwaew, S., Maitland, K., Gomes, E., Pan-Ngum, W., Gesase, S., Silamut, K., Reyburn, H., Joseph, S., Chotivanich, K., Fanello, C.I., Day, N.P., White, N.J., Dondorp, A.M., 2012. Diagnosing severe falciparum malaria in parasitaemic African children: a prospective evaluation of plasma PfHRP2 measurement. PLoS Med 9, e1001297.

Hoffmann, A., Pfeil, J., Alfonso, J., Kurz, F.T., Sahm, F., Heiland, S., Monyer, H., Bendszus, M., Mueller, A.K., Helluy, X., Pham, M., 2016. Experimental Cerebral Malaria Spreads along the Rostral Migratory Stream. PLoS Pathog 12, e1005470.

Hommel, M., Elliott, S.R., Soma, V., Kelly, G., Fowkes, F.J., Chesson, J.M., Duffy, M.F., Bockhorst, J., Avril, M., Mueller, I., Raiko, A., Stanisic, D.I., Rogerson, S.J., Smith, J.D., Beeson, J.G., 2010. Evaluation of the antigenic diversity of placenta-binding *Plasmodium falciparum* variants and the antibody repertoire among pregnant women. Infect Immun 78, 1963-1978.

Hviid, L., Jensen, A.T., 2015. PfEMP1 - A Parasite Protein Family of Key Importance in *Plasmodium falciparum* Malaria Immunity and Pathogenesis. Adv Parasitol 88, 51-84.

Jespersen, J.S., Wang, C.W., Mkumbaye, S.I., Minja, D.T., Petersen, B., Turner, L., Petersen, J.E., Lusingu, J.P., Theander, T.G., Lavstsen, T., 2016. *Plasmodium falciparum* var genes expressed in children with severe malaria encode CIDRalpha1 domains. EMBO Mol Med 8, 839-850.

Josling, G.A., Petter, M., Oehring, S.C., Gupta, A.P., Dietz, O., Wilson, D.W., Schubert, T., Langst, G., Gilson, P.R., Crabb, B.S., Moes, S., Jenoe, P., Lim, S.W., Brown, G.V., Bozdech, Z., Voss, T.S., Duffy, M.F.,

548   2015. A *Plasmodium falciparum* Bromodomain Protein Regulates Invasion Gene Expression. Cell Host

549   Microbe 17, 741-751.

550 Joyner, C., Barnwell, J.W., Galinski, M.R., 2015. No more monkeying around: primate malaria model systems

551   are key to understanding *Plasmodium vivax* liver-stage biology, hypnozoites, and relapses. Front Microbiol

552   6, 145.

553 Kalanon, M., Bargieri, D., Sturm, A., Matthews, K., Ghosh, S., Goodman, C.D., Thiberge, S., Mollard, V.,

554   McFadden, G.I., Menard, R., de Koning-Ward, T.F., 2016. The *Plasmodium* translocon of exported

555   proteins component EXP2 is critical for establishing a patent malaria infection in mice. Cell Microbiol 18,

556   399-412.

557 Karmodiya, K., Pradhan, S.J., Joshi, B., Jangid, R., Reddy, P.C., Galande, S., 2015. A comprehensive

558   epigenome map of *Plasmodium falciparum* reveals unique mechanisms of transcriptional regulation and

559   identifies H3K36me2 as a global mark of gene suppression. Epigenetics Chromatin 8, 32.

560 Lapp, S.A., Mok, S., Zhu, L., Wu, H., Preiser, P.R., Bozdech, Z., Galinski, M.R., 2015. *Plasmodium knowlesi*

561   gene expression differs in ex vivo compared to in vitro blood-stage cultures. Malaria J 14, 110.

562 Maier, A.G., Cooke, B.M., Cowman, A.F., Tilley, L., 2009. Malaria parasite proteins that remodel the host

563   erythrocyte. Nat Rev Microbiol 7, 341-354.

564 Manning, L., Laman, M., Davis, W.A., Davis, T.M., 2014. Clinical features and outcome in children with severe

565   *Plasmodium falciparum* malaria: a meta-analysis. PloS One 9, e86737.

566 Maude, R.J., Barkhof, F., Hassan, M.U., Ghose, A., Hossain, A., Abul Faiz, M., Choudhury, E., Rashid, R., Abu

567   Sayeed, A., Charunwatthana, P., Plewes, K., Kingston, H., Maude, R.R., Silamut, K., Day, N.P., White,

568   N.J., Dondorp, A.M., 2014. Magnetic resonance imaging of the brain in adults with severe falciparum

569   malaria. Malaria J 13, 177.

570 Mayor, A., Bardaji, A., Macete, E., Nhampossa, T., Fonseca, A.M., Gonzalez, R., Maculuve, S., Cistero, P.,

571   Ruperez, M., Campo, J., Vala, A., Sigauque, B., Jimenez, A., Machevo, S., de la Fuente, L., Nhama, A.,

572   Luis, L., Aponte, J.J., Acacio, S., Nhacolo, A., Chitnis, C., Dobano, C., Sevene, E., Alonso, P.L.,

573   Menendez, C., 2015. Changing Trends in *P. falciparum* Burden, Immunity, and Disease in Pregnancy.

574   New Engl J Med 373, 1607-1617.

575 Mayor, A., Hafiz, A., Bassat, Q., Rovira-Vallbona, E., Sanz, S., Machevo, S., Aguilar, R., Cistero, P., Sigauque,

576   B., Menendez, C., Alonso, P.L., Chitnis, C.E., 2011. Association of severe malaria outcomes with platelet-

577   mediated clumping and adhesion to a novel host receptor. PloS One 6, e19422.

578 McDonald, C.R., Darling, A.M., Conroy, A.L., Tran, V., Cabrera, A., Liles, W.C., Wang, M., Aboud, S., Urassa,

579   W., Fawzi, W.W., Kain, K.C., 2015a. Inflammatory and Angiogenic Factors at Mid-Pregnancy Are

580   Associated with Spontaneous Preterm Birth in a Cohort of Tanzanian Women. PloS One 10, e0134619.

581 McDonald, C.R., Tran, V., Kain, K.C., 2015b. Complement Activation in Placental Malaria. Front Microbiol 6,

582   1460.

583 Medana, I.M., Day, N.P., Sachanonta, N., Mai, N.T., Dondorp, A.M., Pongponratn, E., Hien, T.T., White, N.J.,

584   Turner, G.D., 2011. Coma in fatal adult human malaria is not caused by cerebral oedema. Malaria J, 10,

585   267.

586 Mohanty, S., Mishra, S.K., Patnaik, R., Dutt, A.K., Pradhan, S., Das, B., Patnaik, J., Mohanty, A.K., Lee, S.J.,

587   Dondorp, A.M., 2011. Brain swelling and mannitol therapy in adult cerebral malaria: a randomized trial.

588   Clin Infect Dis 53, 349-355.

589 Moxon, C.A., Chisala, N.V., Wassmer, S.C., Taylor, T.E., Seydel, K.B., Molyneux, M.E., Faragher, B.,

590   Kennedy, N., Toh, C.H., Craig, A.G., Heyderman, R.S., 2014. Persistent endothelial activation and

591   inflammation after *Plasmodium falciparum* Infection in Malawian children. J Infect Dis 209, 610-615.

592 Moxon, C.A., Wassmer, S.C., Milner, D.A., Jr., Chisala, N.V., Taylor, T.E., Seydel, K.B., Molyneux, M.E.,

593   Faragher, B., Esmon, C.T., Downey, C., Toh, C.H., Craig, A.G., Heyderman, R.S., 2013. Loss of

594   endothelial protein C receptors links coagulation and inflammation to parasite sequestration in cerebral

595   malaria in African children. Blood 122, 842-851.

596 Nacer, A., Movila, A., Sohet, F., Girgis, N.M., Gundra, U.M., Loke, P., Daneman, R., Frevert, U., 2014.

597   Experimental cerebral malaria pathogenesis--hemodynamics at the blood brain barrier. PLoS Pathog 10,

598   e1004528.

599 O'Sullivan, J.M., Preston, R.J., O'Regan, N., O'Donnell, J.S., 2016. Emerging roles for hemostatic dysfunction

600   in malaria pathogenesis. Blood 127, 2281-2288.

601 Padmanabhan, B., Mathur, S., Manjula, R., Tripathi, S., 2016. Bromodomain and extra-terminal (BET) family

602   proteins: New therapeutic targets in major diseases. J Biosci 41, 295-311.

603   Pai, S., Qin, J., Cavanagh, L., Mitchell, A., El-Assaad, F., Jain, R., Combes, V., Hunt, N.H., Grau, G.E.,

604   Weninger, W., 2014. Real-time imaging reveals the dynamics of leukocyte behaviour during experimental

605   cerebral malaria pathogenesis. PLoS Pathog 10, e1004236.

606   Pain, A., Ferguson, D.J., Kai, O., Urban, B.C., Lowe, B., Marsh, K., Roberts, D.J., 2001. Platelet-mediated

607   clumping of *Plasmodium falciparum*-infected erythrocytes is a common adhesive phenotype and is

608   associated with severe malaria. Proc Natl Acad Sci (USA) 98, 1805-1810.

609   Penet, M.F., Viola, A., Confort-Gouny, S., Le Fur, Y., Duhamel, G., Kober, F., Ibarrola, D., Izquierdo, M., Coltel,

610   N., Gharib, B., Grau, G.E., Cozzone, P.J., 2005. Imaging experimental cerebral malaria in vivo: significant

611   role of ischemic brain edema. J Neurosci 25, 7352-7358.

612   Petersen, J.E., Bouwens, E.A., Tamayo, I., Turner, L., Wang, C.W., Stins, M., Theander, T.G., Hermida, J.,

613   Mosnier, L.O., Lavstsen, T., 2015. Protein C system defects inflicted by the malaria parasite protein

614   PfEMP1 can be overcome by a soluble EPCR variant. Thromb Haemost 114, 1038-1048.

615   Potchen, M.J., Kampondeni, S.D., Seydel, K.B., Birbeck, G.L., Hammond, C.A., Bradley, W.G., DeMarco, J.K.,

616   Glover, S.J., Ugorji, J.O., Latourette, M.T., Siebert, J.E., Molyneux, M.E., Taylor, T.E., 2012. Acute brain

617   MRI findings in 120 Malawian children with cerebral malaria: new insights into an ancient disease. AJNR

618   Am J Neuroradiol 33, 1740-1746.

619   Rogerson, S.J., Pollina, E., Getachew, A., Tadesse, E., Lema, V.M., Molyneux, M.E., 2003. Placental

620   monocyte infiltrates in response to *Plasmodium falciparum* malaria infection and their association with

621   adverse pregnancy outcomes. American J Trop Med Hyg 68, 115-119.

622   Rowe, A., Obeiro, J., Newbold, C.I., Marsh, K., 1995. *Plasmodium falciparum* rosetting is associated with

623   malaria severity in Kenya. Infect Immun 63, 2323-2326.

624   Rowe, J.A., Claessens, A., Corrigan, R.A., Arman, M., 2009. Adhesion of *Plasmodium falciparum*-infected

625   erythrocytes to human cells: molecular mechanisms and therapeutic implications. Expert Rev Mol Med 11,

626   e16.

627   Rowe, J.A., Handel, I.G., Thera, M.A., Deans, A.M., Lyke, K.E., Kone, A., Diallo, D.A., Raza, A., Kai, O.,

628   Marsh, K., Plowe, C.V., Doumbo, O.K., Moulds, J.M., 2007. Blood group O protects against severe

629   *Plasmodium falciparum* malaria through the mechanism of reduced rosetting. Proc Natl Acad Sci (USA)

630   104, 17471-17476.

631   Rowe, J.A., Shafi, J., Kai, O.K., Marsh, K., Raza, A., 2002. Nonimmune IgM, but not IgG binds to the surface of

632   *Plasmodium falciparum*-infected erythrocytes and correlates with rosetting and severe malaria. Am J Trop

633   Med Hyg 66, 692-699.

634   Sahu, P.K., Satpathi, S., Behera, P.K., Mishra, S.K., Mohanty, S., Wassmer, S.C., 2015. Pathogenesis of

635   cerebral malaria: new diagnostic tools, biomarkers, and therapeutic approaches. Front Cell Infect Microbiol

636   5, 75.

637   Salinas, J.L., Kissinger, J.C., Jones, D.P., Galinski, M.R., 2014. Metabolomics in the fight against malaria.

638   Memorias do Instituto Oswaldo Cruz 109, 589-597.

639   Serghides, L., Kim, H., Lu, Z., Kain, D.C., Miller, C., Francis, R.C., Liles, W.C., Zapol, W.M., Kain, K.C., 2011.

640   Inhaled nitric oxide reduces endothelial activation and parasite accumulation in the brain, and enhances

641   survival in experimental cerebral malaria. PloS One 6, e27714.

642   Seydel, K.B., Kampondeni, S.D., Valim, C., Potchen, M.J., Milner, D.A., Muwalo, F.W., Birbeck, G.L., Bradley,

643   W.G., Fox, L.L., Glover, S.J., Hammond, C.A., Heyderman, R.S., Chilingulo, C.A., Molyneux, M.E., Taylor,

644   T.E., 2015. Brain swelling and death in children with cerebral malaria. New Engl J Med 372, 1126-1137.

645   Souza, R.M., Ataide, R., Dombrowski, J.G., Ippolito, V., Aitken, E.H., Valle, S.N., Alvarez, J.M., Epiphanio, S.,

646   Marinho, C.R., 2013. Placental histopathological changes associated with *Plasmodium vivax* infection

647   during pregnancy. PLoS Negl Trop Dis 7, e2071.

648   Stevenson, L., Laursen, E., Cowan, G.J., Bandoh, B., Barfod, L., Cavanagh, D.R., Andersen, G.R., Hviid, L.,

649   2015. alpha2-Macroglobulin Can Crosslink Multiple *Plasmodium falciparum* Erythrocyte Membrane Protein

650   1 (PfEMP1) Molecules and May Facilitate Adhesion of Parasitized Erythrocytes. PLoS Pathog 11,

651   e1005022.

652   Storm, J., Craig, A.G., 2014. Pathogenesis of cerebral malaria-inflammation and cytoadherence. Front Cell

653   Infect Microbiol 4, 100.

654   Taylor, T.E., Fu, W.J., Carr, R.A., Whitten, R.O., Mueller, J.S., Fosiko, N.G., Lewallen, S., Liomba, N.G.,

655   Molyneux, M.E., 2004. Differentiating the pathologies of cerebral malaria by postmortem parasite counts.

656   Nat Med, 10, 143-145.

657   Turner, L., Lavstsen, T., Berger, S.S., Wang, C.W., Petersen, J.E., Avril, M., Brazier, A.J., Freeth, J.,

658   Jespersen, J.S., Nielsen, M.A., Magistrado, P., Lusingu, J., Smith, J.D., Higgins, M.K., Theander, T.G.,

2013. Severe malaria is associated with parasite binding to endothelial protein C receptor. Nature 498, 502-505.

Warrell, D.A., Looareesuwan, S., Phillips, R.E., White, N.J., Warrell, M.J., Chapel, H.M., Areekul, S., Tharavanij, S., 1986. Function of the blood-cerebrospinal fluid barrier in human cerebral malaria: rejection of the permeability hypothesis. Am J Trop Med Hyg 35, 882-889.

Wassmer, S.C., Combes, V., Grau, G.E., 2011a. Platelets and microparticles in cerebral malaria: the unusual suspects. Drug Discov Today Dis Mech 8, e15-e23.

Wassmer, S.C., Moxon, C.A., Taylor, T., Grau, G.E., Molyneux, M.E., Craig, A.G., 2011b. Vascular endothelial cells cultured from patients with cerebral or uncomplicated malaria exhibit differential reactivity to TNF. Cell Microbiol 13, 198-209.

Wassmer, S.C., Taylor, T., Maclennan, C.A., Kanjala, M., Mukaka, M., Molyneux, M.E., Grau, G.E., 2008. Platelet-induced clumping of *Plasmodium falciparum*-infected erythrocytes from Malawian patients with cerebral malaria-possible modulation in vivo by thrombocytopenia. J Infect Dis 197, 72-78.

Wassmer, S.C., Taylor, T.E., Rathod, P.K., Mishra, S.K., Mohanty, S., Arevalo-Herrera, M., Duraisingh, M.T., Smith, J.D., 2015. Investigating the Pathogenesis of Severe Malaria: A Multidisciplinary and Cross-Geographical Approach. Am J Trop Med Hyg 93, 42-56.

White, N.J., Turner, G.D., Day, N.P., Dondorp, A.M., 2013. Lethal malaria: Marchiafava and Bignami were right. J Infect Dis 208, 192-198.

Yeo, T.W., Lampah, D.A., Gitawati, R., Tjitra, E., Kenangalem, E., McNeil, Y.R., Darcy, C.J., Granger, D.L., Weinberg, J.B., Lopansri, B.K., Price, R.N., Duffull, S.B., Celermajer, D.S., Anstey, N.M., 2007. Impaired nitric oxide bioavailability and L-arginine reversible endothelial dysfunction in adults with falciparum malaria. J Exp Med 204, 2693-2704.

Yeo, T.W., Lampah, D.A., Kenangalem, E., Tjitra, E., Weinberg, J.B., Granger, D.L., Price, R.N., Anstey, N.M., 2014. Decreased endothelial nitric oxide bioavailability, impaired microvascular function, and increased tissue oxygen consumption in children with falciparum malaria. J Infect Dis 210, 1627-1632.

Yeo, T.W., Lampah, D.A., Rooslamiati, I., Gitawati, R., Tjitra, E., Kenangalem, E., Price, R.N., Duffull, S.B., Anstey, N.M., 2013. A randomized pilot study of L-arginine infusion in severe falciparum malaria: preliminary safety, efficacy and pharmacokinetics. PloS One 8, e69587.