A NOVEL ROLE FOR VON WILLEBRAND FACTOR IN THE PATHOGENESIS OF EXPERIMENTAL CEREBRAL MALARIA

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KEY POINTS

1. Experimental cerebral malaria is associated with an early marked increase in plasma VWF levels and accumulation of UL-VWF multimers.
2. Following *Plasmodium berghei* infection, VWF<sup>−/−</sup> mice survive significantly longer compared to wild type controls.

ABSTRACT

*Plasmodium falciparum* malaria infection is associated with an early marked increase in plasma VWF levels, together with a pathological accumulation of hyper-reactive ultra-large VWF (UL-VWF) multimers. Given the established critical role of platelets in malaria pathogenesis, these increases in plasma VWF raise the intriguing possibility that VWF may play a direct role in modulating malaria pathogenesis. To address this hypothesis, we utilized an established murine model of experimental cerebral malaria (ECM), in which wild type (WT) C57BL/6J mice were infected with *Plasmodium berghei* ANKA. In keeping with findings in children with *P. falciparum* malaria, acute endothelial cell activation was an early and consistent feature in the murine model of CM, resulting in significantly increased plasma VWF levels. Despite the fact that murine plasma ADAMTS13 levels were not significantly reduced, pathological UL-VWF multimers were also observed in murine plasma following *P. berghei* infection. To determine whether VWF plays a role in modulating the pathogenesis of CM *in vivo*, we further investigated *P. berghei* infection in VWF<sup>−/−</sup> C57BL/6J mice. Clinical ECM progression was delayed and overall survival was significantly prolonged in VWF<sup>−/−</sup> mice compared to wild type controls. Despite this protection against ECM, no significant differences in platelet counts, or blood parasitaemia levels, were observed between VWF<sup>−/−</sup> and WT mice. Interestingly however, the degree of ECM-associated enhanced blood brain barrier permeability was significantly attenuated in VWF<sup>−/−</sup> mice compared to WT controls. Given the significant morbidity and mortality associated with CM, these novel data may have direct translational significance.

**Keywords**

- Von Willebrand factor; cerebral malaria; *Plasmodium falciparum*; *Plasmodium berghei*
INTRODUCTION

Plasmodium falciparum malaria remains a major cause of morbidity and mortality amongst children in sub-Saharan Africa.\textsuperscript{1-3} Although the biological mechanisms involved in the pathophysiology of severe \textit{P. falciparum} malaria remain poorly understood, previous studies have demonstrated that sequestration of \textit{P. falciparum} infected erythrocytes (IE) within the microvasculature of the brain is important in the development of cerebral malaria (CM).\textsuperscript{4,5} This sequestration involves adhesion of IE to host vascular endothelial cell (EC) surfaces,\textsuperscript{6-8} and is mediated by a variety of specific EC adhesion molecules including CD36, intercellular adhesion molecule-1 (ICAM-1), and thrombospondin.\textsuperscript{9} Moreover, recent studies have also demonstrated that the endothelial protein C receptor (EPCR) also plays an important role in modulating the sequestration of IE.\textsuperscript{10} In addition to IE, sequestration of leucocytes and platelets within the cerebral microvasculature has also been reported in post mortem samples from CM patients.\textsuperscript{11,12}

Von Willebrand factor (VWF) is a large sialoglycoprotein synthesized within EC and megakarocytes.\textsuperscript{13} VWF circulates in normal plasma as a series of heterogeneous multimers, and plays a critical role in primary haemostasis by mediating platelet adhesion to exposed collagen at sites of vascular injury.\textsuperscript{14} The multimeric composition of plasma VWF plays a key role in determining its functional activity. In particular, high molecular weight multimers (HMWM) of VWF demonstrate enhanced binding affinities for both collagen and platelets, and are therefore more efficient in mediating platelet recruitment.\textsuperscript{14} Following synthesis within EC, VWF is either constitutively secreted into the plasma, or else stored within specific intracellular organelles known as Weibel-Palade (WP) bodies.\textsuperscript{15} This WP-stored VWF is enriched in HMWM, and is actively secreted following EC activation.

In previous studies we and others have demonstrated that plasma levels of VWF antigen (VWF:Ag) and VWF propeptide (VWF:pp) are markedly elevated in African
children with severe *P. falciparum* malaria.\textsuperscript{16-20} This observation has subsequently been confirmed in other studies that enrolled both children and adult patients with either *P. falciparum* or *P. vivax* infections, from a number of different geographical regions.\textsuperscript{21,22} Interestingly, a study of healthy volunteers infected with *P. falciparum* has also shown that the increase in plasma VWF:Ag and VWF:pp levels is present from a very early stage following the onset of blood stage infection.\textsuperscript{23} Collectively these data demonstrate that marked EC activation, with consequent VWF secretion from WP bodies, constitutes an early hallmark of *P. falciparum* malaria infection.

In addition to the marked increase in plasma VWF:Ag observed in patients with malaria infection, severe *P. falciparum* infection is also associated with a pathological accumulation of ultra-large VWF (UL-VWF) multimers in the plasma of patients.\textsuperscript{17,21} The molecular mechanism(s) responsible for the presence of these UL-VWF multimers remains unclear. Importantly however, only modest reductions in plasma ADAMTS13 levels have been reported in malaria-infected patients.\textsuperscript{17,21,22} Nevertheless, the combination of markedly elevated VWF:Ag levels and hyper-reactive UL-VWF multimers in the plasma, raises the intriguing possibility that VWF may play a novel role in the pathogenesis of *P. falciparum* malaria. This hypothesis is supported by a number of recent independent observations. First, plasma VWF:pp levels in children with severe malaria have been shown to correlate with other established biochemical markers of malaria severity, including plasma lactate levels.\textsuperscript{16} In addition, plasma VWF levels also correlate inversely with platelet count, and with overall clinical outcome.\textsuperscript{16,24} Second, de Mast *et al* have demonstrated that in patients with *P. falciparum* infection, a significant proportion of plasma VWF is circulating in an active confirmation that promotes platelet GpIb binding.\textsuperscript{24} This observation is important since platelet adhesion and accumulation have been implicated in facilitating the adhesion of *P. falciparum* IE to activated EC.\textsuperscript{25-27} Finally, in a shear-based assay we have recently shown
that platelet-decorated UL-VWF strings on the surface of activated EC can also recruit trophozoite-stage *P. falciparum IE*.  

The early increase in plasma VWF levels and circulating UL-VWF multimers observed following *P. falciparum* infection poses a challenge in defining whether VWF directly contributes to the development of human CM, or whether increased VWF levels merely constitute a secondary epiphenomenon associated with EC activation. In this study, we have sought to further investigate the putative role of VWF in malaria pathogenesis *in vivo* using an established murine model of experimental cerebral malaria (ECM).
MATERIALS AND METHODS

Murine Studies

All mouse experiments were performed in compliance with Irish Medicines Board regulations, and were reviewed and approved by the Trinity College Dublin BioResource Ethical Committee. VWF\textsuperscript{-/-} mice were initially obtained from Jackson Laboratories (Bar Harbor, USA). These VWF\textsuperscript{-/-} mice were on a C57BL/6J background and have previously been characterized.\textsuperscript{29,30} Wildtype (WT) C57BL/6J and VWF\textsuperscript{-/-} mice were bred and maintained in-house under standard pathogen-free conditions. All experiments were performed on mice aged 8 to 10 weeks. Blood samples were collected from either tail vein, or by cardiac puncture, into acid citrate dextrose (ACD) anticoagulant (85mM trisodium citrate, 65mM citric acid, 100mM glucose) (Sigma, Ireland). Platelet counts were measured using a Sysmex haematology analyser (KX-21N). To prepare platelet-poor plasma, blood samples were centrifuged at 1500 g for 15 min at 20\textdegree C, aliquoted and stored at -80\textdegree C until use.

P. berghei Survival Studies

Mice were infected by intraperitoneal injection of 2x10\textsuperscript{6} Plasmodium berghei ANKA. Following inoculation, malaria progression was monitored using a previously validated clinical scoring system to assess ECM.\textsuperscript{31} In brief, mice were initially reviewed on a daily basis following P. berghei infection. From Day +5 post-infection, all mice were assessed every 12 hours. Clinical progression of ECM was determined by examining for the appearance of the following signs: hunched posture, ruffled fur, wobbly gait, limb paralysis, convulsions, and coma. Each sign was awarded a score based upon clinical severity (0 or 1). Animals with severe ECM (total cumulative scores >4) were sacrificed by cervical dislocation according to ethical guidelines, and day of death was deemed to be the following day. Blood
P. berghei parasitaemia levels were monitored by examination of Giemsa-stained (VWR International Inc.) thin blood smears obtained from tail vein bleeds.

**Determination of plasma VWF antigen (VWF:Ag) and collagen binding activity (VWF:CB)**

Plasma VWF:Ag levels were measured using a previously described enzyme-linked immunosorbent assay (ELISA). In brief, Maxisorp plates (Nunc, Denmark) were coated with rabbit anti-human VWF antibody (Dako, Denmark) in 50mM carbonate buffer (pH 9.6). Previous studies have demonstrated that these polyclonal anti-human VWF antibodies cross-react with murine VWF and can be used to measure murine plasma VWF:Ag levels. After blocking with 3% bovine serum albumin (Sigma, Ireland) test samples were then added at appropriate dilutions. Bound murine VWF was detected using horseradish peroxidase–conjugated (HRP) rabbit anti-human VWF antibody (Dako, Denmark). Following washing, HRP substrate 3,3’,5,5’-Tetramethylbenzidine (TMB; Substrate Reagent Pack, R&D Systems, UK) was added, and the reaction subsequently stopped with 50μL 1M H₂SO₄. Absorbance was read at 450nm using a VERSAmax microplate reader (Molecular Devices, UK). Normal plasma pooled from twenty WT C57BL/6J mice was used throughout as normal reference plasma. Plasma VWF:Ag levels in infected mice were expressed as a percentage of normal baseline murine VWF:Ag levels.

Similarly, VWF collagen binding activity (VWF:CB) was also determined using a previously described ELISA. Briefly, ninety-six well microtiter plates (Thermo Scientific) were coated with type III collagen derived from human placenta (Sigma-Aldrich) at a final concentration of 40 μg/mL in sodium carbonate/bicarbonate buffer (0.035M NaHCO₃, 0.015M Na₂CO₃, pH 9.6). After washing with imidazole buffer (0.12M NaCl, 0.02M imidazole, 0.005M citric acid, pH 7.3), the plates were blocked using 3% BSA in imidazole for 1 hour at room temperature. Following further washing with imidazole buffer, test
samples were added and incubated for 2 hours at 37°C. After a final washing, bound VWF was detected using HRP–conjugated rabbit anti-human VWF antibody (Dako, Denmark) as described above. Plasma VWF:CB was expressed as a percentage of uninfected controls.

**Plasma VWF multimer analysis**

The multimeric structure of plasma VWF was analyzed by electrophoresis using 1.8% agarose gels prepared from SeaKem® HGT(P) Agarose (Lonza, ME USA) in separating buffer (200mM tris, 100mM glycine and 0.1% SDS, pH 9.0). Stacking gels (0.75% agarose) were prepared from the same agarose and stacking buffer (70mM tris, 5mM EDTA, 0.1% SDS pH 6.7). All gels were run in a Bio-Rad mini-gel electrophoresis system, with an outer buffer of 50mM Tris, 75mM glycine, and an inner buffer of 100mM Tris, 150mM glycine 0.1% SDS for 150 minutes at 50 volts. After electrophoresis, protein was transferred to PVDF membranes (Immobilon-FL, Millipore, MA, USA), blocked with 5% BSA, and incubated with rabbit anti-human VWF antibody (Dako, Denmark) as previously described.37 After thorough washing, membranes were finally incubated with goat anti-rabbit-HRP (Santa Cruz Biotechnology, USA). Bound antibody was detected using the SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, Ireland). The membrane was then exposed to autoradiography (x-ray) film (Fujifilm; Fisher Scientific, Ireland) and the films were developed using the AGFA CP1000 automatic film-developing system (AGFA, Germany). To objectively quantify differences in VWF multimer composition, densitometry of typical individual lanes was performed using ImageJ software (Image Processing and Analysis in Java) as previously described.17
**Plasma VWF propeptide, angiopoietin 2, and ADAMTS13 activity levels**

Murine plasma VWF:pp concentration was determined by ELISA assay using a combination of a mouse-specific 349.3 capture antibody, and horseradish peroxidase-linked 349.2 antibody. Both antibodies were kind gifts from Dr. Bob Montgomery (Blood Research Institute, BloodCenter of Wisconsin, US). Mouse angiopoietin-2 (Ang-2) plasma levels were measured by commercial ELISA according to manufacturer’s instructions (R&D Systems Quantikine ELISA immunoassay; R&D Systems, UK). Finally, plasma ADAMTS13 activity was also assessed using a commercial FRETS-VWF73 assay (Peptides International Inc) as previously described.

**Blood Brain Barrier permeability**

Blood brain barrier (BBB) permeability in WT and VWF$^{-/-}$ mice was investigated at baseline prior to inoculation with *P. berghei* using Evans blue dye. In addition, BBB permeability was repeated at Day +5 following *P. berghei* infection. Briefly, in keeping with previous studies, 2% Evans blue dye (Sigma, Ireland) in PBS was infused via lateral tail vein injection, and then allowed to circulate for 1 hour. Mice were anaesthetised with an I.P. injection of 2.5% tribromethanol, and 40mL of normal saline was cardiacally perfused. To quantify extravasation of Evans blue across the BBB, murine brains were removed and weighed. Cerebral tissues were then homogenised and incubated in 50% Trichloroacetic acid (TCA). Following centrifugation (14,000 rpm for 30 minutes at 4°C), supernatants were plated on a 96-well plate and absorbance was measured at 620 nm. Results were quantified according to a standard curve and expressed as ng Evans blue/ng brain.
Data presentation and statistical analysis

All experimental data and statistical analysis were performed using the GraphPad Prism program (Graphpad Prism version 5.0 for Windows; GraphPad Software, Inc. San Diego, CA). Data were expressed as mean values ± standard error of the mean (SEM). To assess statistical differences, data were analysed using Student’s unpaired 2-tailed $t$ test. ECM clinical scoring data were assessed by two-way ANOVA analysis. Finally, mouse survival data were compared using a Log-rank (Mantel-Cox) Test. For all statistical tests, $P$ values <0.05 were considered significant.
RESULTS

Increased plasma VWF:Ag and VWF:CB levels in experimental cerebral malaria

In order to further investigate the role of VWF in malaria pathogenesis, we used a previously described mouse model of ECM, in which C57BL/6J mice are infected with *P. berghei* ANKA. In keeping with previous findings in children with severe *P. falciparum* malaria, we observed a significant increase in plasma VWF:Ag levels in WT C57BL/6J mice following *P. berghei* infection (Figure 1A). By Day +5 after inoculation, median plasma VWF:Ag levels were increased approximately 2.4 fold (101.9% at Day 0 versus 242.3% at Day +5; \(P<0.001\)), which was similar in magnitude to the increase in plasma VWF:Ag levels observed in children with cerebral malaria. Interestingly, a significant increase in plasma VWF:Ag levels was already evident within 24 hours following *P. berghei* inoculation. This early increase in plasma VWF levels occurred before the appearance of significant blood parasitaemia levels (Figure 1B), and prior to the onset of clinical signs (Figure 1C). Again in keeping with our previous findings in children infected with *P. falciparum*, plasma VWF collagen binding activity (VWF:CB) levels were also significantly elevated in C57BL/6J mice following *P. berghei* infection (113.7% at Day 0 versus 266.6% at Day +5; \(P<0.01\)) (Figure 1D). Furthermore, the magnitude of the increase in VWF:CB observed in the murine ECM model was again similar to that previously observed in patients with severe malaria. In contrast to the significant increases in plasma VWF:Ag and VWF:CB levels, we observed no significant increase in plasma VWF:pp levels in C57BL/6J mice following *P. berghei* infection (Supplementary data - Figure 1A). The molecular mechanism(s) underlying the observation that the plasma VWFpp:VWFAg ratio is not increased in the murine model of ECM remains unclear, but likely relates at least in part to the fact that the plasma half-life of VWF:pp is reduced in C57BL/6J mice (Supplementary data - Figure 1B).
Endothelial cell activation is a feature of experimental cerebral malaria

Acute EC activation plays a critical role in the pathogenesis of *P. falciparum* malaria. The marked increase in plasma VWF:Ag and VWF:CB observed following *P. berghei* infection suggests that EC activation is also an early feature in mice prior to the development of CM. Angiopoietin-2 (Ang-2) is another glycoprotein stored within WP bodies and secreted into plasma following EC activation. In addition, significantly elevated plasma Ang-2 levels have been reported in patients with severe *P. falciparum* malaria, where Ang-2 levels were also shown to correlate with clinical outcome. In keeping with the human data, a marked increase in murine plasma Ang-2 levels was observed in C57BL/6J mice following *P. berghei* infection (Figure 2). For example, plasma Ang-2 levels by Day +3 following *P. berghei* infection were 56.8ng/mL versus only 24.1ng/mL at Day 0 (*P*<0.001). Collectively, these data confirm that marked EC activation and WP body exocytosis, which together represent early hallmarks of *P. falciparum* malaria infection in human patients, also constitute prominent features in the murine model of ECM.

Severe *P. berghei* malaria influences plasma VWF multimer composition

In addition to markedly elevated VWF:Ag and VWF:CB activity levels, pathological UL-VWF multimers have also been reported in the plasma of children with CM. Consequently, VWF multimer analysis and densitometry were assessed in C57BL/6J mice following *P. berghei* infection. In keeping with our previous findings in children, accumulation of abnormal UL-VWF multimers in the plasma was also a feature of murine ECM (Figure 3A and Supplementary Figure 2). UL-VWF multimers were not present in the plasma of uninfected mice, but were observed from Day +3 following *P. berghei* infection. Normally, UL-VWF multimers secreted from WP bodies following EC activation are rapidly proteolysed by the zinc metalloprotease ADAMTS13. Previous studies have reported modest reductions in plasma ADAMTS13 activity levels in children with *P. falciparum* malaria.
infection. In contrast, we observed no significant change in murine plasma ADAMTS13 activity following *P. berghei* infection (Figure 3B). In addition, despite the presence of circulating UL-VWF, peripheral blood film examination demonstrated no significant features of microangiopathic hemolytic anaemia (MAHA) (Figure 3C). In summary, these findings demonstrate that marked elevations of plasma VWF levels, coupled with the appearance of pathological UL-VWF multimers in the plasma, represent consistent features in human and experimental murine CM.

**VWF deficient mice are protected against experimental cerebral malaria**

To determine whether increased plasma VWF levels represent simply a biomarker of acute EC activation in ECM, or whether elevated plasma VWF and/or UL-VWF multimers are directly involved in mediating the pathogenesis of ECM, we studied *P. berghei* infection in VWF<sup>-/-</sup> mice compared to WT. Using a previously validated clinical scoring algorithm to assess experimental malaria progression, we found that the clinical features of ECM progressed significantly more slowly in the VWF<sup>-/-</sup> mice compared to WT mice (*P* = 0.001) (Figure 4A). Importantly however, VWF<sup>-/-</sup> mice still developed typical neurological signs, and died from ECM rather than severe anaemia. In addition, overall survival was significantly prolonged in VWF<sup>-/-</sup> mice compared to WT mice (*P* = 0.01) (Figure 4B). For example, whereas only approximately 30% of WT mice survived until Day +6 following *P. berghei* infection, more than 80% of the VWF<sup>-/-</sup> mice remained alive.

**Thrombocytopenia in ECM is independent of plasma VWF**

Significant thrombocytopenia is common feature in patients with *P. falciparum* malaria. This thrombocytopenia is predominantly due to increased platelet clearance. A putative role for UL-VWF in contributing to this enhanced platelet clearance has been proposed. Furthermore, recent studies have identified novel roles for platelets in killing intraerythrocytic
malaria parasites, and in modulating host immune responses during the early stages of *P. falciparum* infection. Consequently, to investigate potential mechanisms through which VWF<sup>−/−</sup> mice were protected against ECM, daily platelet counts were performed in WT and VWF<sup>−/−</sup> mice following *P. berghei* infection. In keeping with observations in human patients, significant thrombocytopenia was a consistent feature in this murine model (Figure 4C). At Day +4 following inoculation, mean platelet count had fallen by approximately 70% in WT mice. Interestingly, significant thrombocytopenia was also observed in VWF<sup>−/−</sup> mice infected with *P. berghei* (Figure 4C). Importantly, despite the differences in clinical malaria progression, no significant differences in platelet counts were observed between VWF<sup>−/−</sup> and WT mice. Finally, we observed no significant differences in blood *P. berghei* parasitaemia levels between VWF<sup>−/−</sup> mice and WT mice respectively (Figure 4D).

**ECM-induced blood brain barrier permeability is attenuated in VWF deficient mice**

VWF is expressed abundantly by cerebral ECs. In addition, endothelial VWF has recently been shown to play an important role in modulating permeability of the BBB under different pathological conditions. Although the underlying biological mechanisms remain poorly understood, enhanced permeability of the BBB has also been implicated in the pathogenesis of CM. We therefore investigated BBB permeability in VWF<sup>−/−</sup> mice compared to WT mice following infection with *P. berghei*. In keeping with previous studies, we observed no significant difference in BBB permeability to Evans blue dye between VWF<sup>−/−</sup> and WT mice prior to *P. berghei* inoculation (Figure 5). By Day +5 following infection, BBB permeability was markedly elevated in WT (1.8ng/mg at Day 0 versus 4.7ng/mg at Day +5; *P*<0.001). Interestingly, although BBB was also affected in VWF<sup>−/−</sup> mice at Day +5, the increase in permeability was significantly lower than that observed in WT mice (*P*<0.05).
DISCUSSION

C57BL/6 mice infected with \textit{P. berghei} ANKA typically develop a complex neurological syndrome involving clinical features similar to those observed in human CM.\textsuperscript{40,41,59} These include ataxia, paralysis, seizures and coma. Depending upon the dose of \textit{P. berghei}, infected mice typically die within 6 to 10 days. Although there are important differences between human CM and the ECM model,\textsuperscript{60,61} both \textit{P. falciparum} and \textit{P. berghei} infections are associated with pronounced effects on EC biology.\textsuperscript{40,41,62,63} In this study, we demonstrate that \textit{P. berghei} infection mirrors the activated EC phenotype observed in African children with severe \textit{P. falciparum} malaria, with significant increases in plasma VWF:Ag and VWF:CB levels.\textsuperscript{16-20} In addition, and in keeping with previous data from human volunteers infected with \textit{P. falciparum},\textsuperscript{23} these increased plasma VWF levels were observed from a very early stage following \textit{P. berghei} infection. Thus, the early acute EC activation and WP body secretion which constitute hallmarks of \textit{P. falciparum} infection in humans are replicated in this ECM model. Interestingly, \textit{P. falciparum}-infected humans and \textit{P. berghei}-infected mice both exhibit increased plasma VWF levels, before the number of infected erythrocytes had reached 0.1%. The biological mechanism underlying this very early EC activation remains unknown,\textsuperscript{64} however further studies using this murine model may enable definition of the biological mechanisms through which early EC activation in response to malaria infection is modulated.

Previous studies have demonstrated that severe \textit{P. falciparum} malaria is associated with accumulation of abnormal UL-VWF multimers in the plasma.\textsuperscript{17,21} In this study, we further demonstrate that plasma UL-VWF multimers also constitute a feature of the murine model of ECM. The molecular mechanisms responsible for this UL-VWF accumulation have not been defined. However marked exocytosis of UL-VWF multimers from WP bodies following acute EC activation is likely to represent an important contributing factor. Nevertheless, UL-VWF
multimers secreted from WP bodies typically undergo rapid proteolysis by ADAMTS13 on EC surfaces. Following *P. berghei* infection, we observed no significant reduction in murine ADAMTS13 activity as assessed by FRETS-VWF75 assay. In contrast, previous studies did report a mild reduction in plasma ADAMTS13 activity (using both FRETS-VWF75 and full length VWF cleavage assays) in children and adult patients with severe malaria compared to healthy controls.\textsuperscript{17,21,22} Importantly however, residual plasma ADAMTS13 levels in African children with CM still remained above 60%, which should be sufficient to prevent the accumulation of hyper reactive UL-VWF multimers.\textsuperscript{17} In summary therefore, it seems likely that the ability of ADAMTS13 to cleave VWF *in vivo* is reduced in both humans and mice with CM. Although, a number of putative inhibitors of ADAMTS13 have been identified,\textsuperscript{65-69} we previously showed that those described to date could not explain the accumulation of UL-VWF multimers observed in children with CM.\textsuperscript{17} Furthermore, this combination of UL-VWF multimers with normal plasma ADAMTS13 levels has also been recently been reported in other conditions including patients with sickle cell disease.\textsuperscript{70} Further studies to investigate how UL-VWF multimers accumulate in the murine model of ECM despite the presence of ADAMTS13 may therefore be useful in elucidating novel molecular mechanisms involved in both the physiological and pathological regulation of ADAMTS13 activity *in vivo*. Interestingly, different murine strains demonstrate significant variability in susceptibility to malaria infection.\textsuperscript{41,71} The genetic modifiers responsible for this inter-strain variation remain unclear, but plasma VWF:Ag levels vary markedly between different in-bred mouse strains.\textsuperscript{72} In addition, plasma ADAMTS13 activity levels are reduced in some strains (including C57BL/6) due to the insertion of a retrotransposon element into the *ADAMTS13* gene.\textsuperscript{73,74}

Given the key role played by VWF in modulating platelet adhesion and aggregation, together with the accumulating data demonstrating that platelet-endothelial cell interactions play a critical role in malaria biology,\textsuperscript{25-27} we hypothesized that VWF may play a novel role in
modulating the pathophysiology of malaria. This hypothesis was supported by previous *in vitro* experiments in which we showed that platelet-decorated UL-VWF strings could recruit *P. falciparum* IE to endothelial cell surfaces under shear stress conditions. In this study, we demonstrate that VWF<sup>-/-</sup> mice are significantly protected against *P. berghei* infection compared to WT controls. This survival difference was not explained by a difference in *P. berghei* parasitaemia levels, which were similar in both VWF<sup>-/-</sup> and WT mice. Significant thrombocytopenia is a common feature of both human and murine malaria, and has been attributed to a number of mechanisms, including sequestration and enhanced clearance by the spleen. Moreover, recent studies have described a role for platelets in modulating the innate host defense during the early stages of malaria infection. Interestingly, although previous studies suggested that the UL-VWF might be important in mediating this malaria-associated thrombocytopenia, we observed no significant difference in *P. berghei*-induced thrombocytopenia in VWF<sup>-/-</sup> compared to WT mice. Collectively, these data suggest that enhanced platelet clearance in ECM is not influenced by the elevated plasma VWF:Ag levels, nor by circulating UL-VWF multimers. In addition, VWF<sup>-/-</sup> mice are clearly protected against ECM through a platelet-independent mechanism.

Severe malaria infection has been associated with enhanced permeability of the BBB. In addition, recent studies have demonstrated that VWF plays an important role in regulating the BBB. For example, significantly attenuated BBB permeability was observed in VWF<sup>-/-</sup> mice compared to WT controls in a hypoxia/reoxygenation model, and also in an experimental model of status epilepticus. Although the molecular mechanisms through which VWF influences BBB integrity remain poorly understood, a significant increase in expression of the endothelial tight junction protein claudin-5 was demonstrated in the cerebral microvascular EC of VWF<sup>-/-</sup> mice compared to WT controls. In this study, we found that *P. berghei* infection was associated with a significant increase in BBB permeability in both WT and
VWF−/− mice. Interestingly however, the degree of ECM-associated enhanced BBB permeability was significantly attenuated in the VWF−/− mice compared to WT controls. Further studies will be required to fully elucidate the mechanisms through which VWF protects BBB permeability, and to determine the importance of this BBB effect in modulating the improved ECM survival observed in VWF−/− mice.

VWF synthesis is essential for the normal formation of WP bodies within EC. Consequently, VWF−/− mice are not only deficient in plasma and platelet VWF, but also do not possess WP bodies. Importantly, in addition to VWF and VWF:pp, WP bodies also normally store other proteins including P-selectin, Ang-2 and osteoprotegrin. Whether the absence of WP bodies may be important in attenuating ECM in VWF−/− mice remains unclear. However, previous studies with an inhibitory P-selectin aptamer suggested that P-selectin does not play a major role in modulating the enhanced BBB protection observed in VWF−/− mice. In this study we have shown that plasma Ang-2 levels are also significantly increased in murine ECM. This observation is consistent with previous data from children with severe *P. falciparum* malaria. Interestingly, Ang-2 has also been shown to function as an autocrine regulator by sensitizing EC to activation by tumour necrosis factor (TNF), and thus may play a role in modulating the early acute EC activation associated with both *P. falciparum* and *P. berghei* infections. In addition, Ang-2 has also been reported to influence BBB permeability. Despite the absence of WP bodies, baseline plasma Ang-2 levels in the VWF−/− mice were not significantly different compared to those in WT C57BL/6 controls (means 23.1 +/- 5.4 versus 22.8 +/- 3.2 ng/ml respectively).

In conclusion, we demonstrate that early significant EC activation represents a consistent feature of the murine ECM model. In keeping with our previous observations in children with severe *P. falciparum* malaria, this EC activation results in a marked increase in plasma VWF levels, together with a pathological accumulation of hyper-reactive UL-VWF multimers in
plasma. Although the pathobiology underlying CM remains poorly understood, it is complex and involves multiple different mechanisms (including EC activation, IE sequestration; platelet and leucocyte recruitment; cytokine secretion; innate and adaptive immune responses; alteration in EC and BBB permeability). Given this complexity, we postulate that VWF may influence malaria pathogenesis through a number of different mechanisms (Figure 6). For example, during the early stages following malaria infection, formation of UL-VWF strings on activated EC surfaces may be important in recruiting platelets. Subsequently, platelet-bound VWF may play a role in modulating microvasculature sequestration by recruiting malaria-infected erythrocytes, and also by binding to granulocytes and activated monocytes respectively.\textsuperscript{77,78} In addition, VWF may also be involved in the later stages of CM pathogenesis. For example, VWF plays an important role in regulating leucocyte extravascation, and as we have shown, also influences BBB permeability in CM. Defining the roles of VWF and/or UL-VWF multimers in this setting may offer novel therapeutic opportunities.
AUTHORSHIP


Conflict-of-interest disclosure:

J.S.O’D has served on the speaker’s bureau for Baxter, Bayer, Novo Nordisk, Boehringer Ingelheim, Leo Pharma and Octapharma. He has also served on the advisory boards of Baxter, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim and Pfizer. J.S.O.D has also received research grant funding awards from Baxter, Bayer, Pfizer and Novo Nordisk.

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FIGURE LEGENDS

Figure 1. Plasma VWF:Ag and VWF:CB levels are increased in ECM
Following I.P. inoculation with 2 x10^6 P. berghei ANKA parasites, whole blood samples were collected from WT C57BL/6J mice by cardiac puncture. (A) Plasma VWF:Ag levels were then measured at each time point by ELISA. All ELISAs were performed in triplicate, and results presented represent the mean values ± SEM unless otherwise stated (* P<0.05, ** P<0.01, *** P<0.0001 respectively). (B) Peripheral blood P. berghei parasitaemia levels were determined from Giemsa-stained smears (n =16; mean values shown). (C) ECM progression was monitored using a previously validated clinical scoring algorithm (n =16; mean values shown). (D) Plasma VWF:CB activity levels were also determined by ELISA as detailed in the ‘Materials and Methods’.

Figure 2. Early endothelial cell activation is a feature of ECM
Ang-2 is another protein stored within endothelial cell Weibel Palade bodies. To further assess WP body exocytosis in WT C57BL/6J mice following P. berghei infection, plasma Ang-2 levels were measured at specified time points using a commercial ELISA.

Figure 3. Severe P. berghei malaria influences plasma VWF multimer composition
(A) Plasma VWF multimer distribution during the course of malaria infection was assessed by non-reducing agarose gel electrophoresis. Typical multimer gels from example mice are presented. From Day +3 following P. berghei infection, abnormal UL-VWF multimers were consistently observed in murine plasma. Densitometric scanning of multimer gels was also performed, with individual panels corresponding to each lane. The horizontal axis is optical density. Abnormal UL-VWF multimers are present from Day +3 through Day +5, (highlighted by bands beyond the arbitrary red line) that are not present prior to P. berghei
infection. (B) Plasma ADAMTS13 activity levels were measured by FRETS-VWF73 proteolysis assay. All assays were performed in triplicate, and results presented represent the mean values (ns = not significant). (C) Typical Giemsa-stained peripheral blood film from a WT C57BL/6J mouse at Day +5 following *P. berghei* infection. Infected erythrocytes (IE) and thrombocytopenia are evident, but there are no significant features of microangiopathic haemolytic anaemia.

**Figure 4. VWF deficient mice are significantly protected against ECM**

To investigate whether VWF may be directly involved in the pathogenesis of ECM, *P. berghei* infection in VWF<sup>−/−</sup> mice was investigated. (A) Following inoculation with *P. berghei*, clinical phenotype and progression in VWF<sup>−/−</sup> mice (n=10) and WT C57BL/6J mice (n=16) were compared using a validated clinical scoring algorithm. Results presented represent the mean values ± SEM. (* P<0.05, ** P<0.01, *** P<0.0001 respectively). (B) In addition, overall survival in WT (n=16) and VWF<sup>−/−</sup> (n=10) mice infected with 2 x 10<sup>6</sup> *P. berghei* parasites was also determined and analysed by Log-rank (Mantel-Cox) test. (C) Since VWF plays a critical role in modulating platelet adhesion and aggregation, sequential platelet counts were performed in VWF<sup>−/−</sup> mice and WT C57BL/6J mice following *P. berghei* infection. (n = 4-5 mice per time point). (D) Peripheral blood *P. berghei* parasitaemia levels were determined following inoculation in both VWF<sup>−/−</sup> (n=10) and WT C57BL/6J mice (n=10) at specified time points from Giemsa-stained smears. Results presented illustrated represent the mean values ± SEM.
**Figure 5. ECM-induced blood brain barrier permeability is attenuated in VWF deficient mice**

BBB permeability was assessed following a lateral tail vein infusion of Evans blue dye. Since Evan’s blue binds to murine albumin, it does not normally cross an intact BBB. Prior to *P. berghei* inoculation (Day 0), baseline BBB permeability was determined in WT C57BL/6J mice (black circles; n=5) and compared to that of VWF⁻/⁻ mice (blue squares; n=6). Subsequently, BBB permeability was then reassessed in WT C57BL/6J mice (black circles; n=6) and VWF⁻/⁻ mice (blue squares; n=6) at Day +5 following *P. berghei* infection.

**Figure 6. Schematic diagram illustrating the proposed mechanisms through which VWF is involved in malaria pathogenesis**

EC activation and release of WP body contents constitute common early features in both human and murine malaria. This results in the secretion in the release of UL-VWF multimers into the plasma (1) and a marked increase in plasma VWF levels. VWF may influence malaria pathogenesis through a number of different mechanisms. First, UL-VWF strings on the surface of activated EC recruit and sequester platelets within the microvasculature (2). These tethered platelets cause further EC activation, and thus more WP body secretion. In addition, the platelet-decorated VWF may be important in modulating further sequestration by recruiting both malaria-infected erythrocytes, and also by binding to granulocytes and activated monocytes respectively (3). The VWF-mediated sequestration of platelets, infected erythrocytes and leucocytes leads to further enhanced EC activation. During the later stages of CM pathogenesis, VWF may be important in regulating EC permeability, BBB permeability, and leucocyte extravasation (4). Finally, given that platelets play a critical role in the development of microvasculature occlusion, we postulate that VWF may also be important in this context (5).
REFERENCES


Figure 3

(A) Western blot of HMW and LMW proteins across different days post-infection.

(B) Graph showing ADAMTS13 activity (%).

(C) WT blood film Day +5 showing infected erythrocyte and platelet.
Figure 6

Increased EC permeability

FLOW

WP Body  Monocyte  Neutrophil  Infected RBC  Platelet  Von Willebrand Factor
A novel role for von Willebrand factor in the pathogenesis of experimental cerebral malaria

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