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VON WILLEBRAND FACTOR, OSTEOPROTEGERIN AND THE PROTEIN C PATHWAY IN THE PATHOGENESIS OF EXPERIMENTAL CEREBRAL MALARIA

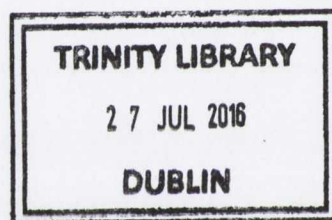


A thesis submitted to the University of Dublin, Trinity College,
for the Degree of Doctor of Philosophy in the School of Medicine.

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Submitted September 2015

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Declaration

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

Niamh O'Regan*, Kristina Gegenbauer*, Jamie M. O'Sullivan, Sanaz Maleki, Teresa M. Brophy, Niall Dalton, Alain Chion, Padraic G. Fallon, Georges E. Grau, Ulrich Budde, Owen P. Smith, Alister G. Craig, Roger J.S. Preston and James S. O'Donnell

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MARKED ELEVATION IN PLASMA OSTOPROTEGERIN (OPG) CONSTITUTES AN EARLY AND CONSISTENT FEATURE OF CEREBRAL MALARIA

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COAGULOPATHY IN *PLASMODIUM BERGHEI* MALARIA

Gegenbauer K., O'Regan N., O'Sullivan J., Preston R., Gleeson E., Chion A., Brophy T., Smyth O., O'Donnell J.

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Summary

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 (ULVWF) 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 and pathological ULVWF multimers were also observed. To determine whether VWF plays a role in modulating the pathogenesis of CM *in vivo*, we further investigated *P. berghei* infection in VWF^{-/-} mice. Clinical ECM progression was delayed and overall survival was significantly prolonged in VWF^{-/-} mice compared to WT controls. Interestingly, the degree of ECM-associated enhanced blood brain barrier permeability was significantly attenuated in VWF^{-/-} mice compared to WT controls. To further investigate the underlying molecular mechanisms, we have studied other genetically modified mice strains in which either the ADAMTS13, or the ASGPR gene have been knocked out. Given the significant morbidity and mortality associated with CM, these novel data may have direct translational significance.

OPG has been shown to upregulate expression of adhesion molecules on EC surfaces, and thereby facilitate leucocyte adhesion. Given the key role of EC activation in the pathogenesis of severe *P. falciparum* malaria, we have investigated plasma OPG levels in children with CM. To further understand the relationship between WPB secretion and malaria, we have further investigated OPG levels in an established murine model of experimental CM. We demonstrate for the first time that severe *P.*

falciparum malaria is also associated with a marked increase in plasma OPG levels. Interestingly, we observed a similar marked increase in plasma OPG levels in a murine model of ECM. In this murine model we further demonstrated that the increase in plasma OPG develops at an early stage following malaria infection. Indeed, plasma OPG levels were significantly increased prior to the presence of detectable levels of parasitemia, and well before the onset of any clinical signs. This finding is consistent with previous studies in which elevations in plasma VWF levels were observed at a surprisingly early stage in both human volunteers infected with *P. falciparum*, and in mice infected with *P. berghei*. Furthermore, plasma OPG level increase progressively through the course of infection, and correlate with other established markers of outcome in patients with severe malaria. Notwithstanding any putative role of OPG in malaria pathogenesis, our combined human and animal data support the hypothesis that plasma OPG levels may constitute a novel biomarker with prognostic significance in malaria.

Infection with *P. falciparum* is associated with thrombocytopenia, decreased levels of anticoagulant clotting factors, and thus generation of thrombin. Whether coagulopathy is involved in the pathogenesis of malaria still remains unknown. Recently published data indicates EPCR plays a role in *P. falciparum* malaria. The molecular mechanisms by which coagulopathy and the protein C pathway contribute to CM remain unknown. We sought to examine if the mouse model of CM could be used to study malarial-induced coagulopathy, and whether the protein C pathway was important in this context. APC administration has been shown to elicit beneficial effects in other murine disease models. Our findings have demonstrated that injection of an EPCR function blocking antibody significantly shortened the survival of *P. berghei* infected mice. Moreover, when APC was pre-administered to WT mice they demonstrated significantly enhanced survival. These novel data suggest a key role for the protein C pathway in malaria infection.

Abbreviations

ACD: Acid citrate dextrose

ADAMTS13^{-/-}: ADAMTS13 deficient mice

ADAMTS13: A Disintegrin and Metalloprotease with Thrombospondin repeats

Ang-2: Angiopoietin-2

APC: Activated protein C

APTT: Activated Partial Thromboplastin Time

ASGPR^{-/-}: ASGPR deficient mice

ASGPR: Asialoglycoprotein Receptor

AT: Antithrombin

BBB: Blood brain barrier

BSA: Bovine Serum Albumin

β2-GPI: β2-Glycoprotein I

Ca²⁺: Calcium

CM: Cerebral malaria

DIC: disseminated intravascular coagulation

EC: Endothelial cells

ECM: Experimental cerebral malaria

ELISA: Enzyme-linked immunosorbent assay

EPCR: Endothelial cell protein C receptor

ER: Endoplasmic reticulum

FIX: Factor IX

FIX: Factor IX

FIXa : Activated Factor IX

FVII: Factor VII

FVIII: Factor VIII

FVIIIa: Activated FVIII

FX: Factor x

FX: Factor X
 FXa: Activated Factor x
 Gal: Galactose
 GalNAc: N-acetylgalactosamine
 GlcNAc: N-acetylglucosamine
 GPIb α : Platelet glycoprotein Ib α
 GPIIb-IIIa: Platelet glycoprotein IIb/IIIa
 Hb: Haemoglobin
 HMWM: High Molecular Weight Multimers
 HUVECs: Human Umbilical Vein Endothelial cells
 I.P.: Intraperitoneal
 I.V.: Intravenous
 ICAM-1: Intercellular adhesion molecule-1
 IE: Infected erythrocytes
 LMWM: Low Molecular Weight Multimers
 MAHA: Microangiopathic haemolytic anaemia
 mAPC: murine APC
 NFI: No febrile illness
 NMFI: non-malaria febrile illness
 OPG: Osteoprotegerin
P. falciparum: *Plasmodium falciparum*
 PC: Protein C
 PCR: Polymerase Chain Reaction
 PfEMP1: *Plasmodium falciparum* erythrocyte membrane protein 1
 PSGL-1: P-selectin glycoprotein ligand 1
 PT: Prothrombin Time
 rADAMTS13 : Recombinant ADAMTS13
 RANKL: Receptor Activator of NF κ B Ligand

RBC: Red blood cell

RDTs: Rapid Diagnostic Tests

rVWF : Recombinant VWF

sEPCR: Soluble EPCR

sTm: Soluble Thrombomodulin

TAT complexes: Thrombin-antithrombin complexes

TBE: Tribromoethanol

TCA: Trichloroacetic acid

TF: Tissue factor

TFPI: Tissue factor pathway inhibitor

Tm: Thrombomodulin

TMB: 3,3',5,5'-Tetramethylbenzidine

tPA: Tissue-type plasminogen activator

TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand

TSP-1: Thrombospondin-1

TTP : Thrombocytopenia Purpura

ULVWF: Ultra large VWF

UM: Uncomplicated malaria

VCAM-1: Vascular adhesion molecule-1

VWD: von Willebrand Disease

VWF^{-/-} mice: VWF deficient mice

VWF: von Willebrand Factor

VWF:Ag von Willebrand Factor Antigen

VWF:CBA von Willebrand Factor Collagen Binding Activity

VWF:pp: von Willebrand Factor propeptide

WPB: Weibel-Palade Bodies

WT: Wild type

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1. Introduction

1.1 Malaria: the causative agent

Malaria remains a major cause of morbidity and mortality in the tropics with an estimated 200 million clinical cases and approx. 600,000 deaths per annum (Murray *et al*, 2012; Carter *et al*, 2005; Mung'Ala-Odera *et al*, 2004; World Health Organization, 2013). The causative agent of malaria are single celled eukaryotic organisms of the genus *Plasmodium*. Just over 120 different *Plasmodium* species which have been identified so far. All of these species share host-specificity characteristics. Human malaria is attributed to several *Plasmodium* species (including *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*) (Carter & Mendis, 2002). More recent findings suggest *P. Knowlseyi* is also capable of causing morbidity in humans (Pain *et al*, 2008). Infection with *P. falciparum* leads to the most virulent disease and is responsible for the majority of fatal malarial infections. Children in holoendemic areas of sub-Saharan Africa under 5 years are particularly at high risk (World Health Organization, 2013). In endemic areas, partial clinical immunity typically develops during the first 5-10 years of life. In spite of this, African children account for more than 90% of malaria-related mortality. Consequently, severe *P. falciparum* malaria continues to constitute a leading cause of childhood death worldwide.

1.2 The disease burden of malaria

The wide variation in the burden of the disease between different regions of the world has been attributed to several factors, such as variation in species of parasite and differences in levels of socio-economic development (World Health Organization, 2013). Malaria in pregnancy is a serious public health problem adding an enormous burden of low birth weight, maternal anaemia and contributing to mortality in

developing countries (Desai *et al*, 2007). As well as a high burden of morbidity and mortality, malaria limits productivity in endemic areas of the world. This infection inflicts substantial costs for governments and communities in most disease-endemic countries of the world (Gallup & Sachs, 2001).

1.3 Life cycle of the malaria parasite

The life cycle of *Plasmodium* is complex and involves the parasite switching between two hosts, and completing both sexual and asexual development. Malaria parasites are transmitted to the human host by an infected female *Anopheles* mosquito, which transfers infectious sporozoites contained within the salivary glands into the peripheral circulation of the host (Mouchet, 1999). Once within the circulation, the sporozoites immediately invade the hepatocytes of the liver. Here they undergo numerous rounds of asexual multiplication over a period of 5 to 7 days. This replication produces thousands of merozoite forms of the parasite. When the hepatocyte ruptures, these new merozoites are released into the circulation and subsequently invade the red blood cells (RBC) establishing the intra-erythrocytic cycle. Following invasion of these RBC, ring stage parasites may lie dormant for up to 15 hours (Markus, 2011). Subsequent to this hypnozoite period, there is a dramatic increase in metabolic and biosynthetic activities within the infected cell. Upon infection of RBC, they next develop into ring- and trophozoite-stage forms. Moreover, parasites develop further and trophozoites undergo asexual replication inside the erythrocyte to become erythrocytic schizonts. At this stage the schizont divides to produce daughter cells. Following rupture of the erythrocytes these daughter merozoites are released to subsequently infect other RBC to carry on the asexual erythrocytic cycle (Gaur *et al*, 2004). With the release of merozoites, toxic metabolic waste products from replication are released into the blood stream resulting in the fever associated with all malarial infections (Parroche *et al*, 2007).

Some merozoites do not mature and become schizonts due to specific environmental signals but instead rather differentiate into male and female sexual forms (Matuschewski, 2006). These forms of parasite are available from the blood stream to feeding female *Anopheles* mosquitos and fertilise the mosquito midgut forming zygotes. These zygotes become ookinetes which are motile and migrate through the mosquito gut wall to form oocysts (Matuschewski, 2006). Replication occurs within the oocyst forming thousands of sporozoites. These sporozoites are released and move to the salivary glands where they await injection into another human host (Matuschewski, 2006), thereby completing the *Plasmodium* life cycle. This life cycle is depicted in Figure 1.1.

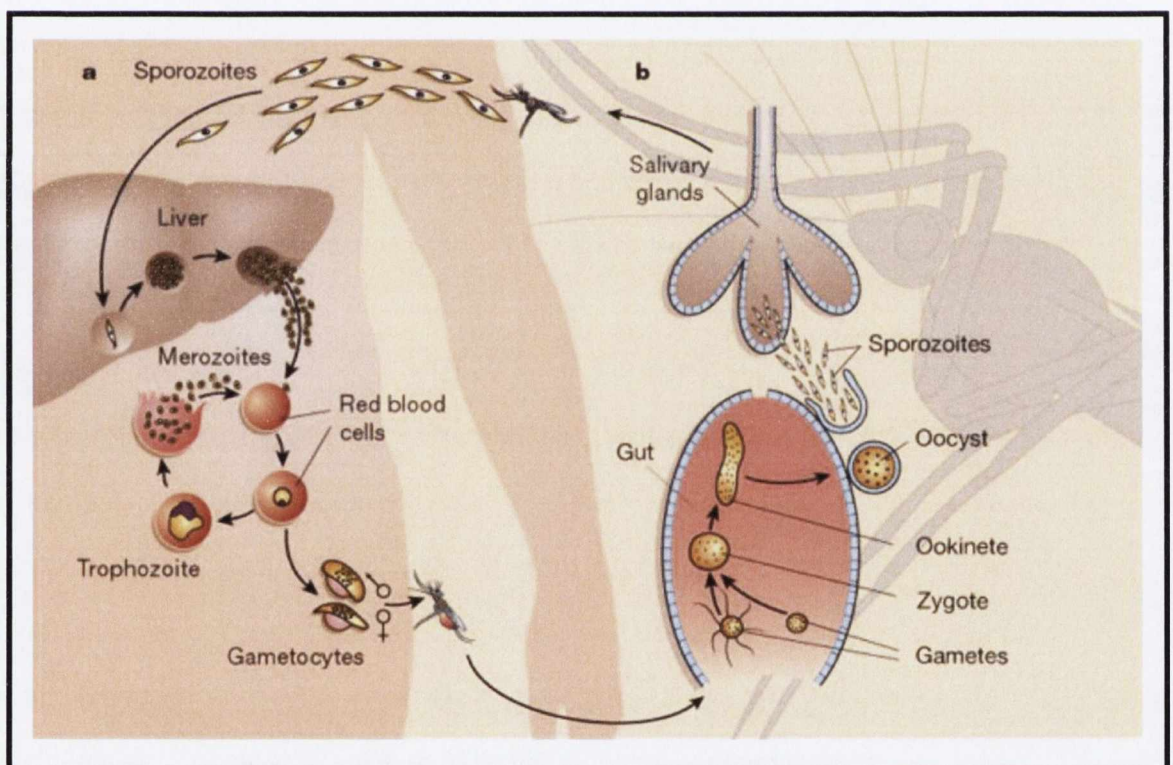


Figure 1. 1 *Plasmodium* life cycle

Schematic overview of the *Plasmodium* life cycle in (A) the human host and (B) the mosquito vector. This figure was adapted from Nature Publishing Group (Wirth, 2002).

1.4 Manifestation of malaria

1.4.1 General pathophysiology

The classic fever-like symptoms of malaria occur when parasites erupt from host RBC releasing pro inflammatory by-products into the circulation (Parroche *et al*, 2007). Patients will often seek treatment for non-specific symptoms including fever, headache, malaise and vomiting prior to developing malaria specific symptoms. Thus, it can be problematic differentiating non malarial febrile illnesses from malarial infection (Luxemburger *et al*, 1998). Dysregulated inflammatory responses and adherence of infected erythrocytes (IE) in the microvasculature are implicated in the pathogenesis of severe *P. falciparum* malaria.

1.4.2 Cerebral malaria

Cerebral malaria (CM) is a complication of severe infection seen in ~1% of children with *P. falciparum* infection causing a diffuse encephalopathy, characterised by ataxia, seizures, hemiplegia, and eventually coma and death (Molyneux *et al*, 1989; Brewster *et al*, 1990). CM is defined by the detection of *P. falciparum* parasites and unarousable coma assessed by verbal and motor responses and rated by the paediatric Blantyre Coma Score (1 or 2 on a scale of 5) with no other identifiable cause for fever or loss of consciousness, such as meningitis (Molyneux *et al*, 1989; Taylor *et al*, 2004). In children with CM, long-term cognitive and neurologic impairment is a frequent complication (Van Hensbroek *et al*, 1997; Boivin, 2002; Boivin *et al*, 2007; Birbeck *et al*, 2010). While linked to higher parasitemias, these are not a prerequisite for CM (Gravenor *et al*, 1998). Although the underlying pathogenesis remains unclear, CM is associated with sequestration of infected erythrocytes, leucocytes and platelets in the brain microvasculature, together with a marked increase in pro-inflammatory cytokine expression and activation of vascular endothelium (Rogerson *et al*).

Sequestration of IE in the microvasculature is thought to play a key role in parasite survival, by preventing splenic clearance (Newton & Krishna, 1998). Previously, it has been demonstrated that sequestration involves adherence of IE to endothelial cell (EC) surfaces. *P. falciparum* expresses different parasite proteins including *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) on the surface of IE that promote the cytoadherence in the microvasculature (Baruch *et al*, 1995). PfEMP1 can modulate the interaction between IE and host cell receptors present on the vascular endothelium to avoid detection and clearance by the spleen (Craig & Scherf, 2001) (Figure 1.2). Moreover, a number of specific receptors expressed on EC surfaces play vital roles in regulating PRBC adhesion, including CD36, thrombospondin, thrombomodulin, E-selectin, P-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 (VCAM-1) (Newbold *et al*, 1997; Turner *et al*, 1994). Expression of these receptors varies considerably between different vascular beds, and can be regulated in response to inflammatory cytokines (e.g. interleukin-1 and TNF- α) (Poher, 1988). Thus, EC activation plays a critical role in regulating IE cytoadherence (Turner *et al*, 1998). Nevertheless, the molecular mechanisms contributing to the pathophysiology of malarial infection remain poorly understood. It is thought that CM results from sequestration of IE in the brain microvasculature, leading to impaired microcirculatory flow and metabolic disturbances. In addition, CM is characterized by a systemic inflammatory response, characterized by high levels of proinflammatory molecules, endothelial activation and blood-brain-barrier dysfunction. It is likely a combination of these processes that contribute to the morbidity and mortality in severe malaria.

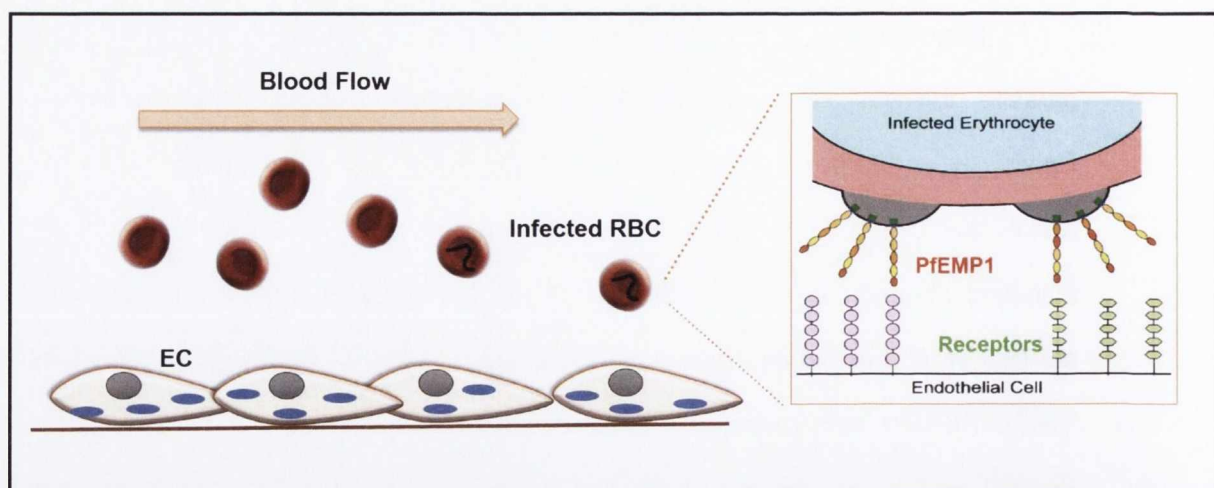


Figure 1.2 PfEMP1 modulates the interaction between IE and host cell receptors present on the vascular endothelium

1.4.3 Immunity

Following infection non-immune patients may develop malaria quickly with rapid parasitic growth causing severe anaemia and death. Although determining whether infection with *P. falciparum* malaria will develop into severe disease is not currently known, previous studies suggest that some interaction between the parasite and host's immune response plays a role (Artavanis-Tsakonas *et al*, 2003; Miller *et al*, 2002; Nyirenda *et al*, 2015). Despite the availability of anti-malarial treatments, the mortality rates remain high particularly with *P. falciparum* infection, and in infected children under the age of 5 years (Armah *et al*, 2007). The innate immune response is involved in limiting the initial phase of parasite replication, providing the host time to develop protective adaptive responses (Stevenson & Riley, 2004; Othoro *et al*, 1999). There are numerous innate immune pathways involved in the recognition and clearance of IE, including the complement system and pattern recognition receptors (e.g. toll-like receptors) (Erdman *et al*, 2008; Silver *et al*, 2010). These pathways are crucial in the control of rising parasitemia levels early in infection.

1.4.4 Sequestration of *P. falciparum*-infected erythrocytes

IE have been demonstrated in autopsies causing congestion in venules and capillaries. Sequestration of IE in the microvasculature is characteristic of all *P. falciparum* infections, including asymptomatic malarial infections and has been seen in patients who died from non-cerebral causes (MacPherson *et al*, 1985; Maneerat *et al*, 1999; Taylor *et al*, 2004; Dorovini-Zis *et al*, 2011). However the level of sequestration is significantly higher in autopsies of patients with CM and correlates with disease severity (MacPherson *et al*, 1985; Riganti *et al*, 1990; Maneerat *et al*, 1999; Dorovini-Zis *et al*, 2011; Ponsford *et al*, 2012). The density and distribution of parasite sequestration helps determine the extent of disease. Sequestration requires remodelling of the host RBC during early parasite development (Marti *et al*, 2004; Hiller *et al*, 2004; Pelle *et al*, 2015). Quantification of parasites in post-mortem tissues, histological assessment, and *in vivo* imaging of rectal mucosa show that dense parasite sequestration occurs in organs outside of the brain. These include lung, heart, kidney, skin and intestine (MacPherson *et al*, 1985; Pongponratn *et al*, 1991; Seydel *et al*, 2006; Milner *et al*, 2013, 2014; Storm & Craig, 2014). In children with CM, sequestration of IE is typically associated with microvascular pathology demonstrated by endothelial damage and perivascular ring haemorrhages. Monocytes and fibrin-platelet thrombi were also associated with IE sequestration and contribute to congestion (Dorovini-Zis *et al*, 2011). This microvascular obstruction can lead to impaired tissue perfusion. Blockade of vessels occurs through the interaction of parasite ligands (e.g. PfEMP1) to endothelial receptors. In addition, there is also adhesion of IE (Roberts *et al*, 1992; Carlson, 1993; Ringwald *et al*, 1993; Chotivanich *et al*, 2004; Arman *et al*, 2007). Moreover, sequestration likely advances tissue hypoxia and metabolic acidosis that is well described in severe malaria infection (Taylor *et al*, 1993; Waller *et al*, 1995; Newton *et al*, 2005).

1.4.5 Endothelial activation

P. falciparum and other malaria infections result in adhesion of IE to microvascular endothelium, and furthermore acute EC activation (Barber *et al*, 2015; Abdi *et al*, 2014). Upregulation of constitutively expressed receptors including ICAM-1 involved in IE cytoadherence, and the release of soluble ICAM-1 shed from the endothelium have been reported in malaria infection and correlate with disease severity (Jakobsen *et al*, 1994; Turner *et al*, 1998). Similarly, other markers of EC activation include soluble factors released from the endothelium such as angiogenic markers angiopoietin-1 and 2 (Yeo *et al*, 2008; Conroy *et al*, 2009; Lovegrove *et al*, 2009). Collectively these data demonstrate that marked EC activation constitutes a hallmark of *P. falciparum* malaria infection.

1.4.6 Blood Brain Barrier Dysfunction

The blood brain barrier (BBB) is a structural and functional barrier that restricts the transport of substances between the blood and central nervous system (Rubin & Staddon, 1999; Ballabh *et al*, 2004). BBB dysfunction was first suggested in CM in an account of the pathological features of malaria (Maegraith, 1948). Since then numerous studies have reported BBB disruption and breakdown in CM (Riganti *et al*, 1990; White *et al*, 2001; Patankar *et al*, 2002; Taylor *et al*, 2004). Previous studies from children in Malawi have shown decreased expression of tight-junction proteins in fatal CM (Brown *et al*). IE have also been shown to increase endothelial barrier permeability *in vitro* (Tripathi *et al*, 2007). These findings are supported by the fact that the cerebrospinal fluid: blood albumin ratio is increased in Malawian children with CM (Brown *et al*). Recently, IE sequestration, fibrinogen extravasation and perivascular haemorrhage have been reported as indicators of BBB (Greiner *et al*, 2015). Based on these pathophysiological findings adjunctive strategies and therapies that could reduce EC activation, down regulate parasite sequestration in the brain microvasculature and preserve BBB integrity could potentially reduce the mortality associated with CM.

1.4.7 Lab Diagnosis

Malaria is diagnosed by detection of IE in the blood. The gold standard for diagnosis is microscopy using a combination of Giemsa's-stained thick and thin blood smears for detection of parasites. Diagnosis of malaria by microscopy has many advantages over other available methods, as it allows for quantification of parasite density and also for differentiation of the species (Bloland, 2001). Distinction between the sexual forms of the parasite, gametocytes and the asexual forms is also distinguished with this method. However, a limitation of microscopy is that it can give false negative results if the blood smears are made when *Plasmodium* parasites are mature, since these are sequestered in the vessels of the internal organs and are not present in peripheral blood. Alternatively, malaria can be detected using Rapid Diagnostic Tests (RDTs) using rapid immunochromatographic techniques to detect specific parasite antigens that are present in the blood of infected individuals. While some RDTs can detect the presence of only *P. falciparum*, others can detect the presence of the other species of *Plasmodium*. The tests are rapid, simple to perform and have good detection limits. However a disadvantage of the method is that parasite antigen continues to circulate after the infection has been cleared, which can lead to false positives. Molecular diagnostic techniques such as Polymerase Chain Reaction (PCR) have also been developed for the identification of malaria parasites in biological samples such as the blood (Vu *et al*, 1995). Although this technique is expensive, it is sensitive and highly specific. This method is not used routinely in areas where malaria is rampant for the diagnosis of malaria. In endemic areas of malaria, it can be difficult to ascertain whether malaria is the actual cause of illness because clinical presentation can overlap with other common childhood febrile illnesses.

1.4.8 Prognosis

Outcomes in severe malaria depend on the type and degree of organ dysfunction. Prognostic indicators are vital in malaria in order to define features

associated with poor outcome. Prognostic indicators could also be beneficial in identifying children in need of referral for more intensive therapy. Original prognostic parameters were defined by Molyneux *et al* in a bedside prognostic index (Molyneux *et al*, 1989). However this prognostic score has subsequently been refined (Helbok *et al*, 2009). The most prominent clinical features associated with poor outcome in severe malaria are coma, respiratory distress, acidosis or convulsions (Bell & Molyneux, 2007; Dondorp *et al*, 2008; Bruneel *et al*, 2003; Jaffar *et al*, 1997). Other prognostic measures of use include hypoglycaemia, severe anaemia, and parasitemia (Molyneux *et al*, 1989; Marsh *et al*, 1995; Waller *et al*, 1995; Van Hensbroek *et al*, 1997). Biomarkers are valuable in severe disease management as well as aiding the formulation of strategies before the onset of disease in asymptomatic malaria. Some examples of biomarkers include lactate dehydrogenase, aldolase, glutamate dehydrogenase and hemozoin formation and have all been investigated for their application in developing malaria detection techniques (Jain *et al*, 2014). However, more studies are needed to evaluate the predictive performance of candidate biomarkers in order to identify suitable candidates for commercialisation. To date, no single marker has been identified and validated that has sufficient sensitivity and specificity to act as a singular predictive measure. Identification of such a biomarker alongside existing measures could be valuable, particularly if an effective adjunctive therapy was made available.

1.5 Experimental models of malaria

Malaria research greatly relies on animal models, as although studies have been carried out with human *Plasmodium* species, they are very limited for ethical reasons (de Mast *et al*, 2007). *In vitro* investigations into human *Plasmodium* species is restricted to culture of blood stages, mosquito infections feedings and infection of primary human hepatocytes. Consequently, it is not currently possible to reproduce the

whole parasite life cycle. This limits studies on host immune responses and mechanisms of disease. To address these limitations and to complement research on human malaria, several animal models are available. Murine models are the most widely used. *Plasmodium Berghei Antwerpen-Kasapa* (ANKA) (*P. berghei*), *P. yoelii*, *P. chabaudi* and *P. vinckei* were initially isolated from rats in Central Africa and have been adapted to grow in laboratory rodents, principally mice and rats (Carlton & Carucci, 2002). Each murine parasite displays different parasite biology and pathogenicity (Sanni *et al*, 2002). These rodent parasites differ in morphology and in outcome of infection. Additionally, lethal and nonlethal strains can be utilised (Wykes & Good, 2009). Following *P. berghei* inoculation, response to infection may vary between different mouse strains, some showing 100% death rate while others rarely die (Garnham, 1973). The use of non-lethal parasites in mice has identified the role of adaptive and innate immunity in mediating clearance and survival from infection. *P. berghei* infects mature RBC and reticulocytes and in C57BL/6 and CBA/T6 mice and is used widely to study the pathogenesis of experimental CM. Conversely, in DBA/2J mice CM resolves and some studies have previously reported that BALB/c mice are resistant to CM. Some strains of *P. chabaudi* and *P. vinckei* infect mature RBC and are used to investigate immune mechanisms and pathogenesis in different mouse strains (Wykes & Good, 2009). *P. chabaudi* infection resolves spontaneously while *P. berghei* infection results in a fulminating lethal parasitemia. Differences are also observed with human malaria parasites, for example *P. falciparum* causes greater mortality than *P. vivax* although both cause serious widespread morbidity. Murine malaria models have provided numerous insights into immunopathogenesis (Lamb *et al*, 2006), and host-parasite interactions (Franke-Fayard *et al*, 2010).

Although there are important differences between human CM and the experimental CM (ECM) model (Franke-Fayard *et al*, 2005; White *et al*, 2010), nevertheless both *P. falciparum* and *P. berghei* infections are associated with effects

on EC biology (Baptista *et al*, 2010; de Souza & Riley, 2002; Carvalho, 2010; Hunt *et al*, 2010). For example human CM and murine ECM share common features such as cytokine production and increased lactate production in the brain (Hunt *et al*, 2010). Conversely, concerns regarding the validity of this model have been raised. Differences include the sequestration of parasitized erythrocytes in human CM, in contrast to the accumulation of leukocytes and platelets in the venules and capillaries of the brain in the murine model (White *et al*, 2010). Despite these differences between human and murine parasites, murine malaria models have proven to be invaluable for malaria research.

1.6 Von Willebrand Factor

von Willebrand factor (VWF) is a large multimeric adhesive glycoprotein that exerts critical functions in maintaining normal haemostasis (Ruggeri & Ware, 1993). First, VWF serves as a molecular bridge mediating platelets binding to sites of vascular injury through exposure of sub endothelial collagen (Figure 1.3). Second, VWF also acts as a carrier molecule for procoagulant factor VIII (FVIII). This interaction prolongs the survival of FVIII in circulation from approximately 2 hours in the absence of VWF, to 12 hours when FVIII circulates in complex with VWF (Lollar, 1991).

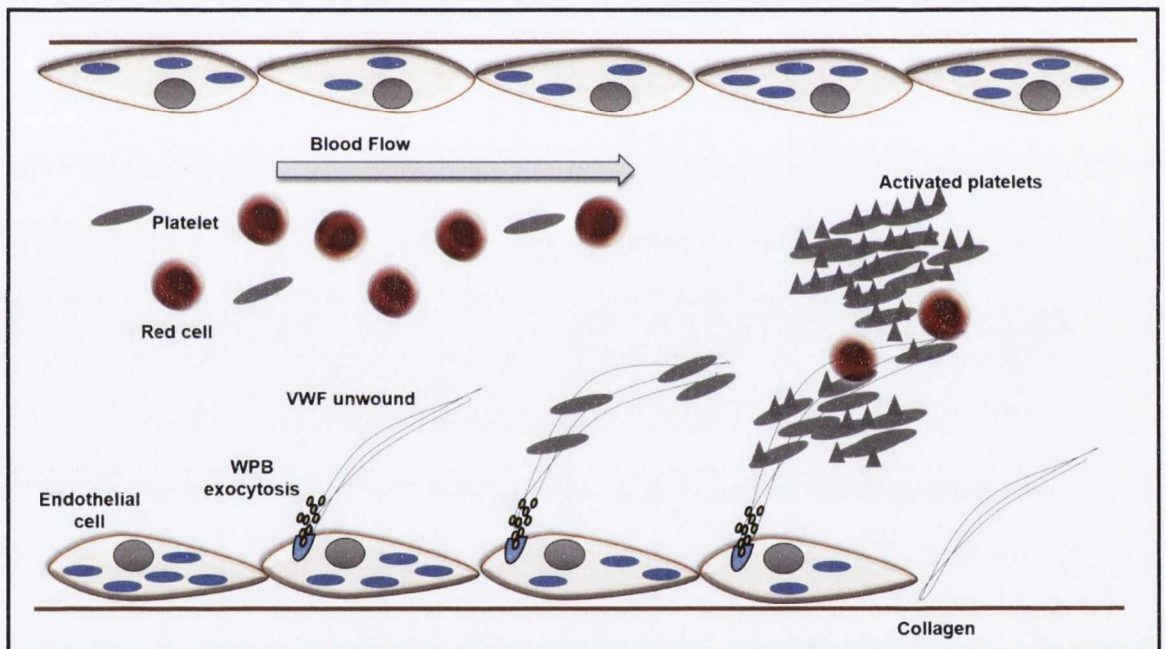


Figure 1. 3 The role of VWF in haemostasis

In normal plasma, VWF circulates at a concentration of $\sim 10\mu\text{g/ml}$, though this can vary over a wide range (from $5\text{-}20\mu\text{g/ml}$). Qualitative or quantitative VWF deficiency is responsible for the most common inherited bleeding disorder, von Willebrand disease (VWD) that occurs in up to 1% of the general population and can be classified into three major groups. Type 1 VWD involves a partial quantitative deficiency of VWF. Type 2 is caused by diverse qualitative defects in the VWF glycoprotein. Finally, type 3 VWD is rare but involves a complete absence of VWF. Due to the variety of functional defects involved, VWD type 2 can be further sub-grouped. Type 2A includes variants with decreased platelet adhesion caused by reduction in high-molecular-weight VWF multimers (HMWM). Type 2B VWD includes VWF variants with gain of function mutations for platelet glycoprotein Ib (GPIb α). Type 2M VWF mutations result in defective adhesion of VWF to platelets despite normal multimer distribution. Finally, type 2N VWD is characterised by decreased affinity for FVIII. Table 1.1 summarises the classification of VWF based on the Scientific and Standardization Committee on von Willebrand factor of the International Society of Thrombosis and Haemostasis (Sadler *et al*, 2006). Conversely, elevated plasma VWF levels are associated with a significant risk of myocardial infarction (Crawley *et al*, 2008), ischaemic stroke (Bongers *et al*, 2006) and venous thromboembolism (Nossent *et al*, 2006).

Type	Description
1	Partial quantitative VWF deficiency
2	Qualitative VWF defects
2A	Decreased VWF-dependent platelet adhesion and a reduction in HMWM
2B	Increased GPIIb α affinity, reduction in HMWM
2M	Defects in VWF-platelet interactions and normal multimers
2N	Reduced FVIII affinity
3	Virtually complete deficiency of VWF

Table 1. 1 Classification of VWD

1.6.1 VWF Biosynthesis

1.6.1.2 VWF gene and tissue expression

The gene encoding VWF is located on chromosome 12 (12p13.2), spanning 178kb of genomic DNA with 52 exons (Ginsburg *et al*, 1985). The primary translational product, pre-pro-VWF encodes 2813 amino acids containing a 22 residue signal peptide, a 741 residue propeptide and a 2050 residue mature protein (Titani *et al*, 1986). In addition, a non-functional VWF pseudogene is located on chromosome 22 (22q11-13) corresponding to exons 23-34 of the VWF gene (Mancuso *et al*, 1991).

In vivo biosynthesis of VWF is limited to EC and megakaryocytes (Jaffe *et al*, 1974; Nachman *et al*, 1977). In addition, Yamamoto *et al* described significant differences in VWF expression across organ-specific vascular beds (Yamamoto *et al*, 1998). The highest mRNA VWF levels were detected in lung, spleen, aorta and brain EC while relatively low levels were found in kidney, gut, and liver micro vessels. Generally, highest VWF expression was demonstrated in venous EC compared to EC

of an arterial origin. These findings were confirmed by immunohistochemical studies in the porcine vascular system where VWF was found to be expressed in all venous EC but VWF was consistently absent from the majority of porcine arterial EC (Rand *et al*, 1987).

1.7 Post translational modification

Prior to secretion the pre-pro-VWF primary translational product undergoes extensive post-translational modification. These include signal peptide cleavage, glycosylation, C-terminal dimerization, N-terminal multimerization and proteolytic cleavage of the propeptide.

1.7.1 N-linked glycosylation

N-linked glycosylation begins as a co-translational event in the endoplasmic reticulum (ER). Each mature VWF subunit contains 12 such sites with an additional 4 sites located within the VWF propeptide (VWF:pp) region (Titani *et al*, 1986). Ten of these sites have found to be occupied (Canis *et al*, 2010). The 14-sugar core unit is rapidly remodelled by a series of glycosyltransferases and glycosidases as the protein passes through the ER (Schwarz & Aeby, 2011). When the protein is correctly folded, the final glucose residue is trimmed facilitating release from the chaperones and signalling that the protein is ready to continue to the Golgi.

1.7.2 Dimerization

Following N-linked glycosylation, pro-VWF subunits dimerize through C-terminal disulphide bonding in the ER. The neutral pH of the ER and the presence of resident chaperones provide an optimal environment for disulphide bond formation. Dimerization is dependent on the prior attachment of N-linked glycan structures. Only dimeric VWF can leave the ER and continue onto the Golgi compartments for further

processing (Wagner *et al*, 1986). Exit from the ER appears to be the rate-limiting step of VWF biosynthesis.

1.7.3 Addition of ABO (H) blood group antigens

The N-linked glycan's of VWF are unusual in that they express covalently linked terminal ABO(H) blood group determinants (Matsui *et al*, 1992). The addition of α 1-2 fucose to the N-glycans termini creates the H-antigen which is the terminal glycan expressed in blood group O individuals (Figure 1.4). This H-antigen serves as an essential precursor for the subsequent development of blood group A or B antigens. The A-antigen (N-acetylgalactosamine) and the B-antigen (galactose) are generated by specific A and B glycosyltransferase enzymes further modifying the H antigen (Yamamoto, 2000). Individuals with the rare Bombay blood group lack the H antigen (Bhende *et al*, 2008) and thus cannot express terminal A or B antigens regardless of their genotype.

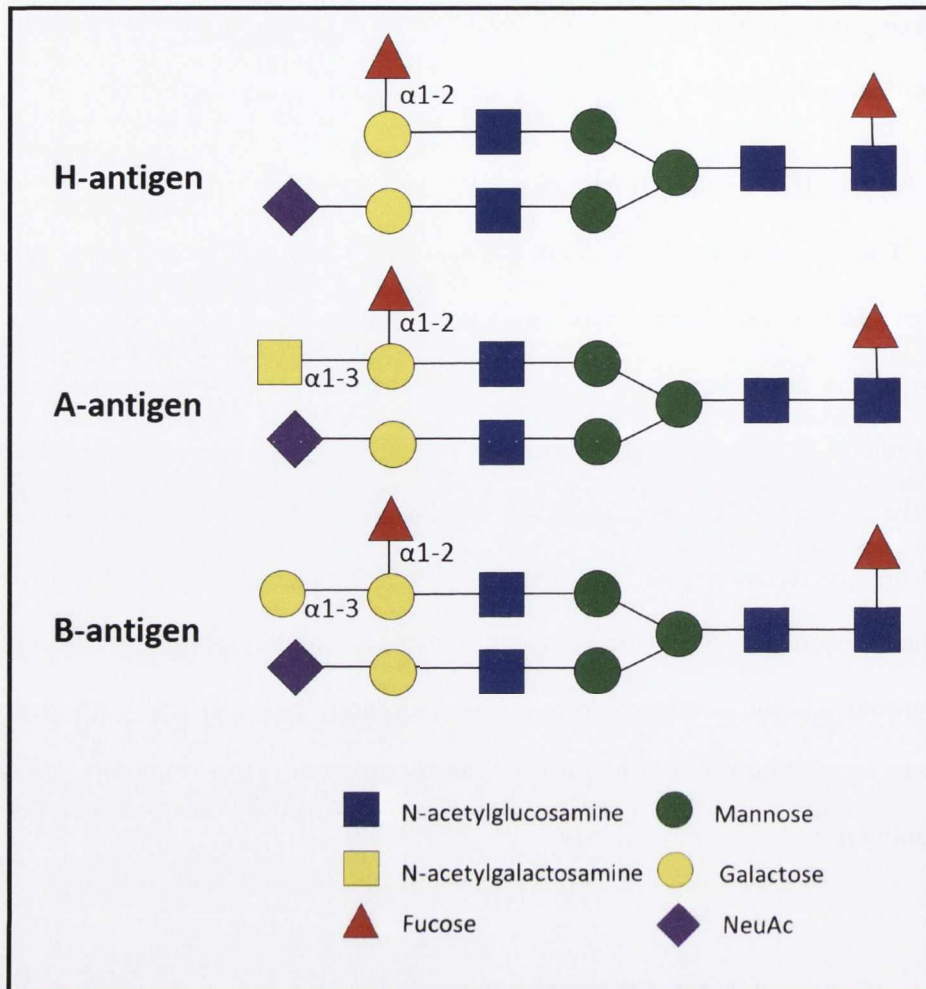


Figure 1. 4 Structure of VWF blood group antigens on VWF N-linked glycans

1.7.4 O-linked glycosylation

O-linked glycosylation occurs in the Golgi and is characterised by the addition of GalNac. This is followed by the addition of $\beta 1-3$ Gal creating core 1 O-glycan. This structure is capped with T-antigen (Samor *et al*, 1989). This structure accounts for 75% of the total O-linked glycan population expressed on VWF. Additionally, a portion of O-glycans are characterised as core 2 structures (Canis *et al*, 2010). Mass spectrometry

analysis has reported the presence ABO(H) blood group antigens on a proportion of these core 2 structures accounting for approximately 1% of the total VWF O-glycan population (Canis *et al*, 2010). The O-linked glycans of VWF are extensive sialylated. VWF contains ten putative O-glycan sites (Samor *et al*, 1989).

1.7.5 Multimerization

Mature VWF circulates as a series of large heterogeneous multimers. VWF assembles into these multimeric structures *via* additional disulphide bridging between N-terminal D'-D3 domains. In keeping with the acidic environment of the Golgi, VWF multimerization is highly pH-dependent. *In vitro* treatment of EC to increase the pH ablates VWF multimerization (Wagner *et al*, 1986). Recent electron microscopy studies have demonstrated that the acidic pH of the trans-Golgi (pH 6.2) enables VWF dimers to form dimeric bouquets (Zhou *et al*, 2011a).

The VWF propeptide (D1D2) is essential for normal VWF multimerization (Mayadas & Wagner, 1989). The propeptide displays intrinsic protein disulphide isomerase activity, and thus functions as a chaperone for VWF multimerization within the Golgi. Vicinal cysteines within the propeptide enable this region to catalyse thiol protein disulphide exchange between adjacent VWF dimers (Mayadas & Wagner, 1992).

1.7.6 VWF domain structure

The VWF translational protein is composed of a series of repeated homologous domains (A-D). Historically the VWF domain structure was given as D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-COOH (N- to C-terminal, Figure 1.5A). Recently however, electron microscopy as well as sequence alignment analysis has provided further details on the structure of VWF resulting in a re-annotation of the domains (Figure 1.5B) (Zhou *et al*, 2012).

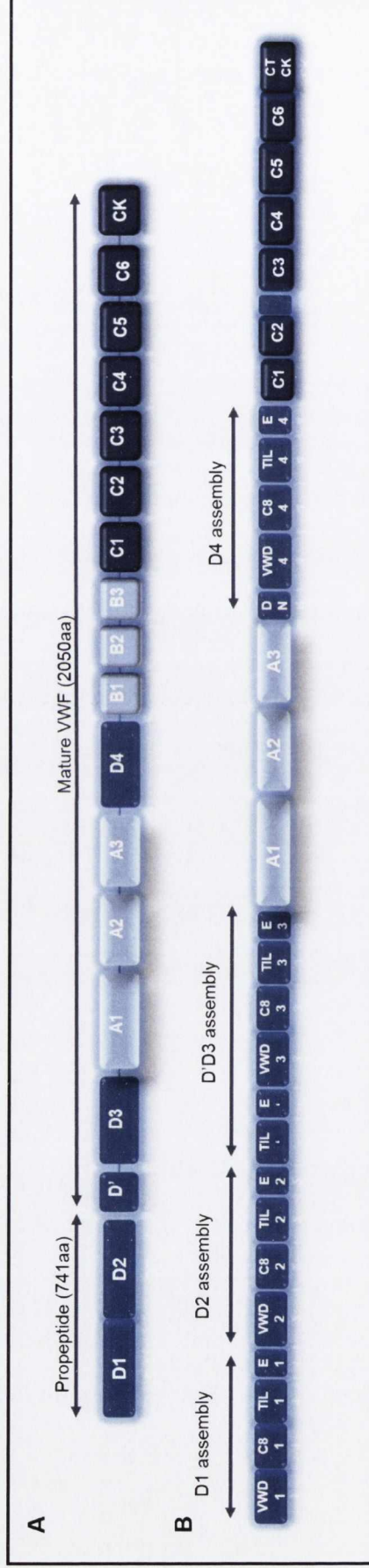


Figure 1. 5 VWF domain structure

(A) Historical annotation of the VWF domains (B) Re-annotated VWF domain architecture

1.7.7 Mature VWF

As a result of the processes detailed above, mature VWF has a multimeric structure and exists as a series of oligomers containing a variable number of subunits, ranging from a minimum of 2 to a maximum of 100. The monomeric size of VWF, taking into account both N- and O-linked glycosylation, is ~250kDa. Thus, mature VWF multimers vary in size from 500kDa to ~10,000kDa (Ruggeri & Zimmerman, 1981).

1.8 VWF storage and secretion

Following biosynthesis, VWF is stored in the Weibel-Palade Bodies (WPB) of EC and in platelet α -granules. Both organelles store HMWM, which is then secreted through a regulated pathway following stimulation. Alternatively however, VWF produced by EC may also undergo constitutive secretion. Approximately 90% of the VWF present in EC was thought to be released *via* this constitutive secretory pathway serving to maintain circulating VWF levels at ~10 μ g/ml (Sporn *et al*, 1986). However, identification of an additional secretory pathway has demonstrated that the majority of EC derived VWF (~80%) actually undergoes basal secretion (Giblin *et al*, 2008). Basal secretion does not require stimulation and occurs from a post-Golgi organelle, presumably the WPB.

Acute stimulated release of VWF involves activation of EC through the action of a variety of agonists including histamine, interleukin-1 and thrombin. VWF stored within WPB is exocytosed in response to endothelial stimulation (Van Mourik *et al*, 2002). Upon activation of EC, hyperactive ULVWF multimers are released into the bloodstream (Wagner *et al*, 1991; Wagner & Bonfanti, 1991). This releasable pool of VWF constitutes ~10% of VWF secreted from the endothelium. EC activation results from stimulation by inflammatory mediators or through vascular injury. Several other agonists for stimulated secretion of VWF have been identified including vasopressin

(Kaufmann *et al*, 2000) and shiga toxin (Huang *et al*, 2010; Nolasco *et al*, 2005). Conversely, other mediators can reduce VWF secretion have also been reported. For example, endothelial derived nitric oxide was shown to limit stimulated secretion of VWF from WPB (Qian *et al*, 2010).

1.8.1 Weibel-Palade Bodies

WPB are elongated rod-shaped, membrane bound secretory organelles specific to EC and thought to originate from the Golgi apparatus. They are approximately 4 μ M in length and 0.1 μ M in diameter, consisting of bundles of tubules embedded in a dense matrix (Weibel & Palade, 1964). During biosynthesis, VWF assemble into helical tubules. These helical tubules are formed with VWF and cleaved VWF:pp (Wagner *et al*, 1982), which are stored in a 1:1 ratio (Sporn *et al*, 1987). The VWF contained within WPB is composed of the highest molecular weight multimers (Loesberg *et al*, 1983). Several other proteins apart from VWF have been identified as constituents of WPB including; P-selectin, interleukin-8, eotaxin-3, endothelin-1, tissue-type plasminogen activator, osteoprotegerin and angiopoietin-2 (Zhang & Li, 2009; Rondaij *et al*, 2006). As such, WPB supply mediators that can be released in response to signalling molecules or stress allowing EC to affect haemostasis, angiogenesis and inflammation.

VWF is a major constituent of these storage organelles, and is a prerequisite for WPB existence as the EC of VWF-deficient animals do not contain WPB (Denis *et al*, 2001; Haberichter *et al*, 2005). Restoration of VWF expression to EC in dogs with Type 3 VWD restores WPB biogenesis (Haberichter *et al*, 2005). Expression of VWF in non-EC induces pseudo-WPB containing VWF, which share their characteristic cigar shape (Voorberg *et al*, 1993; Wagner *et al*, 1991). It has been demonstrated that VWF:pp is required to target VWF to WPB (Wagner *et al*, 1991).

In human embryonic kidney 293 cells, expression of VWF domains D1D2D'D3 is sufficient to store VWF in WPB as removing the A1 domain does not impair formation of elongated storage granules (Michaux *et al*, 2006). Mutations in the

D1D2D'D3 region of VWF that cause various subtypes of VWD affect some of the biosynthesis and WPB formation steps. VWD type 2A is characterised by the absence of large VWF multimers with several type 2A mutations located in the VWF:pp exerting different effects on WPB biogenesis. For example, the mutation Y87S in the D1 domain causes defects of multimer assembly (Rosenberg *et al*, 2002). This mutation when expressed in human EC cause endogenous WPB to become spherical indicating the mutant VWF disrupts the tubular packing of wild-type VWF (Michaux *et al*, 2006). Therefore, mutagenesis of VWF has indicated the roles the D domains play in VWF targeting and tubular packaging. It also demonstrates that multimerization is not required for the formation of WPB and VWF can be stored in WPB that are not elongated. The structural requirements in platelet α -granules have not yet been investigated but are likely to be related.

Exocytosis from WPB is tightly regulated but can be induced by a variety of agonists to rapidly mediate inflammatory, haemostatic and angiogenic events. Following exocytosis, VWF:pp which is not covalently bound to VWF in WPB is rapidly released (Hannah *et al*, 2005). VWF multimers are released in strings which can remain associated with EC for some time (Huang *et al*, 2008; Michaux *et al*, 2006). The strings can be several millimetres long that branch and form bundles indicating a complex association of several VWF multimers. They are composed of ULVWF (Dong *et al*, 2002) as a result of extracellular neutral pH. The EC surface can bind strings formed from agonists *in vitro* (Valentijn *et al*, 2010; Huang *et al*, 2009) and *in vivo* (Michaux *et al*, 2006; Chauhan *et al*, 2007). These unfurled strings can readily attach platelets as seen in figure 1.6 where human umbilical vein endothelial cells (HUVEC) when stimulated with agonists such as histamine release VWF. These strings can be visualised microscopically when platelets are flowed over the cells.

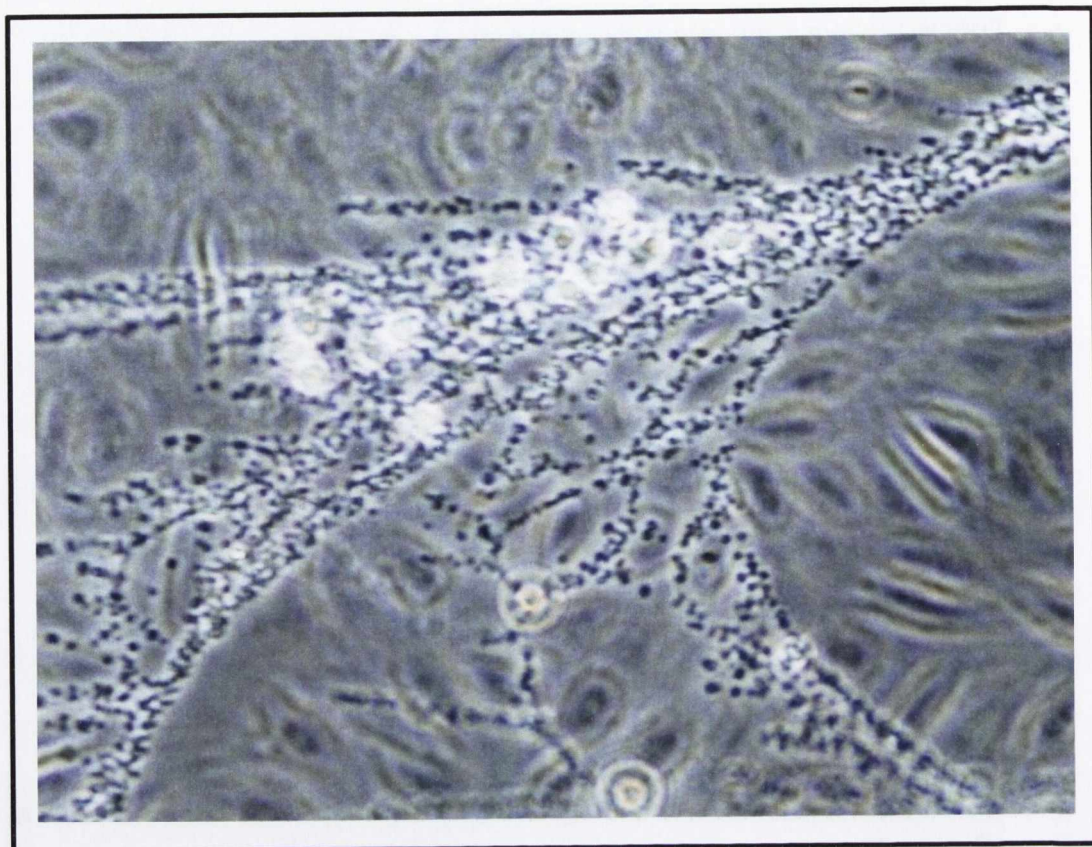


Figure 1. 6 Platelet-decorated strings following WPB exocytosis from histamine treated HUVEC

Angiopoietin-2 (Ang-2) is a secreted glycoprotein that plays roles in angiogenesis, vascular development and also inflammation. Ang-2 is expressed in smooth muscle cells of angiogenic tissue (e.g. placenta) and vascular EC representing another vascular modulator stored within WPB (Fiedler *et al*, 2004). Ang-2 is only incorporated into WPB that lack P-selectin, suggesting that Ang-2 and P-selectin storage are mutually exclusive (Pichiule *et al*, 2004). P-selectin is a transmembrane protein involved in leukocyte adhesion and is expressed on the surface of activated platelets and EC. P-selectin is incorporated into WPB through direct interaction with

VWF (Harrison-Lavoie *et al*, 2006; Barkalow *et al*, 2000; Dole *et al*, 2005). Interestingly, P-selectin can also be incorporated into the α -granules of platelets (Johnston *et al*, 1989). However, while VWF deficiency disrupts P-selectin targeting to WPB, it does not affect targeting of P-selectin to α -granules. In contrast, deficiency of P-selectin does not affect targeting of VWF to WPB (Denis *et al*, 2001). Like P-selectin, osteoprotegerin also appears to be integrated into WPB through direct interaction with VWF (Zannettino *et al*, 2005) and is also co-localised with VWF in platelet α -granules (Chollet *et al*, 2010). Therefore, both P-selectin and OPG are actively stored in WPB as a result of their ability to interact with VWF.

1.8.2 Platelet α -granules

Platelet α -granules are the most abundant platelet secretory organelle, comprising ~10% of the total platelet volume and are essential to normal platelet activity (Blair & Flaumenhaft, 2009). Each platelet contains 50-80 α -granules that range in size from 200-500nm (Frojmovic & Milton, 1982). Following platelet activation, α -granules fuse with the plasma membrane to release their cargo (Polgár *et al*, 2002). Within the α -granules, VWF has been shown to co-localize with other haemostatic proteins including fibrinogen and thrombospondin (Wencel-Drake *et al*, 1985). VWF release is triggered by several different agonists, including ADP, collagen and thrombin and proceeds *via* the regulated secretory pathway. Constitutive secretion of VWF by primary guinea pig megakaryocytes has been demonstrated *in vitro* (Schick *et al*, 1997). However, the significance of constitutive VWF secretion from megakaryocytes in the context of maintaining VWF plasma levels is not known.

1.9 VWF structure and function

1.9.1 FVIII binding

FVIII circulates in non-covalent complex with VWF in plasma. VWF binds to the N-terminal light chain of FVIII (Lollar *et al*, 1988). VWF serves to protect FVIII from premature proteolytic inactivation by activated protein C, stabilises its heterodimer structure, and prolongs its survival in plasma (Weiss *et al*, 1977; Fay *et al*, 1991a). The critical physiological relevance of the VWF-FVIII interaction is highlighted by the markedly reduced FVIII plasma levels in patients with VWD type 3 having virtually undetectable levels, and in patients with a defect in the FVIII-interactive site of VWF (VWD type 2N). Each monomer of multimeric VWF contains a single FVIII binding site. However, within normal plasma the FVIII:VWF stoichiometry is approximately 1:50 (Vlot *et al*, 1995).

1.9.2 Sub endothelium matrix binding

At sites of vascular injury, VWF mediates platelet adhesion to the vascular sub endothelium by binding to collagen. Fibrillar collagens type I and III are most important for VWF binding (Pareti *et al*, 1987). Collagen binding is mediated *via* the A1 and A3 domains of VWF (Cruz *et al*, 1995). Deletion of the A1 domain does not alter collagen binding compared to wild type VWF (Sixma *et al*, 1991). In contrast, deletion of the A3 domain significantly attenuated collagen III binding (Romijn *et al*, 2001). Consequently, the A3 domain is considered the major physiological collagen III binding region (Hoylaerts *et al*, 1997).

1.9.3 Platelet binding

Following vessel injury, VWF binds to sub endothelial collagen and subsequently tethers platelets and supports platelet aggregation to facilitate thrombus formation. Platelets express two distinct receptors for VWF: the GPIb-IX-V complex and the GPIIb-IIIa complex. VWF binding to the GPIb α receptor on non-activated

platelets is important in the initial tethering of platelets to the sub endothelium (Figure 1.7). Once platelets become activated, the GPIIb-IIIa complex becomes an additional high affinity ligand for VWF. This promotes platelet spreading and aggregation, and ultimately the formation of a stable platelet plug. Binding of VWF to platelet receptors is highly dependent on the rate of shear within the vasculature. In the absence of shear stress, VWF adopts a globular, condensed configuration which is non-permissive to GPIIb binding. However shear stress promotes VWF unwinding to form an extended chain that enables interaction with platelet receptors (Siedlecki *et al*, 1996; Ikeda *et al*, 1991). At low rates of shear stress (less than 600 s^{-1}) platelet GPIIb-IIIa can mediate platelet tethering to fibrinogen in a process independent of VWF (Savage *et al*, 1996). In contrast, at higher shear stress VWF is an essential component in platelet tethering. (Savage *et al*, 1998, 1996). Mohri *et al* identified important regions for mediating GPIIb binding residing within the VWF A1 domain, on either side of the disulphide loop (Mohri *et al*, 1988). In addition, a number of VWD type 2M mutations cluster within this VWF binding site for GPIIb. Moreover, Yago *et al* have further demonstrated that specific VWD type 2B mutations can overcome the need for high shear in order for VWF to be able to bind GPIIb (Yago *et al*, 2008). On resting platelets, GPIIb-IIIa does not interact with VWF. However upon platelet activation GPIIb-IIIa displays high affinity binding with VWF.

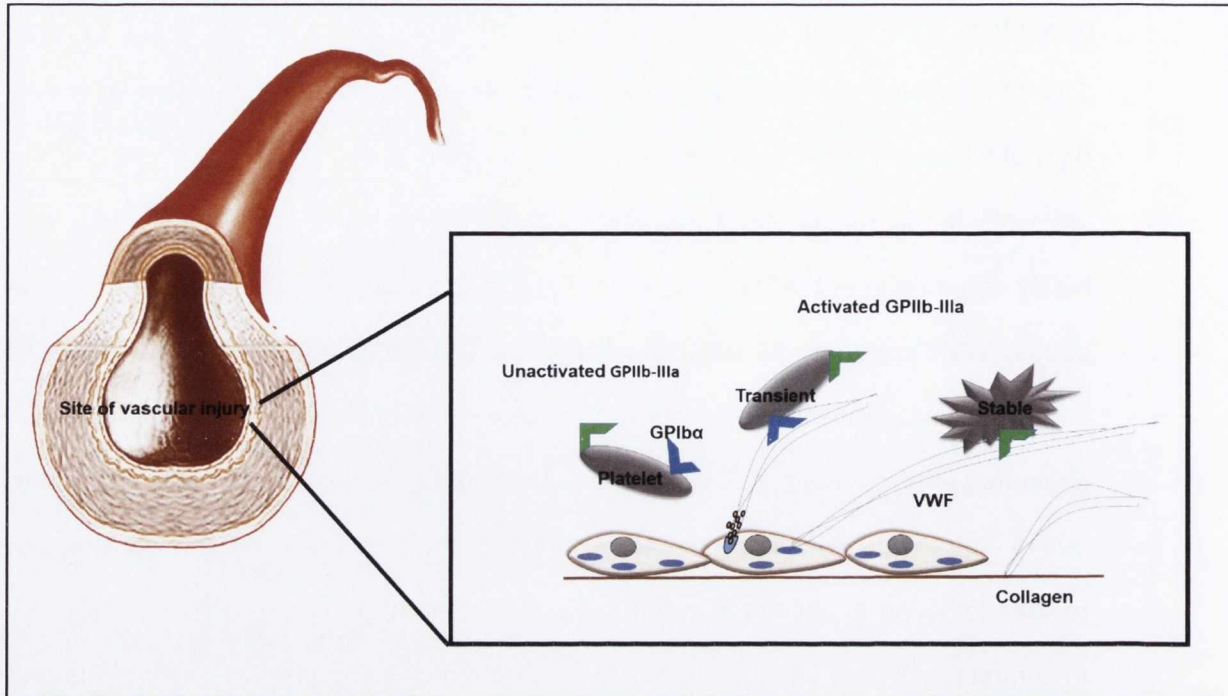


Figure 1. 7 Model of VWF-dependent platelet adhesion

1.9.4 Additional interactions

In addition to binding to proteins of the haemostatic system, VWF is capable of interacting with an array of matrix proteins as well as circulating plasma proteins (Lenting *et al*, 2012; Lenting PJ, Christophe OD, 2015). Though the functional significance of some of these interactions remains unknown, these interactions implicate roles for VWF outside of the haemostatic system. Figure 1.8 illustrates some of binding partners of VWF and the specific domains that regulate their interaction.

Proteins including ADAMTS13 (Zanardelli *et al*, 2009; Feys *et al*, 2009), and two members of the galectin family, galectin-1 and galectin-3 (Saint-Lu *et al*, 2012) are bound to VWF in plasma. β 2-Glycoprotein I (β 2-GPI) also binds to the VWF A1 domain, and competitively inhibits binding to GPIb α , resulting in decreased platelet aggregation (Hulstein *et al*, 2007). VWF can also bind to the adhesive matrix glycoprotein thrombospondin-1 (TSP1) *via* the A3 domain. Interestingly, this interaction

serves to competitively inhibit ADAMTS13-mediated proteolysis of ULVWF strings (Wang *et al*, 2010). Conversely, complement factor H binding to the VWF A2 domain acts to enhance ADAMTS13-mediated cleavage of VWF (Feng *et al*, 2013). VWF also directly interacts with several proteins involved in inflammation. VWF interacts with P-selectin glycoprotein-1 (PSGL-1) and activated β 2 integrins to facilitate leukocyte recruitment (Pendur *et al*, 2006). PSGL-1 promotes initial transient binding, while β 2-integrins participate in stable adhesion. In addition, VWF binds to *Staphylococcus aureus* protein A during endovascular infection which may facilitate colonization (Hartleib *et al*, 2000; O'Seaghda *et al*, 2006).

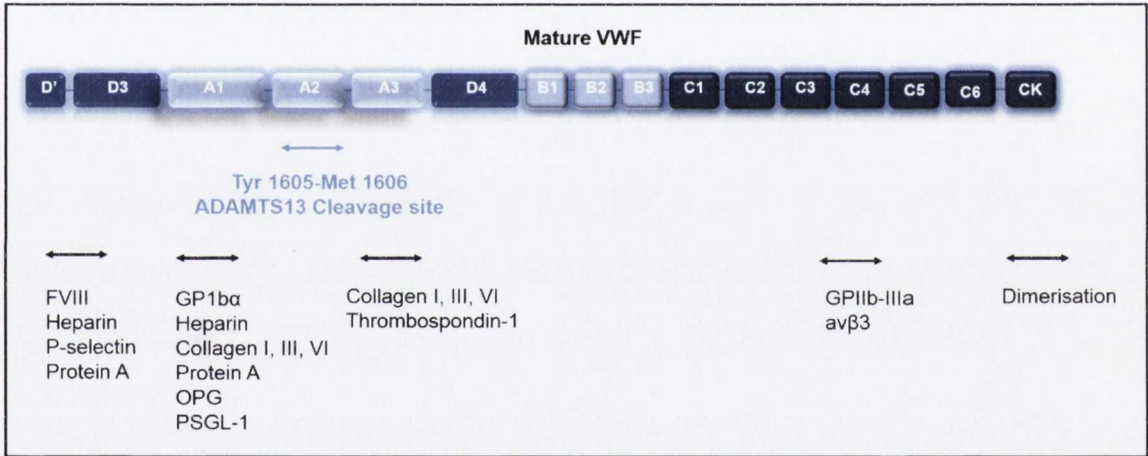


Figure 1. 8 Binding partners of VWF

1.10 VWF and ADAMTS13 in health and disease

Regulation of VWF adhesive activity is critical for maintaining homeostasis in the blood. During secretion from EC, ULVWF remain transiently bound to EC surfaces where they undergo rapid, partial proteolysis into smaller less active VWF multimers

reducing thrombotic potential (Sadler, 2008). ADAMTS13 is a VWF-specific cleaving protease which cleaves the Y1605/M1606 scissile bond in the VWF A2 domain, thereby preventing the accumulation of ULVWF multimers in normal plasma (Levy *et al*, 2005). Deficiency of ADAMTS13 results in the life threatening microangiopathy, thrombotic thrombocytopenic purpura (TTP) (Levy *et al*, 2001). TTP is characterised by the consumption of platelets, as a result of the formation of platelet and VWF rich thrombi in the microvasculature. Most cases of TTP can be attributed to low ADAMTS13 antigen levels or decreased ADAMTS13 activity, due to either a dysfunctional protein or acquired autoantibodies (Luken *et al*, 2005; Sadler, 2008). As a result of this, abnormal ULVWF multimers are present in these patients.

The absence of VWF *in vitro* increases EC proliferation and VWF knockout mice have highly vascularised ears (Starke *et al*, 2011). VWF has also been linked with numerous disease processes including tumour metastasis (Franchini *et al*, 2013). Several animal models have shown that deficiency of ADAMTS13 leads to a prothrombotic state, with the duration of VWF-platelet-endothelium interactions being increased (Banno *et al*, 2006). Furthermore, administration of recombinant ADAMTS13 to deficient mice promoted breakdown of thrombi in arterioles (Chauhan *et al*, 2008). Interestingly, ADAMTS13 deficient mice do not present with TTP like symptoms until challenged with shiga toxin (Motto *et al*, 2005).

Regulation of VWF adhesive potential by ADAMTS13 is not only vital for the control of haemostasis, but also plays a major role in the regulation of VWF function in inflammation. VWF has been shown to participate in the progress of inflammation through adhesion to polymorphonuclear cells and monocytes, thereby contributing to leukocyte tethering and rolling (Pendü *et al*, 2006). VWF acts as a major regulator of leukocyte extravasation during experimental peritonitis (Petri *et al*, 2010) and has been demonstrated to play a regulatory role in cutaneous inflammation. Anti-VWF antibodies were useful in the treatment of cutaneous inflammation without enhancing bleeding

time in mice (Hillgruber *et al*, 2014). Thrombocytopenia is also a major complication of inflammation, and VWF has been suggested as an important mediator of this process (Wagner & Frenette, 2008).

ADAMTS13 levels have been documented to be low in both chronic and acute inflammation (Kremer Hovinga *et al*, 2007; Martin *et al*, 2007). Moreover, an inverse correlation has between high levels of ULWVF and low levels of ADAMTS13 been observed in systemic inflammation (Claus *et al*, 2009). Similarly, high and low levels of ULWVF and ADAMTS13 respectively have been found in ICU patients with systemic inflammation resulting from cardiac surgery or severe sepsis (Bockmeyer *et al*, 2008). The thrombotic state associated with endotoxemia may be due to an imbalance in the expression of both VWF and ADAMTS13; as high and low mRNA levels, as well as high and low blood antigen levels, were found in LPS challenged mice (Mimuro *et al*, 2008). High concentrations of VWF with enhanced functional activity correlating with the rate of haemolysis have also been described in patients with sickle cell disease (Chen *et al*, 2011). Djamiatun *et al* recently also reported elevated VWF levels in dengue infection (Djamiatun *et al*, 2012).

Due to the increasingly apparent role for VWF in inflammation, this protein has become a target in studies (Denis *et al*, 2001; Gandhi *et al*, 2012a; Khan *et al*, 2012). Animal models of disease states have demonstrated that VWF deficiency provides protection against atherosclerosis (Methia *et al*, 2001; van Galen *et al*, 2012) and that ADAMTS13 deficiency is detrimental in myocardial ischemia (Gandhi *et al*, 2012b). Animal studies have also shown that ADAMTS13 has protective properties during ischemic brain damage and reduces VWF-mediated acute cerebral inflammation following stroke (Zhao *et al*, 2009; Fujioka *et al*, 2010).

1.11 VWF and Malaria

Both cerebral and severe malaria patients have decreased ADAMTS13 antigen and activity levels (Larkin *et al*, 2009; Löwenberg *et al*, 2010; Schwameis *et al*, 2015). Similarly, deficiency of plasma ADAMTS13 antigen and activity levels have also been reported in patients infected with *P. vivax* malaria. The molecular mechanism responsible for decreased ADAMTS13 levels in malaria remains to be elucidated (de Mast *et al*, 2009).

Previous studies have demonstrated that plasma VWF antigen (VWF:Ag) (de Mast *et al*, 2007; Larkin *et al*, 2009; Hollestelle *et al*, 2006; Löwenberg *et al*, 2010; Phiri *et al*, 2011; de Mast *et al*, 2009) and VWF:pp (Löwenberg *et al*, 2010; Phiri *et al*, 2011; Conroy *et al*, 2010; Hollestelle *et al*, 2006; de Mast *et al*, 2007) levels are markedly elevated in children with severe *P. falciparum* malaria, consistent with acute EC activation. Indeed, children with CM had VWF:pp levels exceeding those typically observed in fulminant vascular diseases such as TTP. Furthermore, a study of 14 healthy volunteers infected with *P. falciparum* showed that the increased plasma VWF and VWF:pp levels develop soon after the onset of blood stage infection (de Mast *et al*, 2007). Elevated VWF:pp levels inversely correlate with clinical outcomes. Severe *P. falciparum* malaria is associated not only with increased plasma VWF:Ag levels, but an even more marked increase in VWF activity as determined by collagen binding assay (VWF:CB). Moreover, this increase in VWF functional activity is due to the presence of abnormal ULVWF multimers in the plasma of children with severe malaria (Larkin *et al*, 2009) with a correlation between levels of ULVWF and severity of disease (de Mast *et al*, 2007; Hollestelle *et al*, 2006; Conroy *et al*, 2010; Phiri *et al*, 2011). Furthermore, these elevated levels of hyperactive ULVWF that have been described in *P. falciparum* infection, exceed those reported in both acute septicaemia and TTP (van Mourik *et al*, 1999). Consequently, acute EC activation constitutes an early feature of *P. falciparum* infection.

1.12 Severe malaria and WPB exocytosis

Previous studies have demonstrated that plasma lactate levels are markedly elevated in children with severe *P. falciparum* malaria, and that these levels inversely correlate with clinical outcomes. Serum lactate is a strong biomarker of disease severity as is parasite count (Hollestelle *et al*, 2006). This marked increase in VWF:Ag and VWF:pp suggests that WPB exocytosis is occurring during malarial infection (Figure 1.9). The degree of EC perturbation is linked with severity of disease indicated by plasma lactate. Furthermore, significantly elevated plasma Ang-2 levels have been reported in patients with severe *P. falciparum* malaria, where Ang-2 levels were also shown to inversely correlate with clinical outcome (Yeo *et al*, 2008; Conroy *et al*, 2009, 2010; Lovegrove *et al*, 2009; Erdman *et al*, 2011).

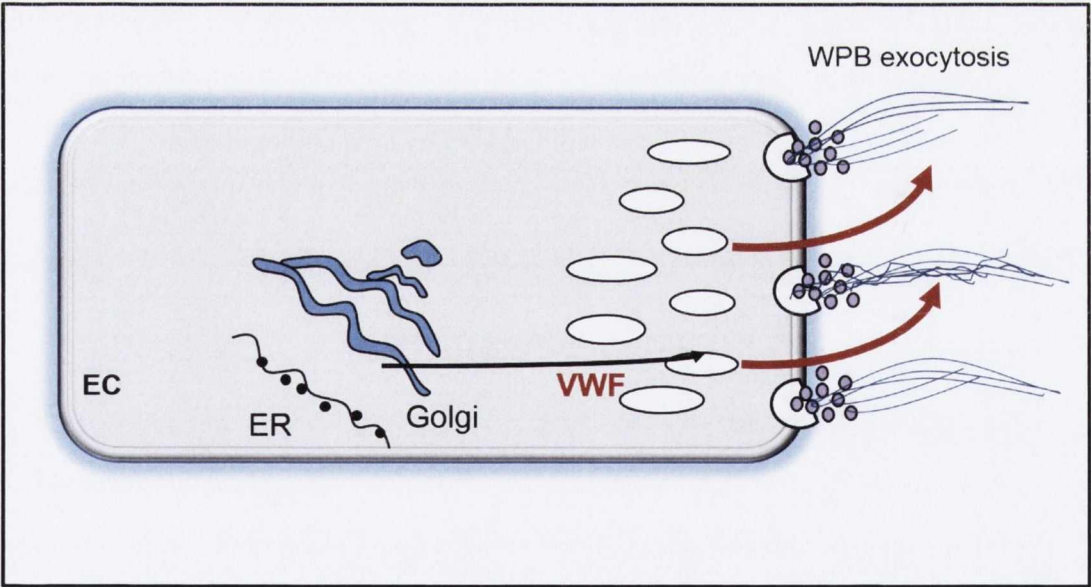


Figure 1. 9 Endothelial cell undergoing exocytosis of WPB in malaria

1.13 ABO phenotype, VWF levels and malaria severity

The ABO blood group system is a critical system for blood group compatibility in transfusion. ABO blood group distribution has evolved along with selective pressure due to *P. falciparum*. Blood group A is associated with a more severe disease phenotype. Blood group O is frequently found in areas that are malaria endemic including South America and Sub Saharan Africa (Cserti & Dzik, 2007). Group O is protective against severe complications of malaria infection. This blood group has ~25% less VWF than other ABO blood groups. In addition, the susceptibility of VWF to proteolysis by the ADAMTS13 was greater for group O VWF than for non-O VWF. This observation supports the hypothesis that VWF may play a role in the pathogenesis of malarial infection.

Strong evidence exists suggesting that ABO phenotype modulates severity of *P. falciparum* malaria. The investigation of ABO is outside the scope of this thesis due to the lack of the ABO blood group system in the murine model of malaria. However, future research into the relationship between ABO blood group phenotypes and malaria pathogenesis may provide a greater understanding of glycobiology in infectious diseases and contribute to a wider insight of malaria pathogenesis.

1.14 VWF in the pathogenesis of malaria

Following endothelial disruption, VWF binds to exposed collagen in the sub endothelial matrix. This anchored VWF undergoes marked conformational changes in response to shear stress exerted by the circulating blood, and can then tether platelets through specific binding of the platelet GP Ib-IX-V receptor. Accumulating evidence suggests that platelet adhesion and aggregation play important roles in facilitating cytoadhesion of *P. falciparum* IE to activated EC. For example, post-mortem studies have shown platelets co-localised with sequestered IE at brain microvascular sites

obstructing vessels (Grau *et al*, 2003). In addition, *in vitro* studies have demonstrated that platelets can modulate the expression of adhesion molecules (e.g. ICAM1) on EC surfaces, and identified platelet-mediated IE adhesion to TNF-activated brain endothelium (Pain *et al*, 2001).

Despite this critical role for platelets, it remains unclear if the pathological ULVWF multimers circulating in the plasma of children with *P. falciparum* play any direct role in mediating the pathophysiology of disease progression. Nevertheless, elegant studies using a novel llama-derived nanobody have demonstrated that a significant proportion of circulating plasma VWF in *P. falciparum* infected patients is present in an active platelet GPIb-binding conformation (de Mast *et al*, 2007). Furthermore, plasma VWF:Ag levels in patients with malaria inversely correlate with platelet count (de Mast *et al*, 2007), and Hollestelle *et al* have previously shown that plasma VWF:pp levels correlate with other established biochemical markers of malaria severity, including plasma lactate (Hollestelle *et al*, 2006).

In recent work, our laboratory previously described an entirely novel mechanism through which VWF can mediate the adhesion and sequestration of IE to activated EC (Bridges *et al*, 2010; López, 2010). In brief, using an *in vitro* laminar flow model, the formation of long platelet-decorated ULVWF strings following HUVEC activation were observed. These findings are in keeping with previous reports. However, Bridges *et al* further demonstrated that these VWF-dependent platelet strings were able to bind fluorescently labelled *P. falciparum* IE in a CD36 dependent manner (Bridges *et al*, 2010). In contrast, VWF-platelet strings did not bind to uninfected erythrocytes. Cumulatively, these data identify a new VWF-dependent mechanism that may contribute to IE sequestration. Moreover, in contrast to classic activation pathways that require *de novo* protein synthesis, pre-formed ULVWF is rapidly released from the WPB stores of activated EC. Thus, formation of VWF-platelet strings on the EC surface may be particularly important in generating a pro-adhesive surface during the early

stages of IE sequestration. We postulate that during the early stages following malaria infection, formation of ULVWF strings on activated EC surfaces may be important in recruiting platelets (Figure 1.10). These tethered platelets result in further EC activation, and more WPB secretion. In addition, platelet-decorated VWF strings play a role in modulating subsequent microvasculature sequestration by recruiting malaria-infected erythrocytes, and also by binding to granulocytes and activated monocytes respectively (Bernardo *et al*, 2005; Pendu *et al*, 2006). The VWF-mediated sequestration of platelets, infected erythrocytes and leucocytes leads to further enhanced EC activation, increased EC permeability, and ultimately results in vessel occlusion.

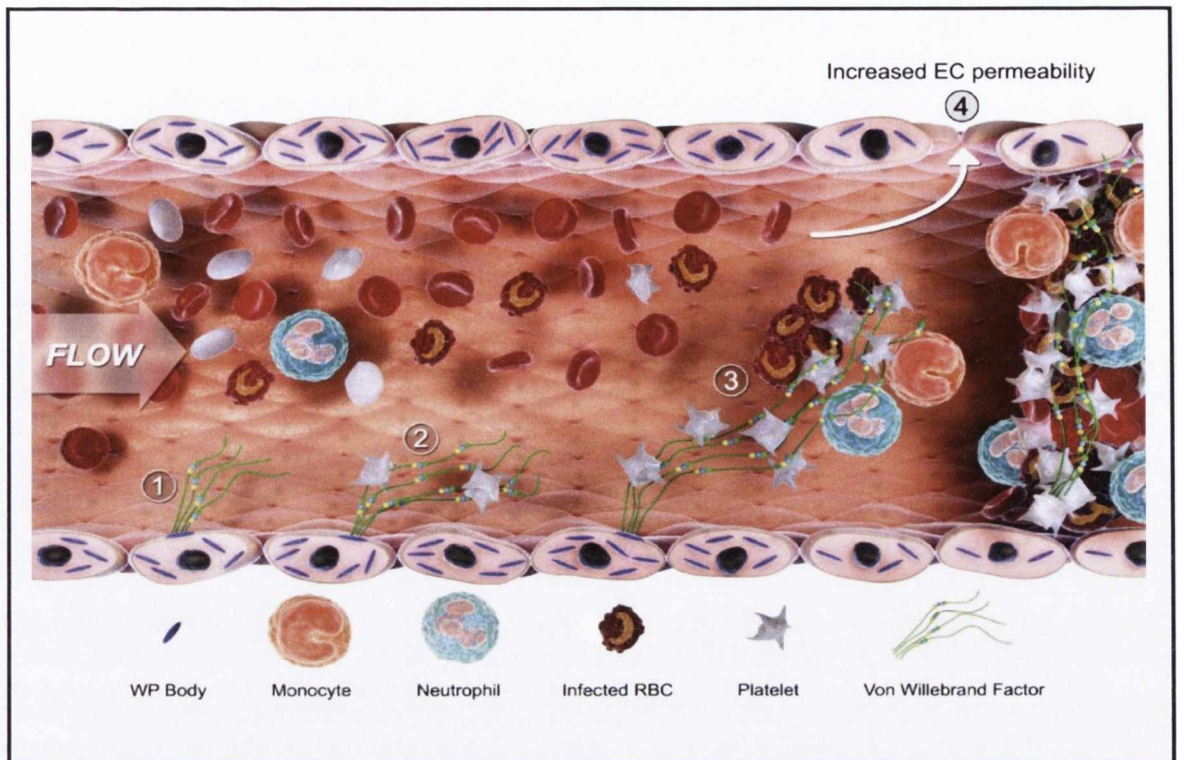


Figure 1. 10 Schematic diagram illustrating the proposed mechanisms through which VWF is involved in malaria pathogenesis

EC activation and release of WPB contents constitute common early features in both human and murine malaria. This results in the secretion in the release of ULVWF multimers into the plasma (1) and a marked increase in plasma VWF levels. ULVWF strings on the surface of activated EC then recruit and sequester platelets within the microvasculature (2). These tethered platelets cause further EC activation, and thus more WPB secretion. In addition, the platelet-decorated VWF strings modulate 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, increased EC permeability (4), and ultimately results in vessel occlusion (5).

1.15 Study objectives

As discussed, our laboratory and others have previously shown that plasma levels of VWF:Ag and VWF:pp are significantly increased in African children with severe *P. falciparum* malaria (Larkin *et al*, 2009; Hollestelle *et al*, 2006; Erdman *et al*, 2011; Phiri *et al*, 2011; Conroy *et al*, 2010). Moreover, a study of healthy volunteers infected with *P. falciparum* has also demonstrated that the elevated plasma VWF:Ag and VWF:pp levels is present from an early stage following infection. Collectively these data demonstrate that marked EC activation, with consequent VWF secretion from WPB, constitutes an early hallmark of *P. falciparum* malaria infection. It is unclear whether early increase in plasma VWF levels and circulating ULVWF multimers observed following *P. falciparum* infection 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 sought to investigate the putative role of VWF in the pathogenesis of malaria *in vivo* using an established murine model of experimental cerebral malaria (ECM). To further investigate the underlying mechanisms of VWF contributing to the pathogenesis of ECM, we wanted to study other genetically modified mice strains.

Previously, Djamiatun *et al* demonstrated that severe dengue fever is associated with exocytosis of WPB with significantly elevated VWF:Ag, VWF:pp and OPG plasma levels (Djamiatun *et al*, 2012). Dengue fever shares many of the key clinical features seen in malaria including EC activation and a pronounced thrombocytopenia. Given the key role of EC activation in the pathogenesis of severe *P. falciparum* malaria, we have further investigated plasma OPG levels in children with CM. To elucidate the relationship between WPB secretion and malaria, we have further examined OPG levels in an established murine model of experimental CM.

Infection with *P. falciparum* is associated with thrombocytopenia, decreased levels of anticoagulant clotting factors, leading to generation of thrombin. Recently, a

novel role for EPCR has been reported in the pathogenesis of *P. falciparum* malaria (Moxon *et al*, 2013; Turner *et al*, 2013). Interestingly, in post-mortem samples from African children with CM, studies have shown marked fibrin deposition and localised to sites of IE sequestration. The brain demonstrates constitutively low expression of both TM and EPCR, and may therefore be particularly vulnerable to CM coagulopathy (Moxon *et al*, 2013). The molecular mechanisms by which coagulopathy and the protein C pathway contribute to CM remain unknown. We sought to examine if the mouse model of CM could be used to study malarial-induced coagulopathy, and to determine whether the Protein C pathway may be involved in the pathogenesis of CM.

2. Materials and Methods

2.1 Patient samples

2.1.1 Blood collection

Recruitment took place at the Queen Elizabeth Central Hospital, Blantyre, Malawi between 2010 and 2011 by Dr. Christopher Moxon. Informed consent was obtained from the parents or legal guardians of all the children enrolled in this study in accordance with the Declaration of Helsinki. Clinical details were recorded for all patients at the time of recruitment, and subsequent analyses were carried out blinded to these details. All patients with malaria received standard supportive and anti-malarial treatment. Children aged 1–12 years were recruited into three categories as follows:

Cerebral malaria cases

Patients (n=146) were recruited when admitted with an initial clinical diagnosis of CM, defined as a Blantyre Coma Score of 2 or less (Molyneux *et al*, 1989), with peripheral *P. falciparum* parasitemia and no other recognisable cause of coma (Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster., 2000).

Uncomplicated malaria samples

Uncomplicated malaria cases (n=52) were recruited from patients with acute febrile illness without signs of organ compromise (Marsh *et al*, 1995).

Febrile controls without malaria

Children with a non-malaria febrile illness (NMFI) (n=48) were recruited from attenders at the Paediatric Accident and Emergency Unit. This group included children not requiring admission, who had a documented febrile illness ($>37.5^{\circ}\text{C}$) with no malaria parasites on a single thick blood film, a negative rapid diagnostic test for malarial

antigens, and no history of having taken any antimalarial medication in the previous two weeks.

Healthy controls

These were well children with no febrile illness (NFI) admitted for elective surgery (n = 26).

2.1.2 VWF:Ag ELISA

VWF antigen (VWF:Ag) was measured by sandwich enzyme-linked immunosorbent assay (ELISA). Maxisorp plates (Nunc, Denmark) were coated with rabbit anti-human VWF antibody (Dako, Denmark) at a final concentration of 6.2µg/mL in 50mM carbonate buffer (pH 9.6), overnight at 4°C. Wells were washed three times with PBS-T (PBS, 0.05% v/v Tween-20) and then blocked for non-specific binding with PBS-T containing 3% bovine serum albumin (BSA; Sigma, Ireland) for 1 hour at room temperature. Wells were again washed three times with PBS-T before test samples at appropriate dilutions were added. Reference plasma (Technoclone, Austria) diluted 1:80-1:2560 (final concentration 0.125-0.0039µg/mL) was used to generate a standard curve. After 2 hour incubation at room temperature wells were washed three times with PBS-T and 100µl of anti-VWF-HRP (Dako, Denmark) diluted 1:500 in PBST (final concentration 2.2µg/mL) was added and incubated for 1 hour at 37°C. Following another 3 PBS-T washes, 100µl of HRP substrate 3,3',5,5'-Tetramethylbenzidine (TMB; Substrate Reagent Pack, R&D Systems, UK) was added to the wells. The reaction was subsequently stopped with 50µL 1M H₂SO₄. Absorbance was read at 450nm using a VERSAmax microplate reader (Molecular Devices, UK).

ELISA samples were tested in triplicate, using two replicate wells for each set of test conditions. The intra-assay and inter-assay coefficients of variation were both less than 10%. A standard curve was generated by plotting VWF concentration (µg/mL) on

the x-axis against optical density (absorbance @ 450nm) on the y-axis. The curve was linearized by performing a logarithmic transformation and the equation of the line and R^2 values were calculated using Microsoft Excel.

2.1.3 OPG ELISA

Human osteoprotegerin (OPG) was measured using the Osteoprotegerin/TNFRSF11B R&D Systems DuoSet® ELISA development system. Maxisorp plates were coated with capture antibody at a working dilution of 2µg/mL in PBS over night at room temperature. Wells were washed for a total of three washes with 400µl PBS-T and blocked with 300µl of reagent diluent (1% BSA in PBS) for 1 hour at room temperature. Wells were washed three times with PBS-T and test samples at appropriate dilutions were added. Recombinant OPG diluted (final concentration 1000-15.625pg/mL) was used to generate a seven point standard curve. After 2 hour incubation at room temperature wells were washed three times with PBS-T and 100µl of detection antibody diluted in PBST (final concentration 200ng/mL) was added and incubated for 2 hours at room temperature. Following incubation wells were washed three times with PBS-T and 100µl of the working dilution of Streptavidin-HRP was added to each well. This was incubated 20 minutes at room temperature avoiding direct light. The plate was again washed three times with PBS-T and 100µl of substrate solution was added to each well. 50µl of the stop solution was added once adequate colour was reached. The optical density was determined at 450nm.

2.1.4 Immunoprecipitation to detect VWF-OPG plasma complexes

Protein G is a cell wall component of group G *Streptococcus* strains which binds the Fc part of an extensive range of immunoglobulins. Protein G coupled to superparamagnetic Dynabeads® (Dynabeads-Protein G, Invitrogen) are therefore suitable for efficient capture of a wide range of immunoglobulins.

The Protein G-coupled magnetic beads were suspended thoroughly to obtain a homogeneous suspension. Plasma was pre-cleared for two hours at room temperature by adding 20µl of beads to 200µl plasma and left to rotate. Following the incubation the samples the beads collected using a magnetic particle collector (New England BioLabs® Inc., Ireland). The supernatant was kept and beads discarded. This plasma was incubated with the antibody against murine OPG (N-20, Santa Cruz Biotechnology, USA) at 2µg/mL overnight at 4°C. Controls included the plasma without antibody and antibody mixed with PBS. Following overnight incubation 20µl of beads were added to the tube and left to rotate for 4 hours at 4°C. Following overnight incubation, 20µl of beads were added to the tube and left to rotate for 4 hours at 4°C. Subsequent to this, the beads were separated from plasma using a magnetic particle collector (New England BioLabs® Inc., Ireland) and washed in triplicate in PBS-T.

Samples were boiled in reducing sample buffer (4X; Life Technologies, UK) with 5% (v/v) 2-mercaptoethanol and analyzed by SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to Polyvinyl-difluoroacetate (PVDF; Immobilon-P; Millipore, Ireland) blocked for 1 hour with 5% BSA-0.05% Tween-20 in PBS, and incubated with rabbit polyclonal anti-human VWF (Dako, Denmark) for 1 hour at room temperature with gentle agitation. Primary antibody was subsequently detected with Goat anti-mouse-HRP (Santa Cruz Biotechnology, USA) and bound antibody was detected using the SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, Ireland) according to the manufacturer's instructions. 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).

2.2 Murine studies

2.2.1 Animal husbandry

Male and female wild type (WT), VWF^{-/-}, ADAMTS13^{-/-} and ASGPR^{-/-} mice were used between 8-10 weeks of age. Mice were housed in individually ventilated and filtered cages under positive pressure (Techniplast, UK). Food and water were supplied ad libitum. 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.

2.2.2 Wild type mice

C57BL/6J mice are the most widely used inbred strain and were the first to have their genome sequenced fully. These mice have been used in a wide variety of research areas which include cardiovascular biology, obesity, genetics and immunology.

2.2.3 VWF^{-/-} mouse model

VWF^{-/-} mice were on a C57BL/6J background and have previously been well characterized (Denis *et al*, 1998, 2001). This strain was developed by a targeted disruption of exons 4 and 5 of the VWF gene (Denis *et al*, 1998). Homozygous VWF^{-/-} mice are viable, fertile and appear normal at birth. They have no detectable plasma, platelet, endothelial VWF or VWF propeptide. These mice also have significantly reduced levels of circulating FVIII (~20% of WT). VWF^{-/-} have prolonged bleeding times and approximately 10% of neonates exhibit spontaneous bleeding episodes. The

haemostatic deficiencies of VWF^{-/-} mice make them suitable for use as a model of type 3 VWD.

2.2.4 VWF^{-/-} mouse genotyping

Heterozygote VWF^{+/-} mice on a C57Bl/6J background initially obtained from Jackson Laboratories (Bar Harbor, USA) were bred and the offspring assessed for VWF^{-/-} homozygosity by PCR. Ear clippings were taken from all offspring at wean age (three weeks old). Ear samples were digested in 150µl DirectPCR Mouse Ear Lysis Reagent (Viagen Biotech, USA), and 0.2mg/mL proteinase K (Sigma-Aldrich, Ireland) at 55°C for 4-5 hours with shaking. Proteinase K was inactivated by heating samples to 85°C for 45 minutes. PCR was carried out on 1µl tail lysate with PrimeSTAR™ HS DNA Polymerase (Takara, Clontech, USA) according to manufacturer’s specifications. (Table 2.1)

Reagent	Amount
5x PrimeSTAR Buffer	10µl
dNTPs	0.2mM
Forward primer	0.2µM
Reverse primer	0.2µM
Template	100ng
PrimeSTAR HS DNA Polymerase	0.5µl
Sterilised dH ₂ O up to	50µl

Table 2. 1 PCR protocol for mouse genotyping using PrimeSTAR DNA polymerase.

A multiplex reaction was set up using the primers outlined in table (2.2) and the PCR conditions detailed in table (2.3).

Primer name	5'-3' Sequence
VWF/GT-s4	GAGGCTGGGTACTATAAGCTC
VWF/GT-as7	GCCATCTTGAAC TTGGATGG
VWF/GT-as10	ATGGAAGGATTGGAGCTACG

Table 2. 2 Primers designed for genotyping VWF^{-/-} mice

Step	Temperature	Time
Hotstart	95°C	2 mins
Denature	95°C	30 seconds
Annealing	56°C	30 seconds
Extension	72°C	1 min/kb
	30 cycles	

Table 2. 3 PCR program used for genotyping VWF^{-/-} mice

1.8% agarose (w/v) was dissolved in Tris Borate EDTA (TBE) buffer (90mM Tris, 90mM Boric acid, 2mM EDTA, pH 8.3) by heating. The gels were poured into a horizontal gel electrophoresis tank (BRL 58 Horizontal Gel Electrophoresis Apparatus minitank; Gibco, UK). Ethidium bromide, which intercalates within the DNA base-pairs was used to visualise the DNA bands by exposure to UV light. DNA samples were

prepared with 10x Loading Dye 4g sucrose, 25mg Orange G; Sigma Aldrich, Ireland, in 10 mLs dH₂O). The samples were run in parallel with 50bp DNA ladder (New England BioLabs, UK). The gels were run at 70V for 45 minutes and a further 10 minutes at 90V and visualised under UV light in a UVP BioSpectrum GelDoc Imaging system. Expectant bands for heterozygous mice are 730bp and 570bp, while WT and homozygous mice have only a single band at 730bp and 570bp, respectively (Figure 2.1).

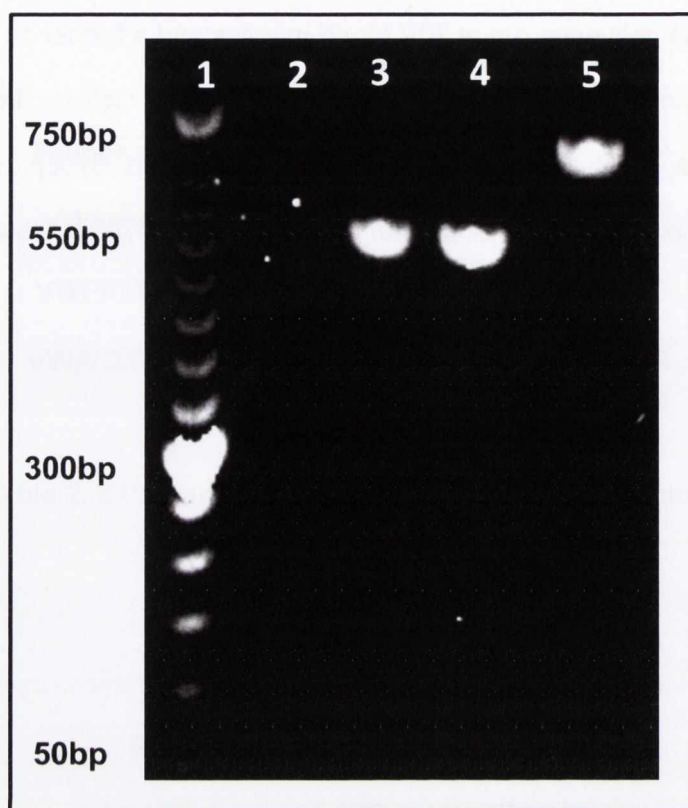


Figure 2. 1 VWF^{-/-} Mouse Genotyping.

Lane 1: 50bp ladder (NEB, UK),

Lane 2: negative control

Lane 3 and 4; homozygous mice with a band size of 570bp,

Lane 5: WT mice display a band size of 730bp. heterozygous mice express both bands

2.2.5 ADAMTS13^{-/-} mouse model

ADAMTS13^{-/-} were developed using a targeting vector designed to replace exons 1 to 6 of the *ADAMTS13* gene and were subsequently backcrossed to a C57BL/6J background (Motto et al 2005). Mice homozygous for this targeted mutation are both viable and fertile. There is no detectable activity of ADAMTS13 enzyme in the plasma of homozygous mice. This strain has a mild procoagulant phenotype using the ferric chloride vascular injury model. VWF-mediated platelet interactions are slightly prolonged when compared to WT using intravital microscopy. This strain shows no spontaneous thrombotic thrombocytopenic purpura.

2.2.6 ADAMTS13^{-/-} mouse genotyping

Heterozygote ADAMTS13^{+/-} mice on a C57BL/6J background (Jackson Labs) were bred and the offspring assessed for ADAMTS13^{-/-} homozygosity by PCR. Ear clippings were taken from offspring at wean age. Ear samples were digested using a quick NaOH DNA isolation method (Truett *et al*, 2000). Briefly, 75µl of 25mM NaOH/0.2mM EDTA was added to each ear clipping. Samples were heated at 98°C for 1 hour. Following cooling 75µl of 40mM Tris HCL (pH5.5) was added prior to centrifuging at 1500 g for 3 minutes. This was diluted 1:10 for PCR. A multiplex reaction was set up using the primers outlined in table (2.4) and the PCR conditions detailed in table (2.5).

Primer	5'-3' Sequence
oIMR8008	AGCCCCAACTCTTGTCTTTTAAT
oIMR8009	GAGTTGCTAGGTTATCAGGAAG
oIMR8010	TGGTTCTAAGTACTGTGGTTTCC

Table 2. 4 Primers designed for genotyping ADAMTS13^{-/-} mice

Step	Temperature	Time
Hotstart	95°C	2 mins
Denature	95°C	10 seconds
Annealing	56°C	15 seconds
Extension	72°C	30 seconds
	35 cycles	

Table 2. 5 PCR program used for genotyping ADAMTS13^{-/-} mice

2% agarose (w/v) was dissolved in TBE buffer (90mM Tris, 90mM Boric acid, 2mM EDTA, pH 8.3) by heating. Gels were prepared as in 2.2.4. The samples were run in parallel with 50bp DNA ladder (Invitrogen, UK). The gels were run at 100V for 1 hour. Expectant bands for heterozygous mice are 290bp and 361bp, while WT and homozygous mice have only a single band at 361bp and 290bp, respectively (Figure 2.2).

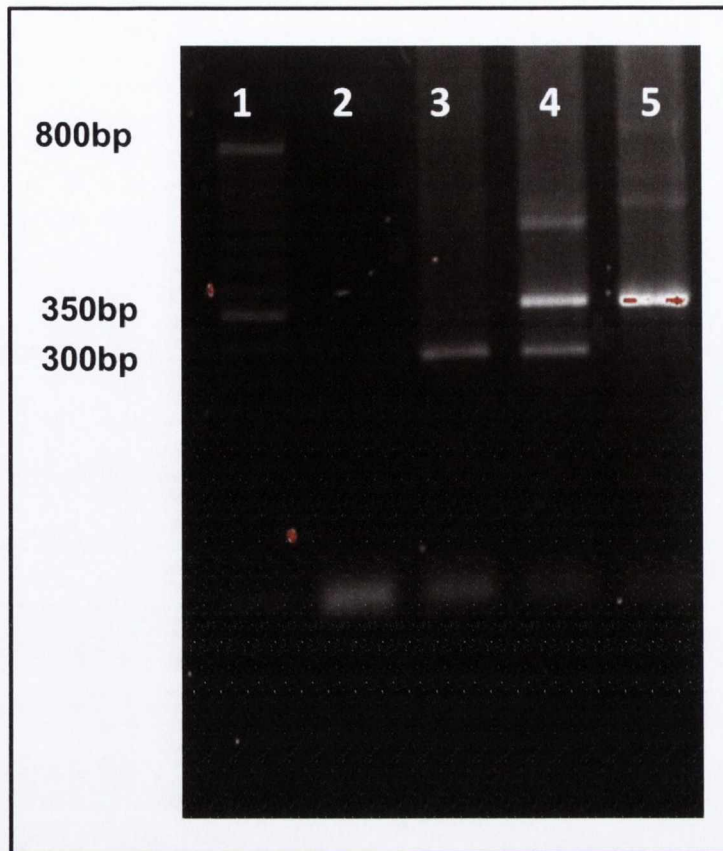


Figure 2. 2 ADAMTS13^{-/-} Mouse Genotyping.

Lane 1: 50bp ladder (Invitrogen, UK),

Lane 2: negative control

Lane 3; homozygous mouse with a band size of 290bp,

Lane 4: heterozygous mouse express both bands 290bp and 361bp,

Lane 5; wild type mice with a band size of 361bp

2.2.7 ASGPR^{-/-} mouse model

ASGPR^{-/-} mice were established using a targeted mutation of the asialoglycoprotein receptor 1 (Asgr1) locus which eradicates endogenous gene expression. Homozygous mice have diminished hepatic clearance of asialoglycoproteins and exhibit different VWF levels with increased plasma VWF. ASGPR1 deficiency is associated with enhanced platelet survival and reduced bleeding times. This strain of mice have an increased susceptibility to infection and mortality from bacteria such as *streptococcus pneumoniae* due to severe intravascular coagulation from impaired clearance of thrombotic components such as platelets and desialyated VWF (Grewal *et al*, 2008; Rumjantseva *et al*, 2009; Soukharev *et al*, 1999).

2.2.8 ASGPR^{-/-} mouse genotyping

Heterozygote ASGPR^{+/-} mice on a C57Bl/6J background (Jackson Labs) were bred and the litters assessed for ASGPR^{-/-} homozygosity by PCR. Ear clippings were taken from offspring at three weeks. Ear samples were digested using a quick NaOH DNA isolation method (Truett *et al*, 2000) similar to ADAMTS13^{-/-} (section 2.2.6). A singleplex reaction was set up, one reaction for WT and one reaction for the mutant allele using the primers outlined in table (2.6) and the PCR conditions detailed in table (2.7).

Primer	5'-3' Sequence
10570	CAG GCT TGG GAG CAG ATA GG
10571	CAG TAG GCC CCA CAC CTT
oIMR3733	CGC CTT CTA TCG CCT TCT TGA
oIMR8444	GCC TGA AGA ACG AGA TCA GC

Table 2. 6 Primers designed for genotyping ASGPR^{-/-} mice

Step	Temperature	Time
1	95°C	2 mins
2	95°C	20 seconds
3	65°C	15 seconds
4	68°C	10 seconds
5	Go to step 2	X 10 times
6	95°C	15 seconds
7	60°C	15 seconds
8	72°C	10 seconds
9	Go to step 6	X 28 cycles
10	72°C	2 mins

Table 2. 7 PCR program used for genotyping *ASGPR^{-/-}* mice

1% agarose (w/v) was dissolved in TBE buffer by heating. Gels were prepared as in 2.2.4. The samples were run in parallel with 50bp DNA ladder (Invitrogen, UK). The gels were run at 100V for 50 minutes. Expectant bands for heterozygous mice are 500bp and 750bp, while WT and homozygous mice have only a single band at 500bp and 750bp, respectively. Due to the singleplex reaction two gels are run for each samples and homozygosity determined (Figure 2.3).

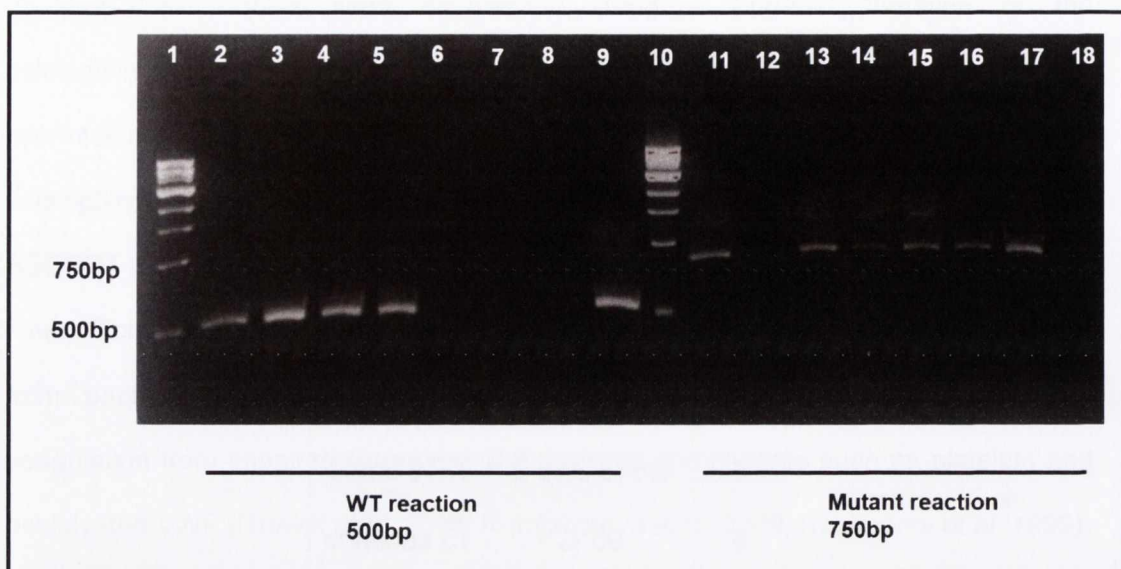


Figure 2. 3 ASGPR^{-/-} Mouse Genotyping.

Lane 1, 10: 50bp ladder (Invitrogen, UK),

Lane 2: heterozygote with WT band at 500bp and corresponding mutant band at 750bp in lane 11,

Lane 3: WT mouse with a band size of 500bp, and no corresponding band in lane 12,

Lane 4, 5: heterozygotes with WT band at 500bp and corresponding mutant band in lane 13 and 14,

Lane 6,7,8; homozygous mice with no bands at 500bp and mutant bands in lane 15,16 and 17,

Lane 9: WT mouse and no corresponding band in lane 18

2.2.9 Anaesthetic selection and preparation

For non-recovery blood collection procedures tribromoethanol (TBE) was selected as a suitable anaesthetic. This compound has numerous advantages over other common inhalation agents. TBE has a quick induction time (2-3 minutes) with short-term surgical anaesthesia lasting approximately 20 minutes without substantial cardiac depression as seen with inhalation agents. Subsequently this allows for sufficient blood volume collection in our studies. TBE has a good margin of safety with low mortality rates of 1% (Papaioannou & Fox, 1993). The stock solution of anaesthetic was prepared by dissolving 2,2,2 tribromoethanol (Sigma-Aldrich, Ireland) in T-amyl alcohol (Sigma-Aldrich, Ireland) to a final concentration of 1.6g/mL. The solution is protected from light and kept at room temperature. A working stock, of 2.5% (v/v) in sterile saline (150mM NaCl) was prepared for injection. The dose used was 0.02mL/g of body weight.

2.2.10 Murine blood collection

2.2.10.1 Blood collection not requiring anaesthetic

Submandibular bleeding of mice

Submandibular bleeding of mice was performed using a lancet. This technique allows 10-200µl blood to be withdrawn without anaesthetic.

Tail micro sampling

A 23 gauge needle was employed to puncture the tail allowing 5-10µl blood to be collected.

2.2.10.2 Blood collection requiring anaesthetic

Subclavical incision

Once the righting and pedal reflexes were lost, mice were placed on their side, the forelimb stretched and a deep incision made in the axilla close to the thorax. The subclavian or axillary arteries were severed and blood was collected into heparin-coated microcontainers (BD Unitech, Ireland).

Cardiac Puncture

Once the righting reflex was gone, a lateral incision through the integument and abdominal wall just beneath the rib cage was made. The liver was carefully separated from the diaphragm so a small incision could be made followed by exposing the whole pleural cavity. After carefully displacing the lungs, a cut was made through the rib cage up to the collarbone. A similar cut was made on the contralateral side. Any tissue connecting to the heart was trimmed away. Using a 1 mL syringe and a 23 gauge needle blood was collected by cardiac puncture into acid citrate dextrose (ACD) anticoagulant (85mM trisodium citrate, 65mM citric acid, 100mM glucose, (Sigma-Aldrich, Ireland) in uninfected mice and on day +3, day +4 and day +5 of malaria infection.

Anticoagulant	Assay
ACD, Heparin	ELISA, Platelet counts
Sodium citrate	APTT, PT

Table 2. 8 Anticoagulants used in blood collection

2.2.11 Plasma preparation

Red blood cell (RBC), Haemoglobin (Hb), and platelets, were all measured on whole blood on a Sysmex haematology analyser (KX-21N). Platelet-poor plasma was prepared by centrifugation at 1500 g for 15 min at 20°C and aliquoted and stored at -80°C until use.

2.2.12 Intravenous delivery

For intravenous (I.V.) delivery, WT or VWF^{-/-} mice were immobilised in a restraint unit. Gentle heat was applied to the tail to encourage vasodilation. The tail veins, which run the length of both lateral aspects of the tail, were inspected for quality and a suitable site was chosen. The tail was stabilized between the thumb and forefinger. Digital pressure served as a tourniquet. The desired compound was administered in sterile PBS in a volume of 100µl via lateral tail vein injection. After removal of the needle, pressure was applied to the site for up to 1 minute. Compounds utilised in these studies are demonstrated in table (2.9).

Compound	Dose	Mouse strain
Histamine	0.5-1mM	WT
Evans Blue	2% in Sterile PBS	WT, VWF ^{-/-}
EPCR blocking Ab	50µg dose in 100µl PBS	WT
Activated Protein C	2 10µg doses in 100µl PBS	WT

Table 2. 9 Different compounds administered throughout by I.V. injection

2.2.13 Cardiac perfusion

Once deeply anesthetised, the animal was taped to the tray with the chest upward. The chest was opened by cutting the diaphragm and a cut was made through the rib cage up to the collarbone. A similar cut was made on the contralateral side. The chest flap was moved away and the left ventricle of the heart was pierced with a 27 gauge needle. Immediately, thereafter, the right atrium was cut and 0.9% saline was perfused in at 2mL per minute to flush blood from the circulatory system. 40mL of normal saline was cardiacally perfused in total.

2.3 *Plasmodium berghei* experimental methods

2.3.1 Microscopical demonstration of *Plasmodium berghei* using Giemsa staining

Blood smears were prepared by placing one drop of blood withdrawn from submandibular bleed or tail micro sampling onto a microscope glass slide and smearing it. After air drying the blood, it was fixed in 100% methanol for 15 seconds. This was left to air dry for 2 to 5 minutes, the slide was then placed into a staining cuvette filled with fresh Giemsa staining solution (Sigma-Aldrich, Ireland) prepared as a 1:5 dilution of the stock in de-ionised water. Slides were allowed to stain for 8 minutes and carefully rinsed with tap water followed by air drying and examination of the smear under a light microscope at 100x magnification with oil immersion (Figure 2.4).

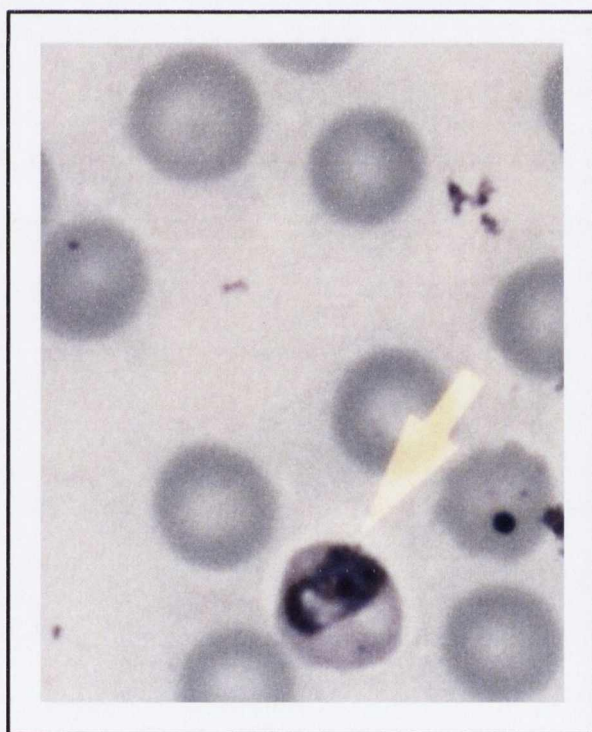


Figure 2. 4 Giemsa stain of blood film.

Yellow arrow highlights *P. berghei* infected erythrocyte

2.3.2 Determination of parasitemia

The malaria parasitemia level reflects the percentage parasitized erythrocytes in the peripheral blood of an infected mouse (or human). A reticulocyte ocular supplied a grid in the eye piece of the microscope with a defined area. In ten different fields of view the number of infected erythrocytes was counted. In one field of view the total number of infected and uninfected erythrocytes were counted. According to the equation below the parasitemia was calculated:

$$\% \text{ Parasitemia} = (\text{total number of infected erythrocytes} / 10 \text{ fields of view}) / (\text{total number of erythrocytes counted}) \times 100$$

2.3.3 *Plasmodium berghei* inoculation

Experimental murine models of cerebral malaria have been widely used to study its pathogenesis. *Plasmodium berghei* ANKA strain is a mouse-specific malaria parasite that causes disease and death in over 95% of wild type C57BL/6 within 6-8 days. Mice can be infected by I.V. or I.P. injection of infected erythrocytes. Typically, blood is first collected from an infected incubator animal with a parasitemia between 1 and 10% and then 10^5 to 10^8 parasites injected into test mice. This methodology allows equal parasite inoculation.

Parasitized erythrocytes used for inoculation were obtained by cardiac puncture from an infected donor mouse. The parasitemia was checked (section 2.3.2) and above 1% was deemed suitable to sub infect the test mice. The infected blood was collected from the donor mouse into heparin coated tubes and diluted to the desired density in sterile PBS. Each test mouse was inoculated I.P with 2×10^6 parasitized red blood cells of *P. berghei* ANKA in a 0.2mL suspension. The day of inoculation was defined as day 0.

2.3.4 Cryopreservation of *Plasmodium berghei*

Blood stages of parasite clones can be stored long term to be used as stocks. 100µl of infected blood was obtained from an infected mouse with a parasitemia of >1% by cardiac puncture. This was mixed with 200µl freezing solution containing 10% glycerol in Alsever's solution (Sigma, Ireland) and instantly frozen in liquid nitrogen in cryotubes.

2.3.5 Survival studies

8-10 week old male and female WT C57BL/6J, VWF^{-/-} C57BL/6J, ADAMTS13^{-/-} C57BL/J or ASGPR^{-/-} C57BL/6J mice were infected I.P. with 2×10^6 *P. berghei* ANKA. Following inoculation, malaria progression was monitored using a previously validated clinical scoring system to assess experimental CM (Amante *et al*, 2007), and overall survival determined. In brief, mice were initially reviewed on a daily basis following *P. berghei* infection. From Day +5 of post-infection, all mice were assessed every 12 hours. Clinical progression of experimental CM 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 attributed a score (0, 0.5 or 1) based upon clinical severity. 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 subsequent day. The daily monitoring was documented as depicted in figure 2.5.

Animal	Day 1	Day 2	Day 3	Day 4	Day 5 AM	Day 5 PM	Day 6 AM	Day 6 PM	Day 7 AM	Day 7 PM	Day 8 AM	Day 8 PM	Day 9 AM	Day 9 PM
No clinical signs														
Hunched posture														
Ruffled fur														
Wobbly gait														
Limb paralysis														
Convulsions														
Coma														
Sum														
Weight														
%Parasites														

Figure 2. 5 Clinical scoring chart for survival studies

2.4 Analysis of Murine VWF

2.4.1 Murine VWF:Ag 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 (Badirou *et al*, 2012; Mohlke *et al*, 1999). 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 20 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.

2.4.2 Murine VWF collagen binding assay

High molecular weight VWF multimers bind to collagen with significantly greater affinity than low molecular weight forms (Sporn *et al*. 1987). VWF:CB of VWF was determined using an ELISA based collagen binding assay (Shida *et al*, 2014). 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) at room temperature for 48 hours. Wells were washed with imidazole buffer (0.12M NaCl, 0.02M imidazole, 0.005M citric acid, pH 7.3) and blocked with 3% BSA in imidazole for 1 hour at room temperature. After washing three times with imidazole buffer, test

samples were added to the plate and incubated for 2 hours at 37°C. Wells were washed with imidazole buffer 3 times and incubated with horseradish peroxidase–conjugated rabbit anti-human VWF Ab (Dako, Denmark) diluted 1:220 in imidazole buffer for 1 hour at room temperature. After washing, bound antibody was detected with TMB (Substrate Reagent Pack, R&D Systems, UK), and the reaction subsequently stopped with 1M H₂SO₄. Absorbance was read at 450nm using a VERSAmax microplate reader (Molecular Devices, UK). Plasma VWF:CB was expressed as a percentage of uninfected controls.

2.4.3 Murine VWF Propeptide

2.4.3.1 Purification of VWF propeptide Antibody

The VWF propeptide (VWF:pp) antibodies in ascites were a kind gift from Dr. Sandra Haberichter (Blood Research Institute, Blood Centre of Wisconsin, Milwaukee, WI 53201). These antibodies were first subjected to lipoprotein separation. Dextran sulphate (Sigma, Ireland) is routinely used to selectively precipitate lipoproteins. Delipidation of the ascites involved mixing the antibody with 10% dextran sulphate and CaCl₂ for 15 minutes at room temperature with gentle agitation. Subsequent to this, the sample was centrifuged at 10,000 g for 10 minutes. The antibody was next dialysed into PBS for 4 hours at room temperature.

Following Delipidation, Montage kit Prosep-A kit (Millipore, Ireland) was used for further antibody purification as per manufacturer's instructions. The spin column was assembled. After loading the plug into the spin column and placing the spin column into a centrifuge tube, the media was equilibrated with 10 mL binding buffer by centrifuging the spin column at 500 × g for 5 minutes. The samples were pre-filtered through a 0.22 µm Steriflip-GP filter device to remove any debris immediately before loading the sample. The filtered samples were diluted 1:1 (v/v) in Binding Buffer. The spin column was centrifuged at 150 × g for 20 minutes. The spin column was washed

and centrifuged as previous to remove unbound contaminants. To purify mouse IgG1 elution involved elution buffer and neutralization buffer to bring the sample to neutral pH. The spin column was again centrifuged for 5 minutes at 500 × g.

Following antibody purification, samples were subjected to electrophoresis on pre-cast gradient gels (4-12% NuPAGE® Bis-Tris Gels; Life Technologies, UK). Samples were mixed with NuPAGE® LDS Sample Buffer (4X; Life Technologies, UK) and incubated at 70°C for 10 minutes before loading onto the pre-cast gels. For reducing conditions, samples were mixed with NuPAGE® LDS Sample Buffer as well as NuPAGE® Sample Reducing Agent (10X; Life Technologies, UK) and were heated to 70°C for 5 minutes. A pre-stained high molecular weight marker (PageRuler Prestained Protein Ladder; ThermoScientific, UK) was run in parallel with all test samples for protein identification based on size. Electrophoresis was carried out at 175V for approximately 45 minutes in NuPAGE® MOPS SDS running buffer for the Bis-Tris gels and NuPAGE® Tris-Acetate buffer for the Tris-acetate gels (Life Technologies, UK). Detection of proteins was performed by coomassie blue staining (section 2.4.5.2).

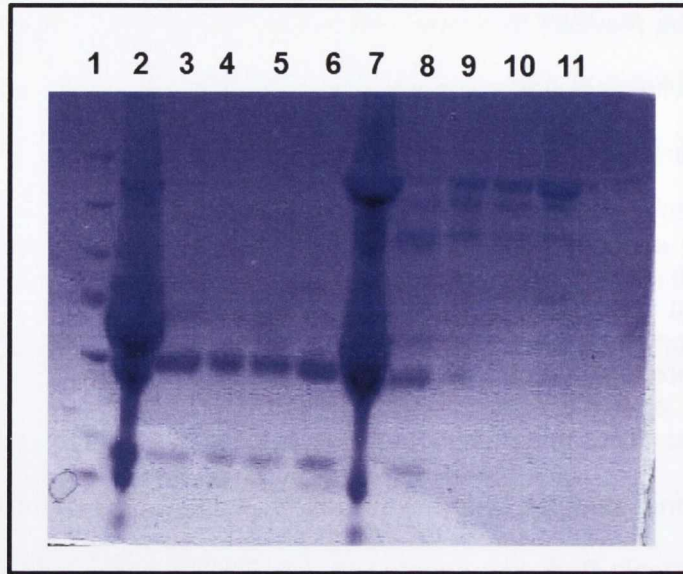


Figure 2. 6 Coomassie of VWF:pp antibody purification

Lane 1: Ladder (PageRuler Prestained Protein Ladder; ThermoScientific, UK)

Lane 2: Antibody prior to purification

Lane 3 & 4: Elution 1 reduced

Lane 5 & 6: Elution 2 off column reduced

Lane 7: antibody before purification

Lane 8 & 9: Elution 1 non-reduced

Lane 10 & 11: Elution 2 non-reduced

2.4.3.2 Murine VWF Propeptide ELISA

Murine plasma VWF:pp concentrations were determined by ELISA (Pruss *et al*, 2011). Polysorp plates (Nunc, Denmark) were coated with a mouse-specific 349.3 capture antibody (0.5 mg/mL) at 2 µg/mL in carbonate coating buffer; 100 µl/well and incubated at 4°C over night. Wells were washed three times with PBS-T (PBS, 0.05% v/v Tween-20) and then blocked for non-specific binding with PBS-T containing 3% bovine serum albumin (BSA; Sigma-Aldrich, Ireland) for 1 hour at room temperature. Plates were again washed three times with 200 µl/well PBS-T. Samples at appropriate dilutions in 3% BSA were added to the wells and incubated at room temperature 2 hours. Murine reference plasma (Sigma, Ireland) was used to generate a standard curve. Plates were washed as described previously. The horseradish peroxidase-linked 349.2 antibody (1.46 mg/mL) was diluted to 1.0 µg/mL in 3% BSA. 100 µl/well was incubated at room temperature 2 hours. Wells were washed three times as previously described and 100 µl of TMB (Substrate Reagent Pack, R&D Systems, UK) was added to the wells. The reaction was subsequently stopped with 50 µL 1M H₂SO₄. Absorbance was read at 450nm using a VERSAmax microplate reader (Molecular Devices, UK).

2.4.4 Histamine dosing

Histamine is an endogenous circulatory mediator that can increase permeability of the capillaries and protein leakage from the vasculature. Histamine (Sigma, Ireland) was therefore used to induce WPB exocytosis. Two different doses of histamine were administered to the mice (0.5mM and 1 mM) by I.V. injection *via* lateral tail vein. At appropriate time points, 3 and 10 minutes, mice were anaesthetised with an intraperitoneal injection of 2.5% tribromoethanol and exsanguinated Subclavical incision (Section 2.2.10.2). Blood samples were collected into lithium heparin, centrifuged, plasma was removed and VWF:Ag level (Section 2.4.1) and VWF:pp level (Section 2.4.3) were determined by ELISA.

2.4.5 Electrophoresis and protein separation

2.4.5.1 VWF multimer gel

The multimeric pattern of VWF can be visualised by electrophoresis of VWF using non-reducing agarose gels. VWF multimer analysis was performed using a Bio-Rad mini gel casting system (Fannin LTD, UK). Biorad glass plates were cleaned thoroughly with methanol before pre-heating at 37°C. 1.8% agarose gels (1.5mm diameter) were prepared by dissolving 0.36g SeaKem® HGT(P) Agarose, Lonza, ME, USA in 20mL of separating buffer (200mM tris, 100mM glycine and 0.1% SDS, pH 9.0). The agarose was dissolved by extensive heating ~80°C before pouring 8mLs per/gel into the pre-heated plates. To facilitate gel setting, the gels were cooled at 4°C for 10 minutes. Stacking gels (0.75% agarose) were prepared by heating 0.15g SeaKem® HGT (P) Agarose in 20mL of stacking buffer (70mM tris, 5mM EDTA, 0.1% SDS pH 6.7). Once dissolved, 4mL of stacking gel were poured on top of the cooled separating gels in the casting unit. A 10 well comb (1.5mm diameter) was inserted immediately before incubating the gels at 4°C to allow them to set.

VWF samples containing ~2ng VWF mixed with 10X reducing SDS-PAGE loading buffer (70mM Tris, pH 6.8, 2.4% SDS, 0.67M urea, 4mM EDTA, 10% glycerol, 0.01% bromophenol blue (Cao *et al*, 2012)) heated at 60°C for 20 minutes and loaded onto gels. The gels were run using Bio-Rad mini-gel electrophoresis tank. The outer chamber of the tank was filled with running buffer (50mM tris, 75mM glycine) while the inner chamber was filled with (100mM tris, 150mM glycine 0.1% SDS). The gels were electrophorised for 150 minutes at 50 volts. After electrophoresis, the transfer of protein to PVDF membranes (Immobilon-FL, Millipore, MA, USA) was carried out overnight at 11 volts in the cold room in transfer buffer (25mM Tris, 192mM glycine and 20% (v/v) methanol). The membranes were then rinsed with PBS-T and blocked for 1 hour at room temperature with PBS-T containing 5% BSA. Finally, the membranes were incubated with anti-VWF pAb (Dako, Denmark) diluted 1:1000 in PBS-T for 1 hour at

room temperature with gentle agitation. After thorough washing 1:10,000 goat anti-rabbit-HRP (Santa Cruz Biotechnology, USA) was incubated at room temperature. Following rigorous washing, bound antibody was detected using the SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, Ireland) according to the manufacturer's instructions. 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). Detection of proteins was performed by both coomassie blue staining and western blotting.

2.4.5.2 Coomassie Staining

Coomassie® (Coomassie® Brilliant Blue, Sigma-Aldrich, Ireland) blue staining was used for the non-specific detection of proteins. After SDS-PAGE, gels were washed with dH₂O and incubated in Coomassie® blue staining solution (0.1% (w/v) brilliant blue (Sigma-Aldrich, Ireland), 20% (v/v) methanol, 10% (v/v) acetic acid) for 40 minutes. Gels were then rinsed in dH₂O and incubated in destain (50% (v/v) methanol, 10% (v/v) acetic acid) for 1 hour with gentle agitation.

2.4.5.3 Western blotting

Following electrophoresis, proteins were transferred to a PVDF membrane (Immobilon-P; Millipore, Ireland) using an electroblot system (Trans-Blot Electrophoretic Transfer Cell, Bio-Rad, Fannin LTD, UK). The PVDF membrane was pre-activated by immersion in methanol. Transfer was performed at 100V for 1 hour in transfer buffer (25mM Tris, 192mM glycine and 20% (v/v) methanol). Following protein transfer, the membrane was blocked for non-specific binding with 3% solution BSA in PBS-T at room temperature. The membrane was washed with PBS-T, and then incubated with rabbit polyclonal anti-human VWF-HRP (Dako, Denmark) diluted 1:20,000 in PBS-T for 1 hour at room temperature with gentle agitation. After thorough washing, bound antibody was detected using the SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, Ireland) according to the

manufacturer's instructions. 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).

2.4.5.4 Densitometry

To objectively quantify differences in VWF multimer composition, densitometry of typical individual lanes was performed using ImageJ software (Image Processing and Analysis in Java). ImageJ is a Java-based image analysis programme which allows the measurement of density profiles, peak heights as well as peak intensity of the band, in this case protein bands. The capacity to quantify the intensity of Western blot bands for the statistical analysis of multiple blots makes densitometry a valuable tool.

2.5 ADAMTS13 Activity

FRET-VWF73 (Peptides International Inc) assay was used to measure plasma ADAMTS13 activity as per manufacturer's guidelines. FRET-VWF73 is a 73 amino acid peptide comprising the ADAMTS13 cleavage site and surrounding amino acids in the VWF A2 domain. The excitation and emission spectra of the chromophores are within the 340-450nm range. Fluorescence is measured every 5 min for 1 hour to determine ADAMTS13 enzymatic activity levels. ADAMTS13 activity in plasma samples is quantified by calculation of the slope for the test sample compared to a standard curve. In these assays, ADAMTS13^{-/-} plasma was used as a negative control as seen in Figure 2.5

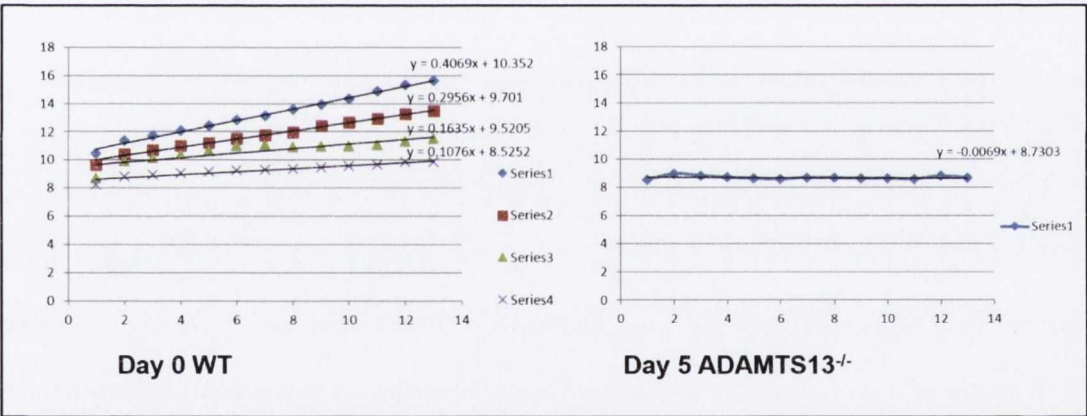


Figure 2. 7 Slope of FRETs from WT mouse versus ADAMTS13^{-/-} mouse

The measurement of ADAMTS13^{-/-} mice remains unchanged for the duration of the experiment.

2.6 Angiopoietin-2 ELISA

Angiopoietin-2 (Ang-2) represents another vascular modulator stored within WPB. Murine Ang-2 was measured using the R&D Systems Quantikine ELISA immunoassay (R&D Systems, UK). The sensitivity of the assay was to 4.98pg/mL. The assay was carried out according to manufacturer's guidelines.

2.7 Murine OPG

Murine OPG was measured using an R&D Systems Mouse Osteoprotegerin/ TNFRSF11B DuoSet® ELISA development system (R&D Systems, UK). This DuoSet is calibrated against highly purified NS0-expressed recombinant mouse OPG/Fc Chimera. The assay was carried out as per manufacturer's specifications.

2.8 Blood Brain Barrier permeability

The blood brain barrier (BBB) is a highly selective barrier formed by brain EC. BBB breakdown is said to be a hallmark of CM (Schofield & Grau, 2005; Pamplona *et al*, 2007). Initially, permeability was investigated in WT and VWF^{-/-} mice 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. Since Evan's blue binds murine albumin, it does not cross a normal intact BBB. In keeping with previous studies (Suidan *et al*, 2013; Noubade *et al*, 2008), 2% Evans blue (Sigma, Ireland) in PBS was administered to the mice *via* injection into the lateral tail vein (section 2.2.12) and allowed to circulate for 1 hour. Mice were anaesthetised with an I.P. injection of 2.5% tribromoethanol, and 40mL 0.9% saline were cardiacallly perfused (section 2.2.13). Figure 2.6 depicts the Evans blue uptake in a mouse with CM. To quantify extravasation of Evans blue across the BBB into cerebral tissues, murine brains were removed and weighed. The cerebral tissues were then homogenised for 8 minutes at a

frequency of 30 Hz, and sonicated for 30 seconds with PBS, and finally incubated in 50% Trichloroacetic acid (TCA). Following centrifugation at 14,000 rpm for 30 minutes at 4°C, supernatant (200 µL) was plated on a 96-well plate and absorbance was measured at 620 nm. Concentrations were extrapolated off a standard curve.

With anaemia and red cell haemolysis during experimental malarial infection haemoglobin could potentially interfere with the readings of Evan's blue absorbance. To avoid haemoglobin contributing to the measurements, 3 day +5 brain samples were collected which had not had Evan's blue administered, the brains were treated similar to the others and the absorbance recorded was subtracted from the day +5 samples before further analysis to exclude any haemoglobin interference.

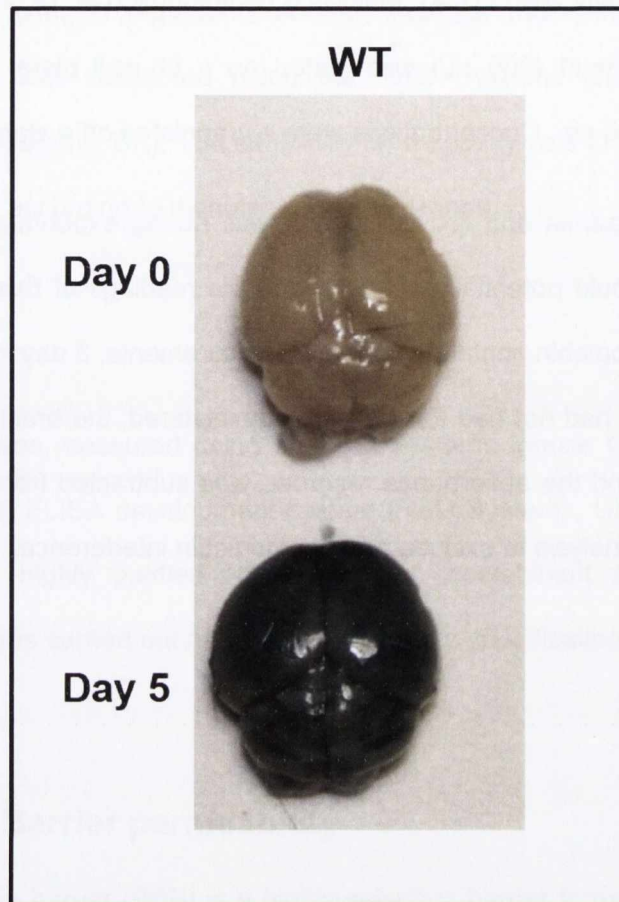


Figure 2. 8 Blood brain barrier breakdown in CM

The BBB remains intact in a healthy mouse so up Evans blue crosses the barrier. In CM, permeability occurs in parallel to neurological features and Evans blue can cross the BBB and bind to albumin.

2.10 Coagulopathy in *P. berghei* ANKA

2.10.1 Activated Partial Thromboplastin Time

Activated Partial Thromboplastin Time (APTT) measures the activity of the intrinsic and common pathways of coagulation. The APTT is the time taken from the addition of calcium to the formation of a fibrin clot.

Murine plasma was diluted with Siemens OV buffer and phospholipids along with a negatively charged surface activator (kaolin, cephalin; C.K. Prest. Stago, Beijing, China) were added and incubated at 37°C for 3 minutes. This results in activation of the contact factors and leads to the generation of FXIa. Upon addition of 0.2mM CaCl₂, FIX is activated by FXIa and FXa is then generated by the tenase complex, in which FVIII serves as a cofactor. The time for fibrin clot formation was recorded using Amelung KC4 Micro Clinical Coagulation Analyzer (Amelung, Trinity Biotech, Ireland).

2.10.2 Prothrombin Time

The Prothrombin Time (PT) in contrast to the APTT measures the activity of the extrinsic and common pathways of coagulation and consequently, is reliant on the functional activity of factors VII, X, V, II and fibrinogen. The PT is a one-stage clotting assay based upon on the time required for a fibrin clot to form after the addition of Tissue Factor, phospholipid and calcium to plasma.

Hemosil recombiplastin 2G was reconstituted as per manufacture's guidelines. This was heated to 37°C. Murine plasma was diluted with saline and left to incubate for 3 minutes at 37°C. Clot was initiated by the addition of the reconstituted recombiplastin (supplying tissue factor and calcium). Time to clot was measured using Amelung KC4 Micro Clinical Coagulation Analyzer (Amelung, Trinity Biotech, Ireland).

2.10.3 Thrombin-antithrombin complexes

Thrombin-antithrombin (TAT) complexes are formed following the neutralization of thrombin by antithrombin III have been used as a surrogate marker for thrombin generation. TAT complexes were measured by ELISA (Abcam, Cambridge, UK) as per manufacturer's guidelines.

2.10.4 Antithrombin

The serine protease inhibitor Antithrombin (AT) is an important inhibitor of thrombin activity. AT III concentration was measured in mouse plasma using an ELISA (Abcam, Cambridge, UK) as per kit instructions.

2.10.5 Fibrinogen

Fibrinogen is a soluble plasma protein that is converted to polymeric fibrin in response to damage to the vascular system. Fibrinogen was quantified by means of radial immunodiffusion (Cruinn Diagnostics Limited, Ireland). Proteins in plasma immunochemically react with specific antibodies within the agarose gel of the plate. Immune complexes are visible as circular precipitation lines known as radial immunodiffusion. The diameter of this precipitation is directly proportional to the concentration of the respective protein in the sample. Conversion to the corresponding concentration is carried out by means of a reference value table.

To perform the assay, 5µl of plasma was dispensed into a well. Once samples were added, the lid was closed and allowed to stand at room temperature. The diameter was measured after 48 hours and read off the reference table.

Diameter (mm)	Percent of Normal (%)	Concentration (g/L)
6.0	100	2.5
6.1	105	2.62
6.2	110	2.74
6.3	115	2.87
6.4	120	3
6.5	125	3.12

Table 2. 10 Fibrinogen table of reference values

2.10.6 Soluble Thrombomodulin

Thrombomodulin (Tm) is predominantly synthesised by vascular EC. Tm is a cell surface receptor and is a vital cofactor for thrombin, which in turn activates protein C. Soluble Tm (sTm) was measured using an R&D Systems Mouse Thrombomodulin/BDCA-3 DuoSet® ELISA development system (R&D Systems, UK).

2.10.7 Soluble EPCR

The endothelial cell protein C receptor (EPCR) functions as an important regulator of the protein C anticoagulant pathway enhancing activation of Protein C by the thrombin-thrombomodulin complex. Soluble EPCR (sEPCR) was quantified by an ELISA kit (antibodies-online GmbH, Germany) as per manufacturer's specifications.

2.10.8 Determining a role for EPCR in *P. berghei* induced CM

To investigate whether EPCR plays a role in experimental CM, mice were injected with an established function blocking antibody against EPCR which blocks the interaction between protein C or APC with EPCR. This antibody has previously been validated by flow cytometry and surface plasmon resonance (Centelles *et al*, 2010).

RCR-16 (kind gift from Prof K. Fukudome) or isotype control antibody (rat IgG2a) were dialysed overnight at 4°C into PBS to remove azide. 50µg of antibody or isotype control were administered I.V. immediately prior to infection with *P. berghei*. Disease progression was then monitored as previously stated (section 2.3.5) and a survival curve was generated.

2.10.9 Activated Protein C treatment of mice with *P. berghei* infection

To explore a role for activated Protein C (APC) in the mouse model of CM, WT mice were treated with two doses of APC. Firstly, 10µg of murine APC (kind gift from Dr. R. Preston) was administered I.V. directly after infection with *P. berghei*. Subsequent to this, another dose of 10µg of APC I.V. was administered four hours after inoculation. Clinical progression was monitored using the previously mentioned clinical scoring algorithm, and overall survival determined.

Following on from this experiment, to further investigate APC as a therapeutic in *P. berghei* infection, mice were treated again with two 10µg doses of APC on Day +3 following initial infection. Again four hours were left between the two treatments. As before, disease progression was monitored as previously stated and a survival curve was generated.

2.11 Data analysis and statistics

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 medians and mean values $\pm$ standard error of the mean (SEM). To assess statistical differences, data were analysed using Student's unpaired 2-tailed *t* test. Experimental CM clinical scoring data were assessed by two-way ANOVA analysis. The Spearman correlation coefficient was used as a measure of linear association between two variables. Finally, mouse survival

data were compared using a Log-rank (Mantel-Cox) Test. For all statistical tests, P values <0.05 were considered significant.

3. Elucidating a role for VWF in the pathogenesis of experimental cerebral malaria

3.1 Introduction

3.1.1 Malaria pathogenesis

Plasmodium falciparum malaria remains a major cause of morbidity and mortality amongst children in sub-Saharan Africa (Murray *et al*, 2012). Although the biological mechanisms involved in the pathophysiology of severe *P. falciparum* malaria remain poorly understood, previous studies have demonstrated that sequestration of *P. falciparum* IE within the microvasculature of the brain is important in the development of CM (Newton & Krishna, 1998; Taylor *et al*, 2004). This sequestration involves adhesion of IE to host vascular EC surfaces (Clark *et al*, 1994; Berendt *et al*, 1994; Grau & De Kossodo, 1994), and is mediated by a variety of specific EC adhesion molecules including CD36, ICAM-1, and thrombospondin (Craig & Scherf, 2001). In addition, recent studies have also demonstrated that the endothelial protein C receptor (EPCR) also plays an important role in modulating the sequestration of IE (Turner *et al*, 2013; Moxon *et al*, 2013). Importantly, in addition to IE, sequestration of leucocytes and platelets within the cerebral microvasculature has also been reported in post mortem samples from CM patients (Grau *et al*, 2003; Patnaik *et al*, 1994). Understanding the molecular mechanisms involved in pathogenesis is not just of basic scientific interest but also of direct clinical importance.

3.1.2 VWF

VWF is a large sialoglycoprotein synthesized within EC and megakaryocytes (Mayadas & Wagner, 1991). 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 (Lenting PJ,

Christophe OD, 2015). 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 (Lenting PJ, Christophe OD, 2015). Following synthesis within EC, VWF is either constitutively secreted into the plasma, or else stored within specific intracellular organelles known as Weibel-Palade bodies (WPB) (Nightingale & Cutler, 2013). This WPB-stored VWF is enriched in high molecular weight multimers (HMWM), and is actively secreted following EC activation.

3.1.3 VWF and malaria

Recent studies have shown that plasma VWF:Ag and VWF propeptide levels are markedly elevated in children with severe *falciparum* malaria (Hollestelle *et al*, 2006; Phiri *et al*, 2011). Moreover elevated VWF:Ag levels inversely correlate with clinical outcomes. Furthermore, a study of 14 healthy volunteers infected with *P. falciparum* showed that increased plasma VWF:Ag and VWF propeptide levels develop shortly after the onset of blood stage infection (de Mast *et al*, 2007). Consequently, acute EC activation constitutes an early and consistent feature of *P. falciparum* infection.

Although the pathophysiology underlying CM includes occlusion of the microvasculature by IE, recent studies more have clearly demonstrated that platelets are also crucial in regulating vessel occlusion. In particular, post-mortem studies of children who died from CM have observed the presence of platelet aggregates obstructing vessels (Grau *et al*, 2003). These data suggest that platelets may be important, and therefore raise the possibility that VWF might be directly involved in the pathogenesis of CM. In addition to acute EC activation and increase of plasma VWF:Ag levels, circulating ULVWF multimers have also been observed in children with severe malaria (Larkin *et al*, 2009). Importantly, platelet-decorated ULVWF strings

have been shown to recruit trophozoite-stage falciparum-IE to EC surfaces in shear-based assays (Bridges *et al*, 2010). Collectively, these emerging data therefore suggest a novel role for VWF in the pathogenesis of CM.

3.1.4 Mouse model of malaria

Experimental murine models of CM have been widely used to study its pathogenesis. *Plasmodium berghei* ANKA strain is a mouse-specific malaria parasite that causes disease and death in over 95% of wild type (WT) C57BL/6 mice within 6-8 days. Previous published literature has demonstrated that this murine model shares many of the key clinical features observed in children with CM (Craig *et al*, 2012; de Souza *et al*, 2010; de Souza & Riley, 2002; Combes *et al*, 2005; Schofield & Grau, 2005). For example, mice infected with *P. berghei* die from a murine cerebral syndrome, associated with paralysis, seizures and coma. Of particular relevance to this project, there is significant sequestration of both leucocytes and *P. berghei*-IE in the cerebral vasculature (Rogerson *et al*; Hunt & Grau, 2003; Baptista *et al*, 2010). In addition, preliminary studies in the murine model have demonstrated evidence of EC activation, confirming the suitability of this model to study the importance and underlying molecular mechanisms of EC activation in the pathogenesis of malaria (Desruisseaux *et al*, 2010; Faille *et al*, 2009). We sought to further investigate the possible molecular mechanism(s) through which VWF might contribute to pathology of malaria using this murine model.

3.2 Clinical features of malaria in C57BL/6J WT mice

3.2.1 Clinical progression of malarial infection in WT mice

The mouse model of CM was initially validated in our laboratory. Following inoculation with *P. berghei* ANKA mice became progressively unwell. Clinical progression was monitored using an established clinical scoring system (Amante *et al*, 2007). This algorithm combines behavioural signs of persistent pain in mice, such as altered posture or behaviour, with signs of experimental CM (ECM). Signs include ruffled fur, hunched posture, wobbly gait, limb paralysis, seizures and coma. According to the scoring algorithm, *P. berghei* infected mice were attributed points (0, 0.5 or 1) for each sign depending on presentation. A score of 2.5 or more was considered indicative of CM. Typically CM started from day +5 post infection (Figure 3.1) (n=16).

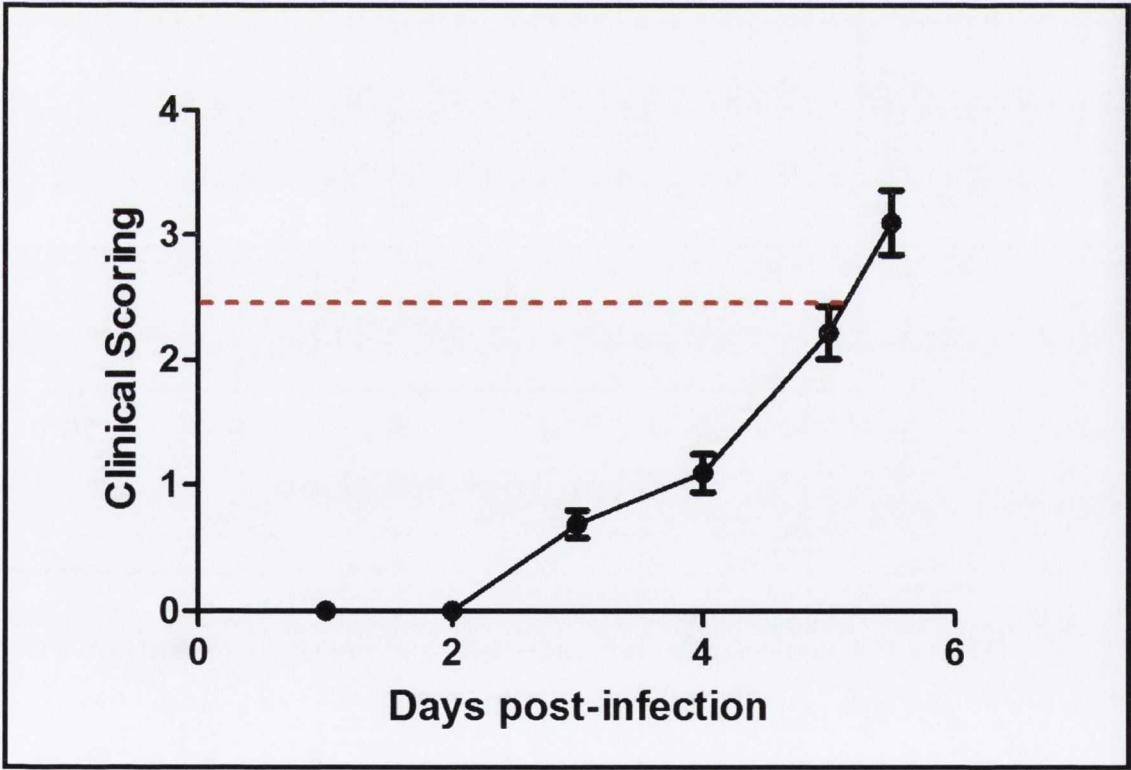


Figure 3. 1 Clinical progression of *P. berghei* infection in WT mice

3.2.2 Percentage survival of WT mice after inoculation with *P. berghei*

In addition to clinical scoring, overall survival of infected mice was also determined. Following infection with 2×10^6 *P. berghei* ANKA mice typically died from a complex neurological syndrome approximately 6 to 8 days post inoculation with *P. berghei* malaria (Figure 3.2) (n=16). Animals with severe ECM (accumulative scores >4) were sacrificed by cervical dislocation according to ethics guidelines, and the day of death was deemed to be the subsequent day. Ethically, death is not to be an endpoint in the study of pathogenesis of malaria.

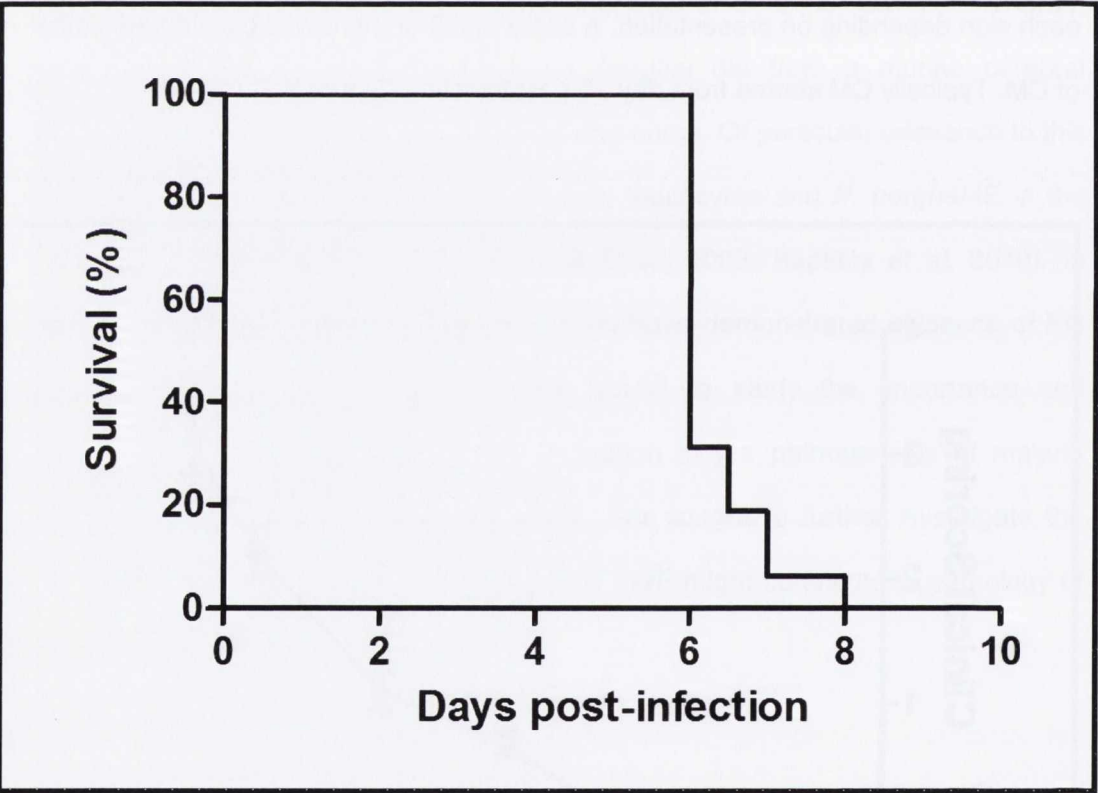


Figure 3. 2 Survival of WT mice after infection with *P. berghei* ANKA

3.2.3 Weight change and parasitemia in WT mice

Following *P. berghei* inoculation, mice were monitored daily for weight loss as well as general distress as per ethical guidelines. Weight decreased over the course of infection, particularly in later stages (Figure 3.3A). In addition, percentage parasitemia was also assessed using Giemsa staining. *P. berghei* parasites were observed in peripheral blood from Day +2 following inoculation (Figure 3.3B) (n=16).

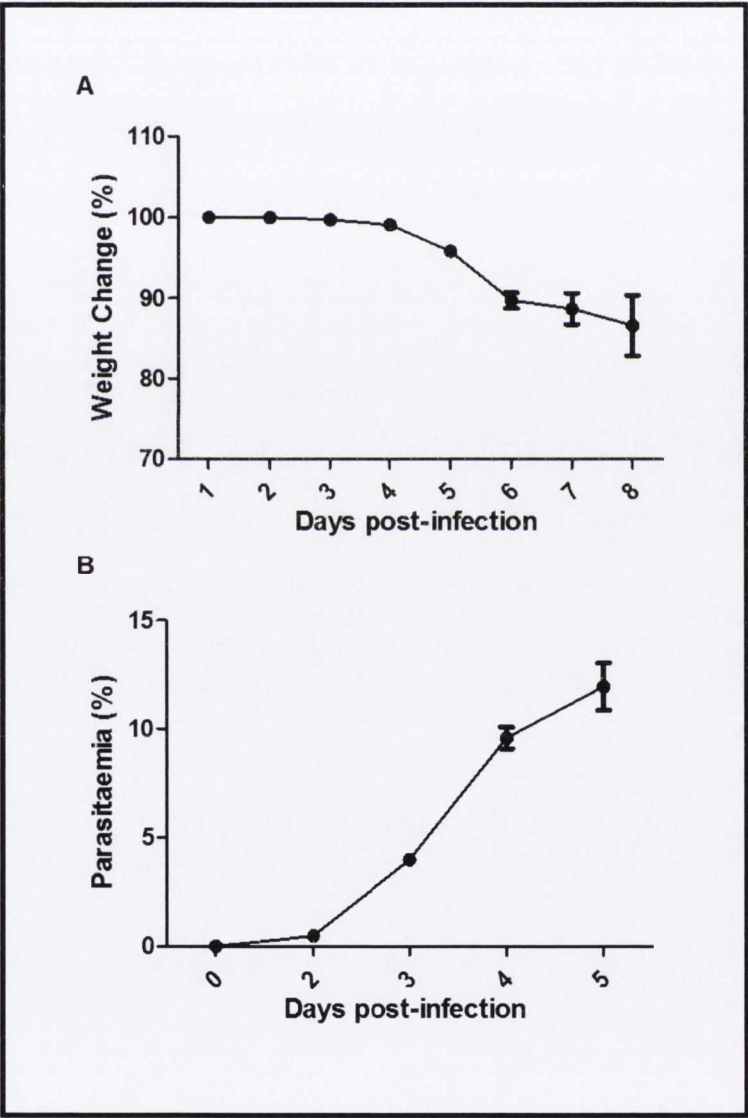


Figure 3. 3 (A) Percentage weight change in WT mice after malarial infection
(B) Percentage parasitemia in WT mice after inoculation with *P. berghei*

3.3 Characterisation of VWF and WPB exocytosis in the experimental model of malaria

3.3.1 Increased plasma VWF:Ag levels in the murine model of ECM

In order to further investigate the role of VWF in malaria pathogenesis, we measured plasma VWF:Ag levels in WT mice following *P. berghei* infection. In keeping with previous findings in children with severe *P. falciparum* malaria (Hollestelle *et al*, 2006; Larkin *et al*, 2009), we observed a significant increase in plasma VWF:Ag levels in mice following *P. berghei* infection (Figure 3.4) (n=4-8). At Day +5 after inoculation, median plasma VWF:Ag levels were increased approximately 2.5 fold (101.9% versus 242.3% at Day 0 and Day +5 respectively; $P \leq 0.001$), which was similar to the magnitude of increase in plasma VWF:Ag levels observed in children with CM (Hollestelle *et al*, 2006). Interestingly, a significant increase in plasma VWF:Ag levels were already evident within 24 hours following *P. berghei* inoculation. This early increase in plasma VWF levels occurred before the appearance of significant blood parasitemia levels, and prior to the onset of clinical signs (Figure 3.1 and 3.3B). This is in keeping with previous studies demonstrating VWF:Ag levels increased early in patients experimentally infected with *P. falciparum* (de Mast *et al*, 2007).

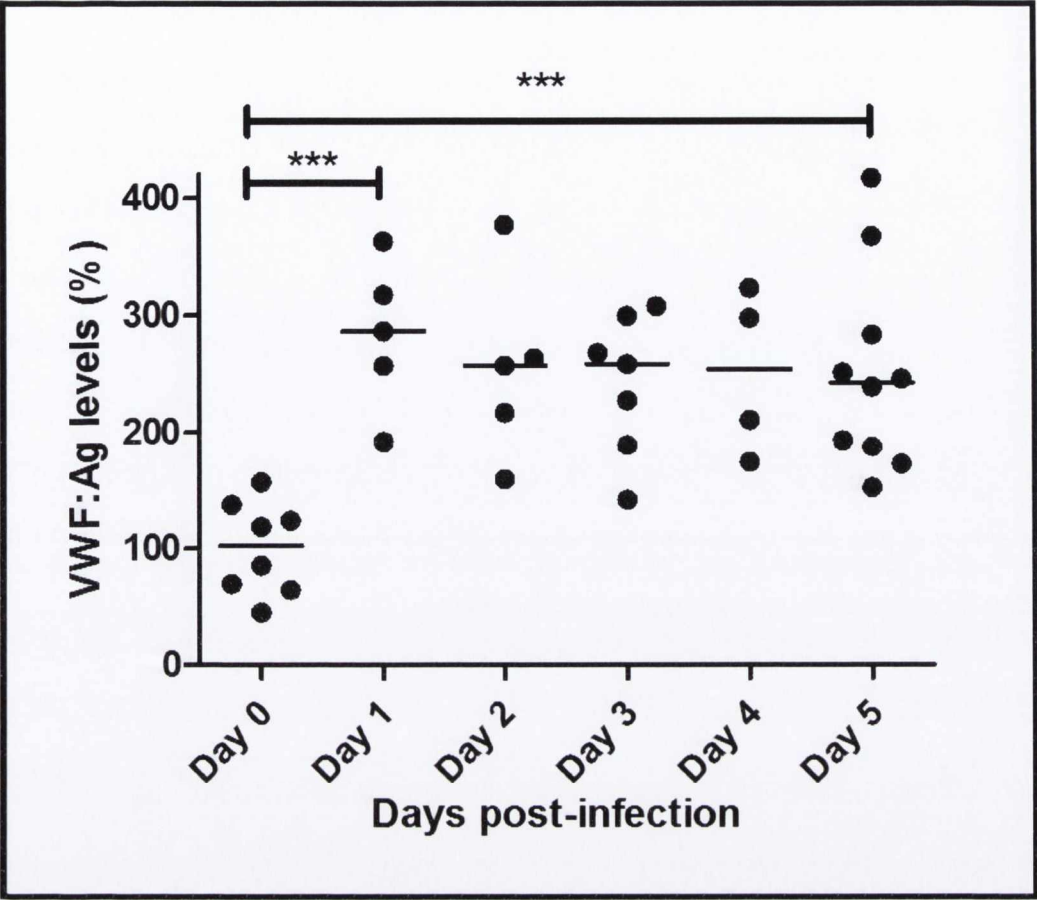
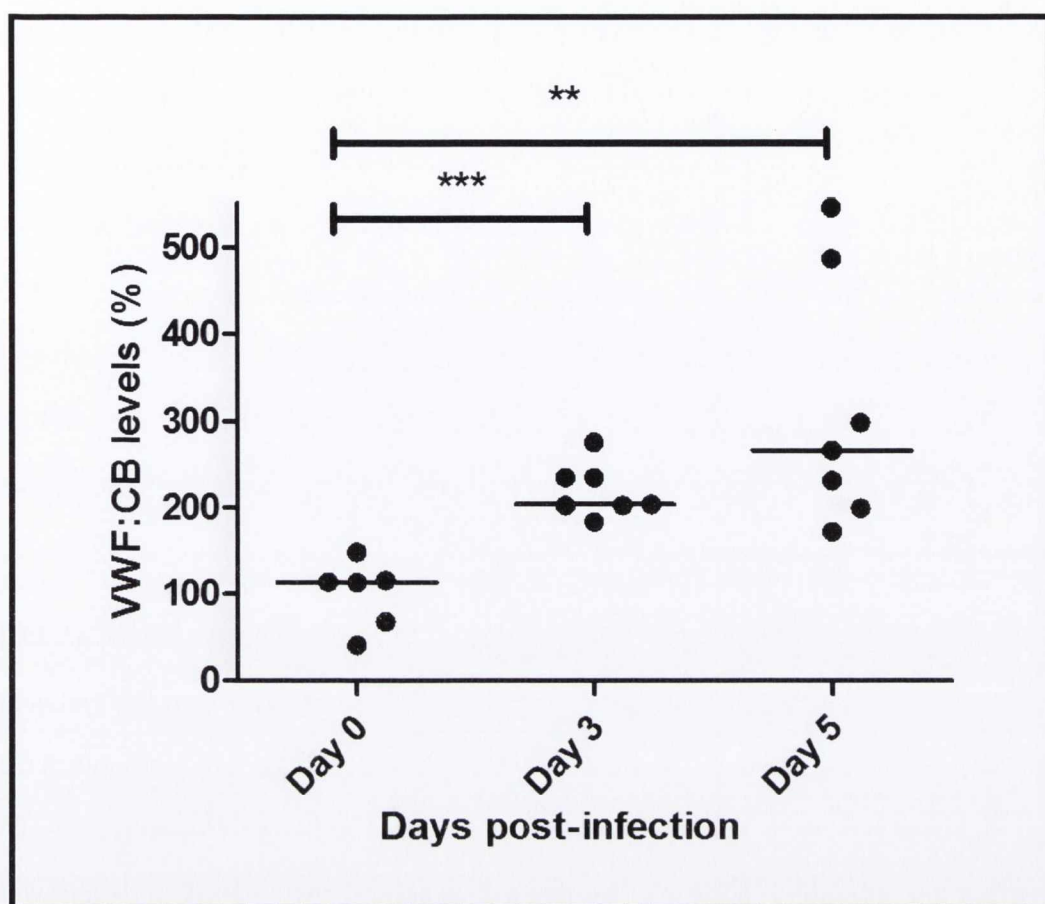


Figure 3. 4 VWF:Ag levels increased after inoculation with *P. berghei* ANKA

3.3.2 Plasma VWF:CB activity is significantly elevated in ECM

VWF collagen binding activity (VWF:CB) is an assay which detects the ability of VWF to bind to collagen. It is well-established that the VWF:CB is particularly sensitive to the presence of circulating high molecular weight VWF multimers (Larkin *et al*, 2009). Again in keeping with our previous findings in children infected with *P. falciparum* (Larkin *et al*, 2009), plasma VWF:CB levels were also significantly elevated in C57BL/6J mice following *P. berghei* infection (113.7% versus 266.6% at Day 0 and Day +5 respectively; $P \leq 0.01$) (Figure 3.5) ($n=6-7$). 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.



3.3.3 Plasma VWF:pp levels are not elevated in murine ECM

The VWF propeptide (VWF:pp) is essential for normal VWF multimerization (Mayadas *et al*, 1989) and functions as a chaperone for VWF multimerization within the Golgi. After cleavage, the propeptide remains non-covalently associated with VWF (Mayadas & Wagner, 1989). Previously, our lab demonstrated an increase in VWF:pp levels indicating acute EC activation as a hallmark of malaria in children (Hollestelle *et al*, 2006). 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 (Figure 3.6A) (n=4-7).

To investigate further the observation that VWF:pp is not increased in ECM mice were treated with I.V. histamine to induce WPB secretion. Following histamine injection, a significant increase in plasma VWF:Ag levels were observed (Figure 3.6B). This increase in plasma VWF:Ag was still detectable 10 minutes following histamine administration (Figure 3.6C) (n=3). In contrast, although plasma VWF:pp levels were also markedly elevated 3 minutes following histamine, plasma levels fell rapidly, suggesting that the plasma clearance of murine VWF:pp is much more rapid than that of human VWF:pp.

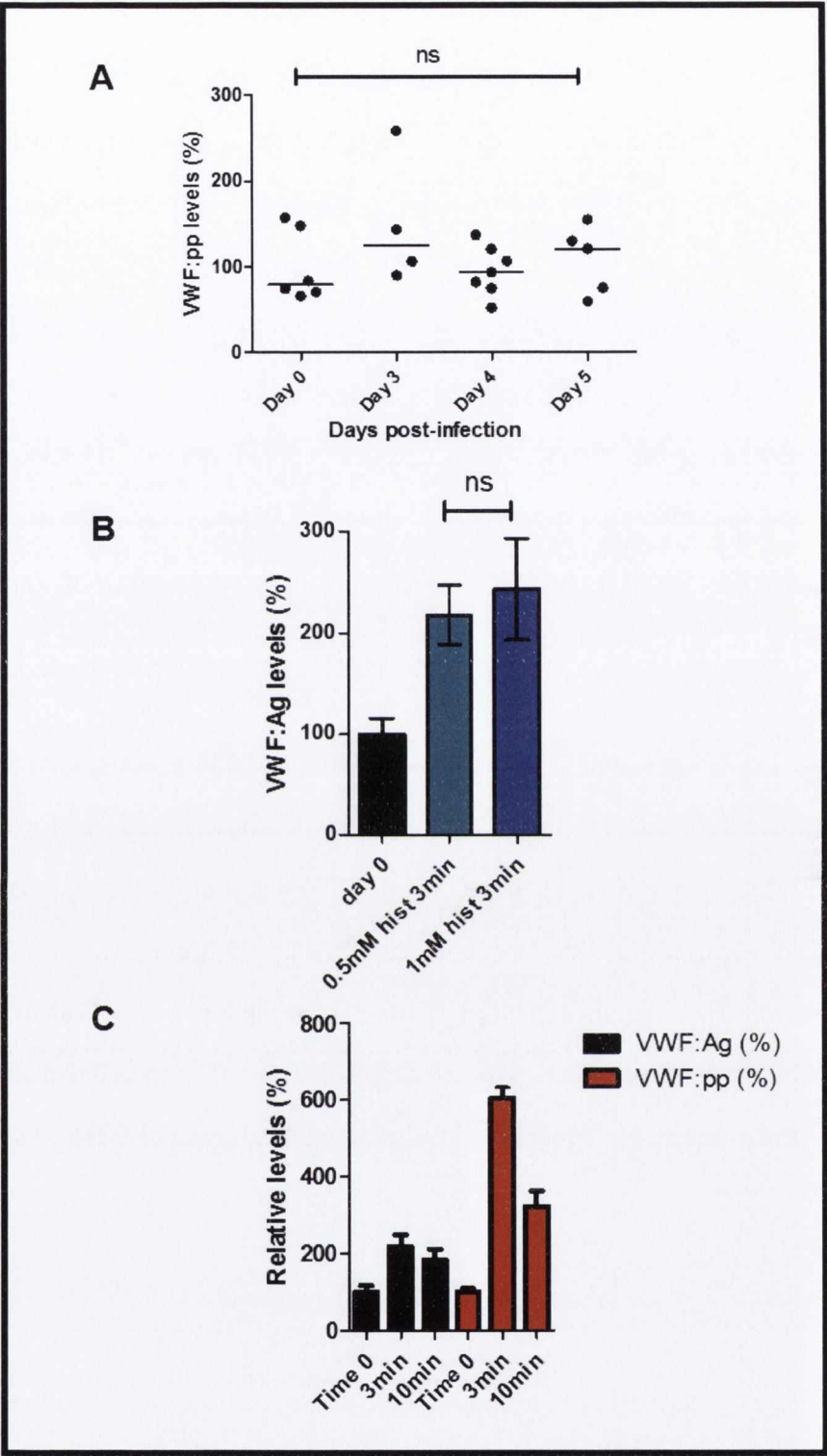


Figure 3. 6 (A) VWF:pp levels remain unchanged during the course of infection
(B) Histamine doses (C) VWF:Ag and VWF:pp levels after histamine
administration

3.3.4 WPB exocytosis during *P. berghei* infection

Acute EC activation plays a critical role in the pathogenesis of *P. falciparum* malaria. EC activation may also be an early feature in mice prior to the development of ECM. Angiopoietin-2 (Ang-2) is approximately a 70kDa secreted glycoprotein that plays roles in angiogenesis, vascular development and also inflammation. During inflammation Ang-2 functions as an autocrine regulator sensitizing EC to the effects of tumor necrosis factor, and consequently increases the expression of adhesion receptors (Fiedler *et al*, 2006). Ang-2 is expressed in smooth muscle cells (e.g. placenta) and vascular EC, representing another vascular modulator stored within WPB (Fiedler *et al*, 2004). 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 inversely correlate with clinical outcome (Yeo *et al*, 2008; Conroy *et al*, 2009, 2010; Lovegrove *et al*, 2009; Erdman *et al*, 2011). 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 3.7) (n=4-6). 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 ($P \leq 0.001$). Collectively, these data suggest marked EC activation and WPB exocytosis, which represent early hallmarks of *P. falciparum* malaria infection in human patients, also constitute prominent features in the murine model of ECM.

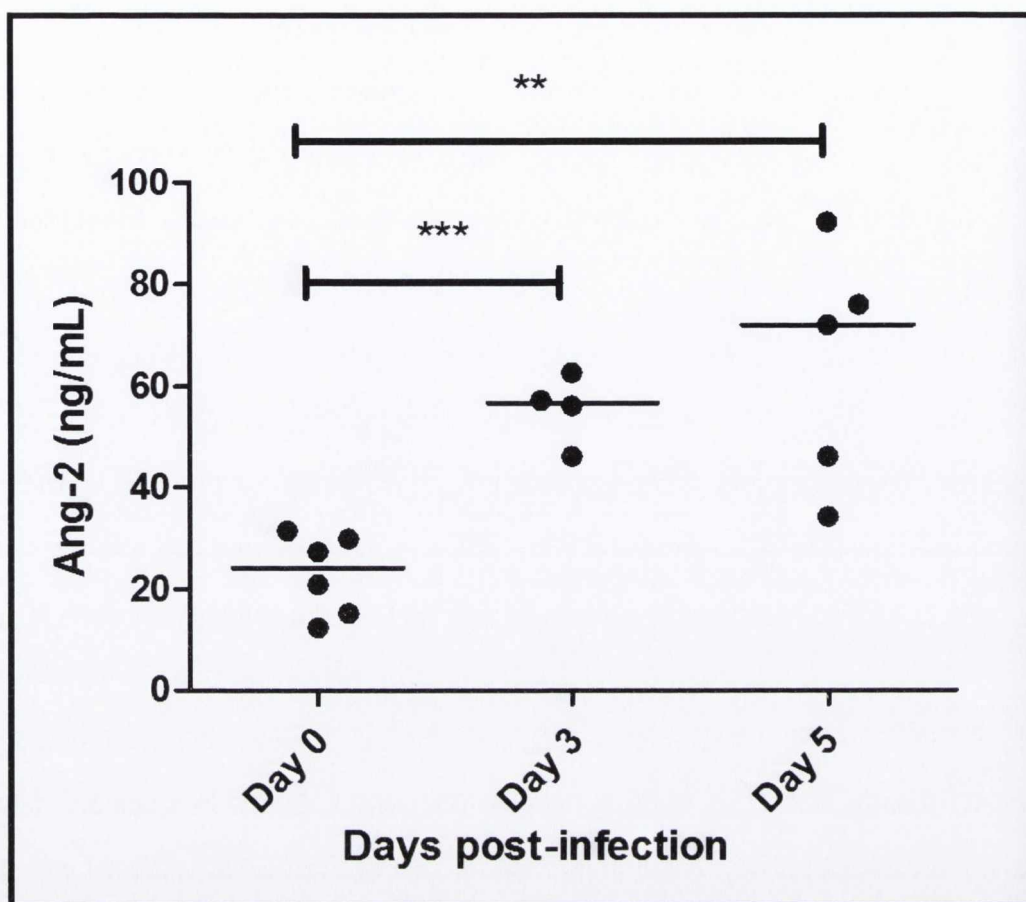


Figure 3. 7 Plasma Ang-2 levels increase correlating with disease progression

3.3.5 Abnormal ULVWF multimers are a feature of ECM

In normal plasma, ULVWF multimers secreted from WPB following EC activation are rapidly proteolysed by the zinc metalloprotease ADAMTS13 (Figure 3.8A). ULVWF multimers have significantly enhanced functional activity. In addition to markedly elevated VWF:Ag and VWF:CB activity levels, pathological ULVWF multimers have also been reported in the plasma of children with CM (Larkin *et al*, 2009). 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 ULVWF multimers in the plasma was also a feature of murine ECM (Figure 3.8B). ULVWF multimers were not present in the plasma of WT mice, but were observed from Day +3 following *P. berghei* infection. To objectively quantify differences in VWF multimer composition, densitometry of typical individual lanes was performed using ImageJ software (Figure 3.8 C, D). In summary, these findings demonstrate that marked elevations of plasma VWF levels, coupled with the appearance of pathological ULVWF multimers in the plasma, represent consistent features in both human and murine severe malaria infection.

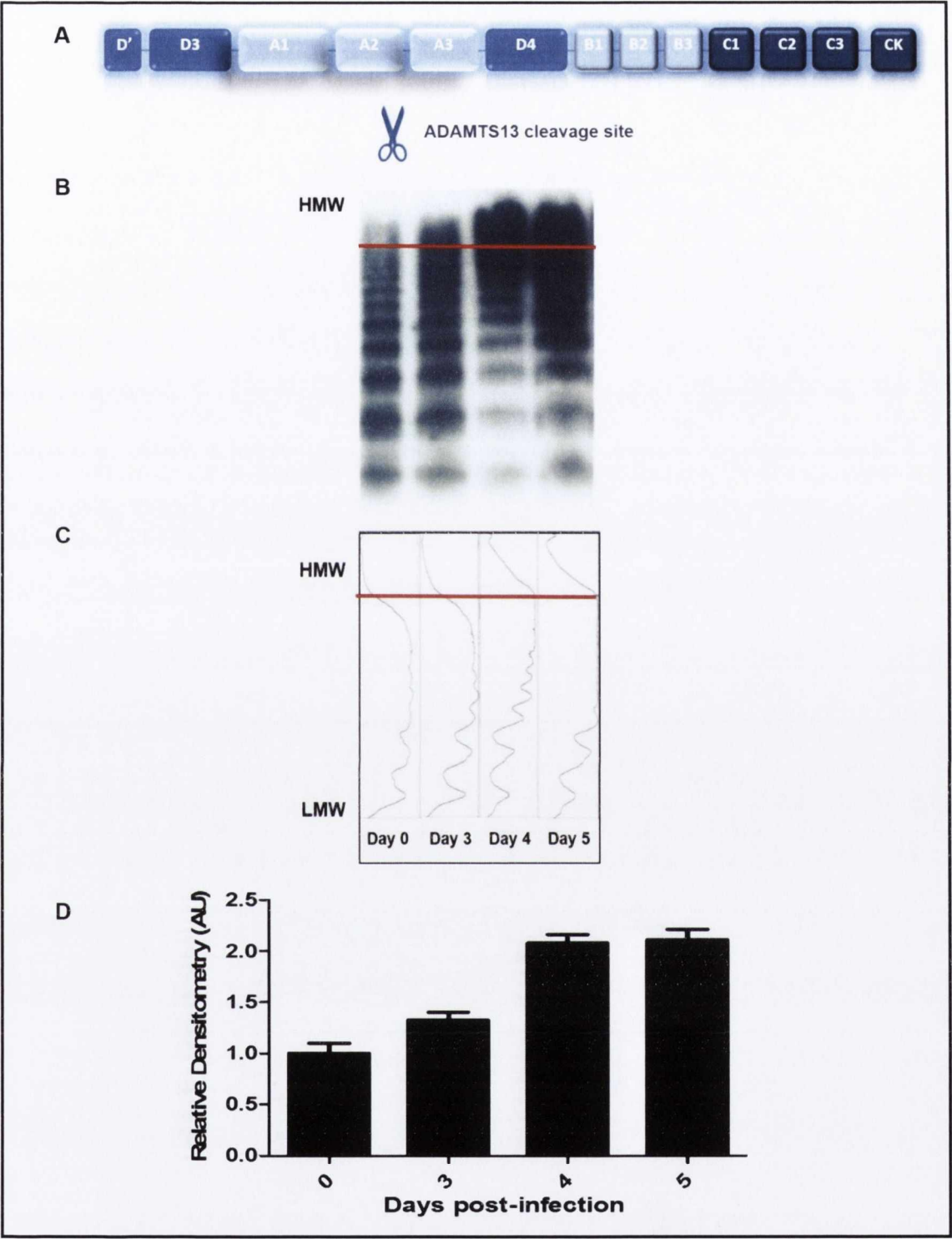


Figure 3. 8 Severe *P. berghei* malaria influences plasma VWF multimer composition

(A) Schematic of VWF and ADAMTS13 cleavage site (B) VWF multimer analysis after *P. berghei* infection (C+D) Densitometry of individual lanes in multimer gel

3.3.6 Elucidating the aetiology of ULVWF

As mentioned in section 3.3.5 ADAMTS13 is a zinc metalloprotease which cleaves haemostatically active forms of VWF. VWF multimerization is a critical determinant of its functional activity. Upon release from EC, VWF exists as elongated strings of ULVWF multimers. Proteolysis by ADAMTS13 results in a heterogeneous population of high molecular weight multimers (HMWM) and low molecular weight multimers (LMWM) of VWF within the circulation. Previous studies have reported modest reductions in plasma ADAMTS13 activity levels in children with *P. falciparum* infection (Larkin *et al*, 2009; de Mast *et al*, 2009; Löwenberg *et al*, 2010). In contrast, we observed no significant reduction in murine plasma ADAMTS13 levels following *P. berghei* infection (Figure 3.9) (n=4-6).



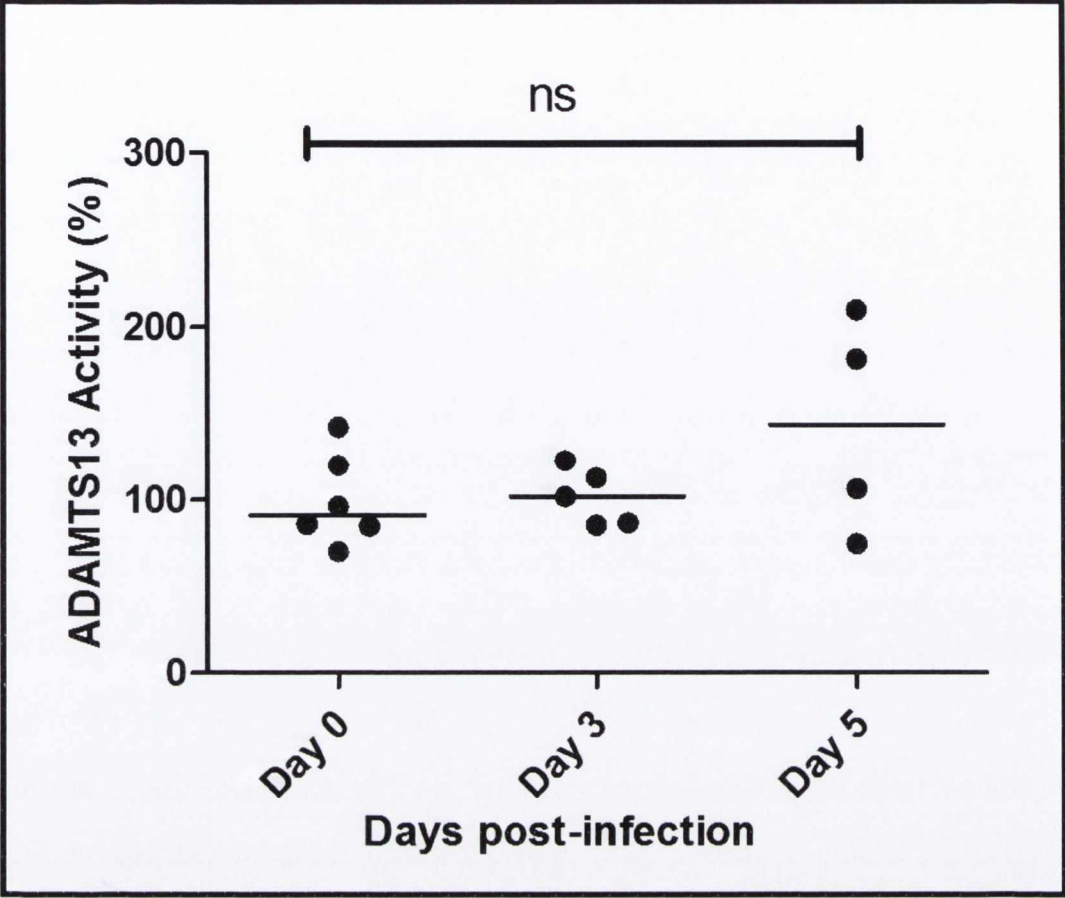


Figure 3. 9 ADAMTS13 activity remains unchanged during *P. berghei* ANKA infection

3.4 The VWF^{-/-} mouse model

Increased plasma VWF levels may represent a biomarker of acute EC activation in ECM. Alternatively, elevated plasma VWF and/or ULVWF multimers may be directly involved in mediating the pathogenesis of ECM. To address this important question, we proceeded to study *P. berghei* infection in VWF^{-/-} mice compared to WT. These VWF^{-/-} mice were on a C57BL/6J background and have previously been well characterized (Denis *et al*, 1998, 2001). This mouse is null for VWF gene with no plasma VWF, no platelet VWF and no WPB.

3.4.1 Clinical phenotype in VWF^{-/-} mouse model

Using a previously validated clinical scoring algorithm (Amante *et al*, 2007) (see section 3.2.1), we assessed malaria progression in VWF^{-/-} mice compared to WT mice following *P. berghei* infection. We observed that the clinical features of ECM progressed significantly more slowly in the VWF^{-/-} mice compared to WT mice (Figure 3.10) (WT n=16, VWF^{-/-} n=10). For example, at Day +5 of ECM VWF^{-/-} mice had a significant delay in clinical progression ($P \leq 0.001$), suggesting that VWF^{-/-} mice are significantly protected.

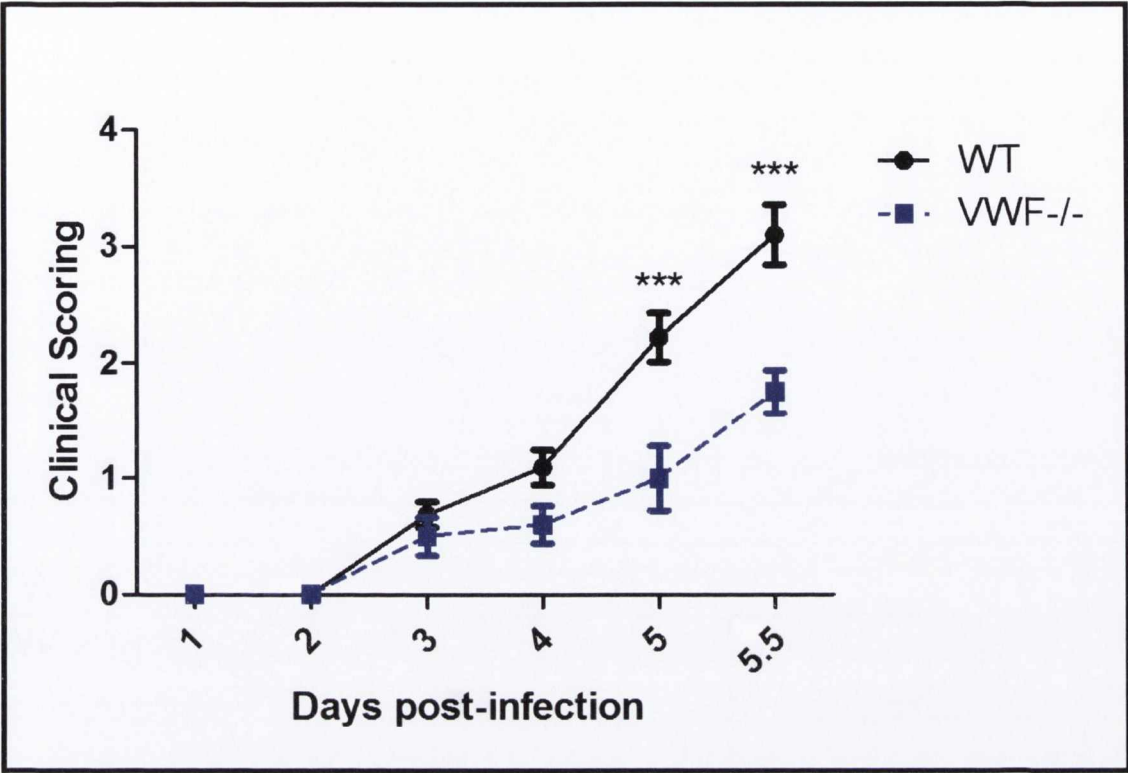


Figure 3. 10 Clinical features of experimental CM progressed significantly more slowly in the VWF^{-/-} mice

3.4.2 VWF^{-/-} mice are protected against experimental CM

In addition to clinical scoring, we assessed overall survival in VWF^{-/-} mice compared to WT. Interestingly, overall survival was also significantly prolonged in VWF^{-/-} mice compared to WT mice (P=0.0106) (Figure 3.11) (WT n=16, VWF^{-/-} n=10). Such that, at Day +6 30% of WT compared to 80% of VWF^{-/-} were still alive. Importantly however, VWF^{-/-} mice still developed typical neurological signs, and died from ECM.

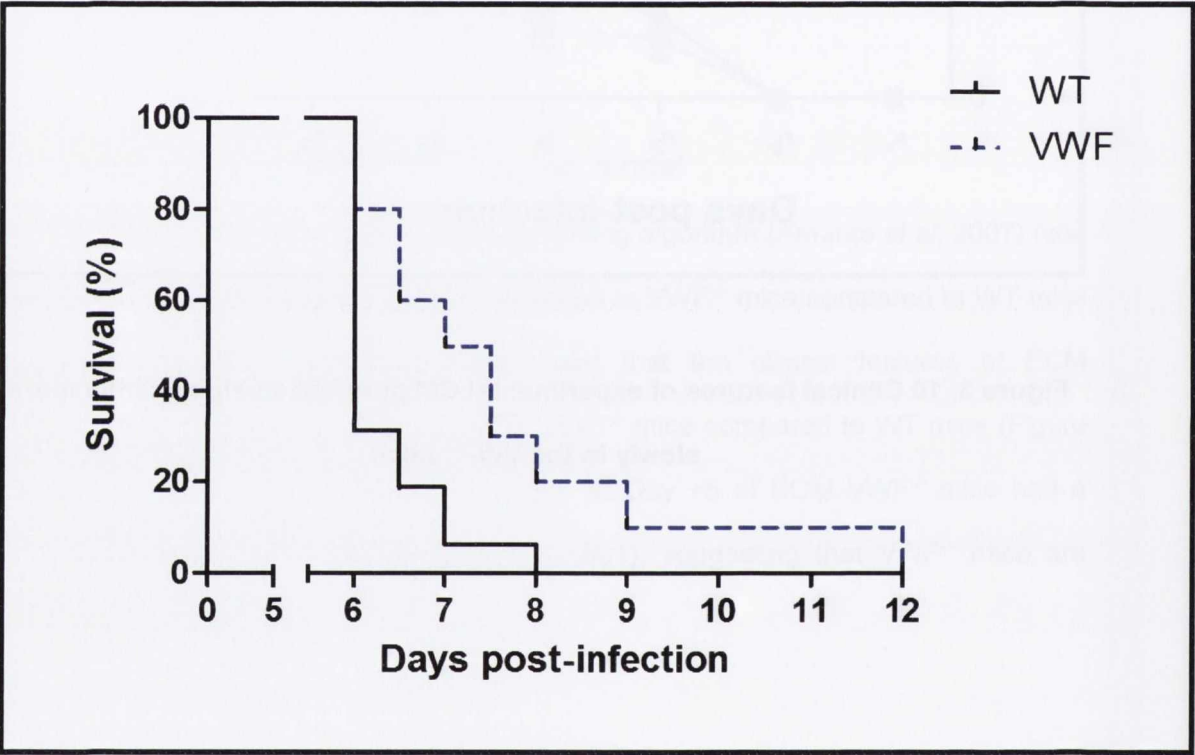


Figure 3. 11 VWF deficient mice are protected against experimental cerebral malaria

3.4.3 Red blood cells and haemoglobin of VWF^{-/-} mice versus WT during *P. berghei* infection

During the blood stage of infection, *P. falciparum* can induce anaemia. Severe malarial anaemia can be a major cause of mortality and morbidity in patients with disease. Red blood cell count (RBC) and haemoglobin concentration (Hb) were monitored during the course of *P. berghei* infection. Notably however, RBC and Hb did not change significantly during the course of infection (n=3-5).

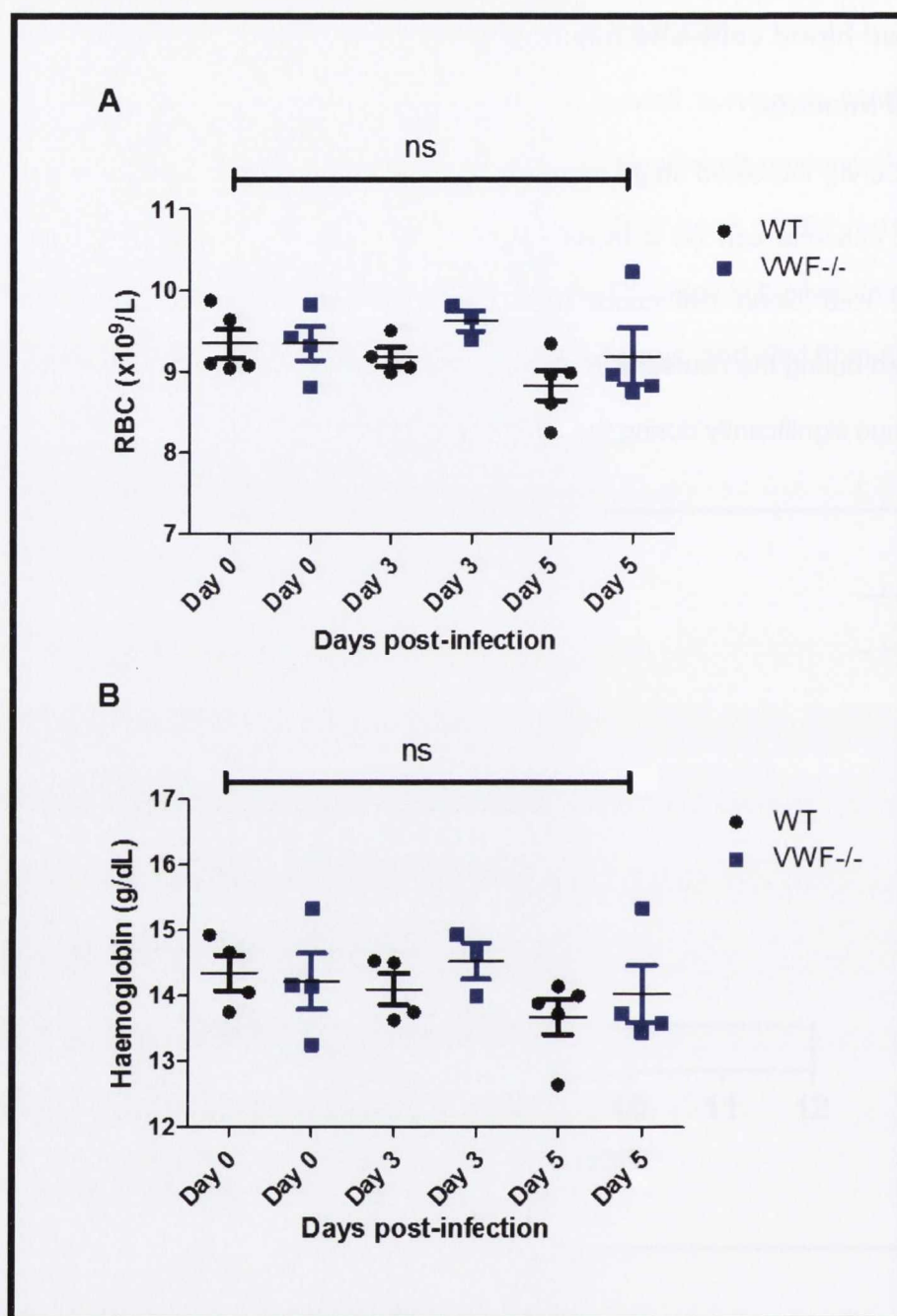


Figure 3. 12 (A) Red cell counts and (B) haemoglobin do not change over the course of infection

3.4.4 Weight and parasitemia in VWF^{-/-} versus WT mice

All infected mice were monitored daily for weight loss as well as general distress as per ethical guidelines. There were no significant differences in weight loss between VWF^{-/-} and WT mice (Figure 3.13A). Importantly, although overall survival was significantly prolonged in VWF^{-/-} mice compared to WT mice, we observed no significant differences in blood *P. berghei* parasitemia levels (Figure 3.13B) (WT n=16, VWF^{-/-} n=10).

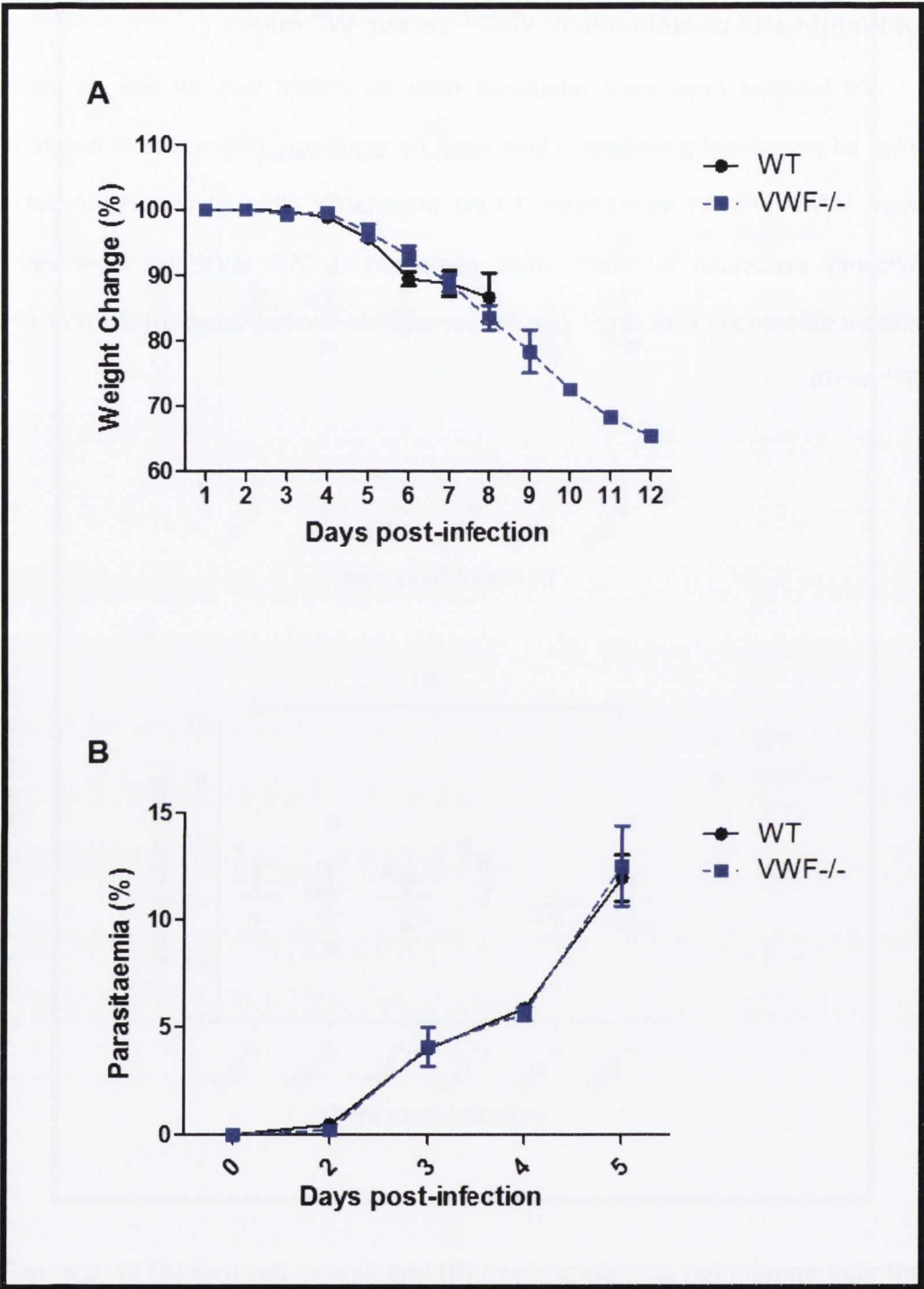


Figure 3. 13 (A) Weight change and (B) parasitemia are not significantly different between both strains

3.4.5 VWF^{-/-} mice lack Weibel-Palade bodies

To elucidate the mechanism through which VWF^{-/-} mice are protected against ECM we further investigated other WPB contents in the murine model of ECM. As previously mentioned, Ang-2 is a vascular modulator expressed in smooth muscle cells (e.g. placenta) and stored within WPB (Fiedler *et al*, 2004).

Previous studies have shown that WPB formation within EC occurs only in the presence of VWF (Denis *et al*, 1998). Thus, these storage organelles are not synthesised in the EC of VWF knockout animals. Consequently, it remains unclear whether the malaria-resistant phenotype observed in VWF^{-/-} mice is due to the absence of plasma VWF, or may be attributable to a secondary absence of WPB. Interestingly, plasma Ang-2 levels were significantly elevated in VWF^{-/-} to a similar degree to WT mice by Day +5 of infection ($P \leq 0.005$) (Figure 3.14) ($n=4-6$). Ang-2 may potentially derive from smooth muscle cells in these animals lacking WPB.

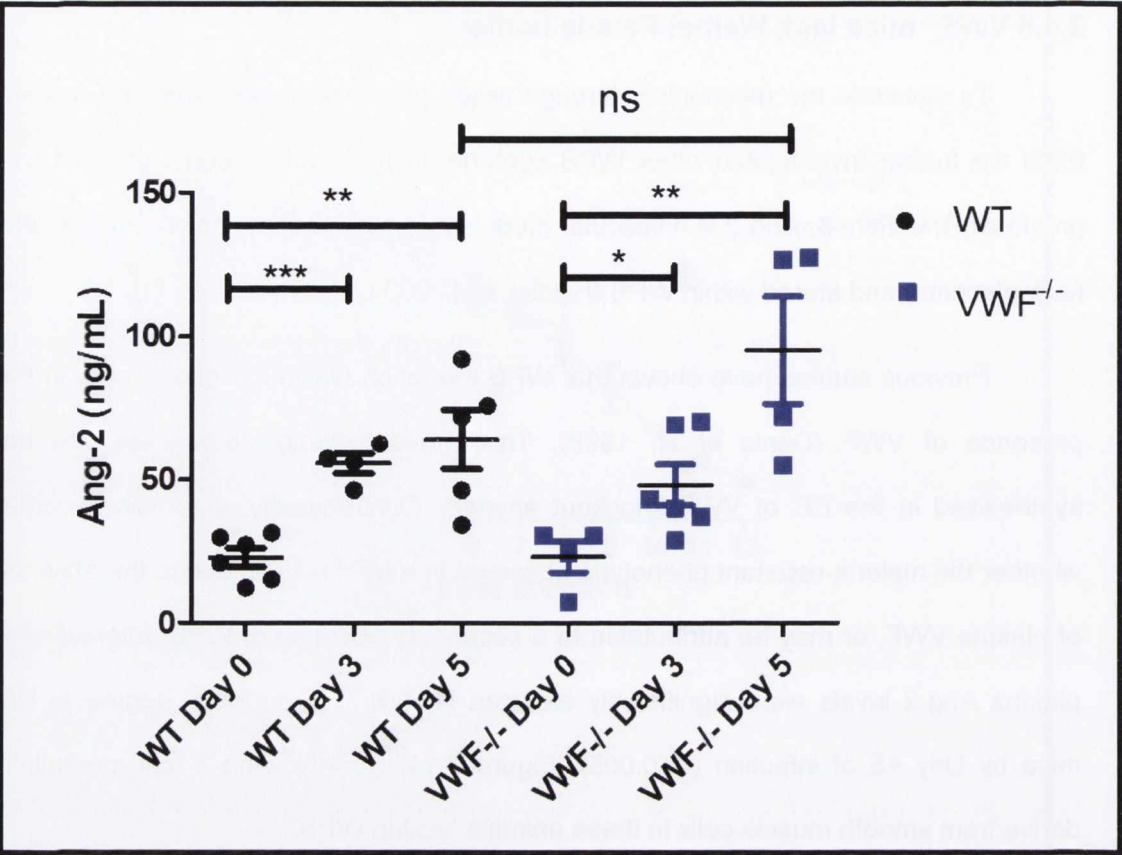


Figure 3. 14 Ang-2 levels increase in VWF^{-/-} mice during the course of *P. berghei* infection

3.4.6 Thrombocytopenia in experimental CM is independent of plasma VWF

Significant thrombocytopenia is common feature in patients with *P. falciparum* malaria (Cox & McConkey, 2010; Faille *et al*, 2009). This thrombocytopenia is predominantly due to increased platelet clearance. A putative role for ULVWF in contributing to this enhanced platelet clearance has been proposed (de Mast *et al*, 2007, 2010). Furthermore, recent studies have identified novel roles for platelets in killing intraerythrocytic malaria parasites (Mcmorran *et al*, 2009), and in modulating host immune responses during the early stages of *P. falciparum* infection (Srivastava *et al*, 2008). Platelet counts were used as indicator of platelet function. Although Platelet counts have limitations with regards to platelet function, blood volume was too limited to carry out platelet aggregation studies.

Consequently, to investigate potential mechanisms through which VWF^{-/-} mice were protected against ECM, daily platelet counts were performed in WT and VWF^{-/-} mice following *P. berghei* infection. In keeping with observations in human patients, significant thrombocytopenia was also a consistent feature in the murine model (Figure 3.15). For example, by Day +4 following inoculation of WT mice, mean platelet count had fallen by approximately 70%. Interestingly, significant thrombocytopenia was also observed in VWF^{-/-} mice infected with *P. berghei* (Figure 3.15) (n=3-5). Importantly, despite the differences in clinical malaria progression, no significant differences in platelet counts were observed between VWF^{-/-} and WT mice.

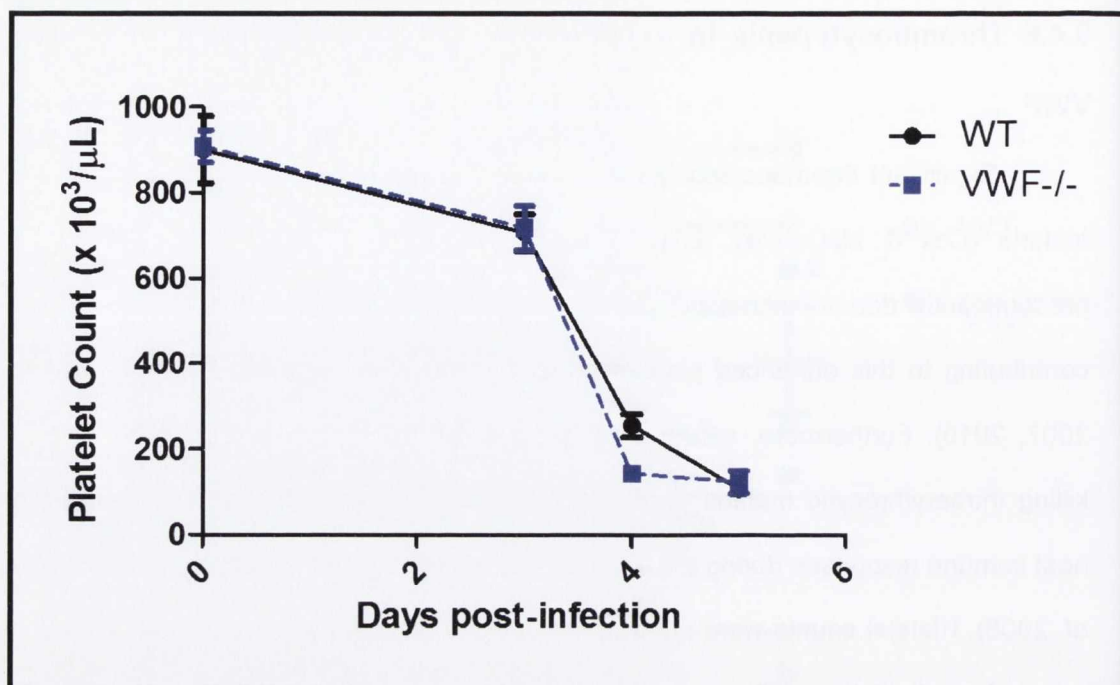


Figure 3. 15 VWF^{-/-} mice are protected against CM in a platelet-independent mechanism

3.4.7 ECM-induced blood brain barrier permeability is attenuated in VWF^{-/-} mice

VWF is expressed abundantly by cerebral EC (Yamamoto *et al*, 1998). In addition, endothelial VWF has recently been shown to play an important role in modulating permeability of the blood-brain barrier (BBB) under different pathological conditions (Noubade *et al*, 2008; Suidan *et al*, 2013). Although the underlying biological mechanisms remain poorly understood, enhanced permeability of the BBB has also been implicated in the pathogenesis of CM (Schofield & Grau, 2005; Pamplona *et al*, 2007). We therefore investigated BBB permeability in VWF^{-/-} 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^{-/-} and WT mice prior to *P. berghei* inoculation (Figure 3.16). By Day +5 following infection, BBB permeability was markedly elevated in WT (1.8ng/mg versus 4.7ng/mg day 0 and day +5 respectively; $P \leq 0.001$). Interestingly, although BBB was also affected in VWF^{-/-} mice at Day +5, the increase in permeability was significantly lower than that observed in WT mice ($P \leq 0.05$) (n=5-6).

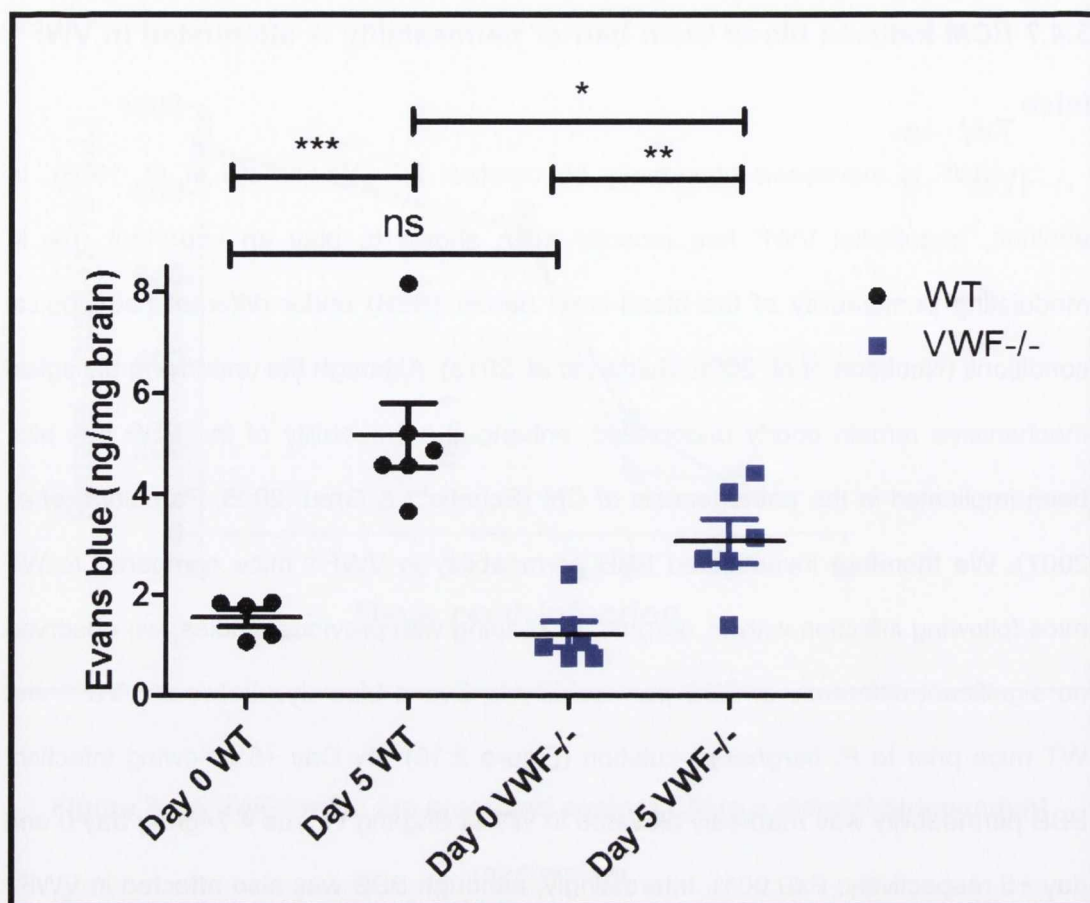


Figure 3. 16 Experimental CM-induced blood-brain barrier permeability is attenuated in VWF^{-/-} mice

3.5 Discussion

C57BL/6J mice infected with *P. berghei* ANKA typically develop a complex neurological syndrome involving clinical features similar to those observed in human CM (Baptista *et al*, 2010; de Souza & Riley, 2002). These include ataxia, paralysis, seizures and coma. Depending upon the dose of *P. berghei*, infected mice typically die within 6 to 10 days. Although there are important differences between human CM and the ECM model (Franke-Fayard *et al*, 2005; White *et al*, 2010), nevertheless both *P. falciparum* and *P. berghei* infections are associated with effects on EC biology (Baptista *et al*, 2010; de Souza & Riley, 2002; Carvalho, 2010; Hunt *et al*, 2010). The mouse model of malaria was established in our group and preliminary experiments were conducted on WT mice to assess disease progression and survival in these mice. All data gathered were consistent with the previous literature. In this murine model the timing and extent of EC activation was examined.

In this study, we demonstrate that *P. berghei* infection is associated with significant increases in plasma VWF:Ag and VWF:CB levels, similar to those previously observed in African children with severe *P. falciparum* malaria (Hollestelle *et al*, 2006; Larkin *et al*, 2009; Conroy *et al*, 2010; Erdman *et al*, 2011; Phiri *et al*, 2011). In addition, and in keeping with previous data from human volunteers infected with *P. falciparum* (de Mast *et al*, 2007), these increased plasma VWF levels were observed from a very early stage following *P. berghei* infection. Thus, early acute EC activation and WPB secretion constitute hallmarks of both *P. falciparum* infection in humans, and of the murine model of ECM. Interestingly, in both humans and in mice infected with *P. berghei*, the increase in plasma VWF levels was first apparent before the number of infected erythrocytes had reached 0.1%. The biological mechanism underlying this very early EC activation remains unknown (MacDonald *et al*, 2001). Further studies using C57BL/6 mice infected with *P. berghei* may provide a useful model through which to define the biological mechanisms through which this early EC activation is modulated. From

data collected, we have shown that plasma Ang-2 levels are also significantly increased in murine CM. This observation is consistent with previous data from children with severe *P. falciparum* malaria (Conroy *et al*, 2010; Erdman *et al*, 2011; Conroy *et al*, 2009; Lovegrove *et al*, 2009; Yeo *et al*, 2008). Interestingly, Ang-2 has also been shown to function as an autocrine regulator by sensitizing EC to activation by tumour necrosis factor (TNF) (Fiedler *et al*, 2006), and thus may play a role in modulating the early acute EC activation associated with both *P. falciparum* and *P. berghei* infections.

Previous studies have demonstrated that severe *P. falciparum* malaria is associated with accumulation of abnormal ULVWF multimers in the plasma (Larkin *et al*, 2009; de Mast *et al*, 2009). In this study, we further demonstrate that plasma ULVWF multimers also constitute a feature of the murine model of CM. The molecular mechanisms responsible for this ULVWF accumulation have not been defined. However marked exocytosis of ULVWF multimers from WPB following acute EC activation is likely to represent an important contributing factor. Nevertheless, ULVWF multimers secreted from WPB typically undergo rapid proteolysis by ADAMTS13 on EC surfaces. We observed no significant reduction in murine ADAMTS13 levels following *P. berghei* infection. In contrast, previous studies did report a significant reduction in plasma ADAMTS13 levels in children and adult patients with severe malaria compared to healthy controls (Larkin *et al*, 2009; de Mast *et al*, 2009; Löwenberg *et al*, 2010). 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 ULVWF multimers (Dong *et al*, 2004; Larkin *et al*, 2009). 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 (Bernardo *et al*, 2004; Bonnefoy *et al*, 2006; Cao *et al*, 2008; Crawley *et al*, 2005; Studt *et al*, 2005), our lab previously showed that these could not explain the accumulation of ULVWF multimers observed in children with CM

(Larkin *et al*, 2009). Interestingly, this combination of ULVWF multimers despite normal plasma ADAMTS13 levels has also been recently been reported in patients with sickle cell disease (Chen *et al*, 2011). Further studies to investigate how ULVWF multimers accumulate in the murine model of CM may therefore be useful in elucidating novel molecular mechanisms involved in the physiological and pathological regulation of ADAMTS13 activity *in vivo*. Cumulatively these findings suggest that marked early elevation in VWF:Ag levels, and the appearance of pathological ULVWF multimers in the plasma, represent hallmarks of both human and murine severe malaria infection.

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, 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 ULVWF strings could recruit *P. falciparum* IE to EC surfaces under shear stress conditions (Bridges *et al*, 2010). In this study, we demonstrate that VWF^{-/-} mice are significantly protected against *P. berghei* infection compared to WT controls. This survival difference was not explained by a difference in *P. berghei* parasitemia levels, which were similar in both VWF^{-/-} and WT mice. Significant thrombocytopenia is a common feature of both human and murine malaria (Cox & McConkey, 2010; Faille *et al*, 2009). Moreover, recent studies have described a role for platelets in modulating the innate host defense during the early stages of malaria infection (Mcmorran *et al*, 2009; Srivastava *et al*, 2008). Interestingly, although previous studies suggested that the ULVWF might be important in mediating this malaria-associated thrombocytopenia (de Mast *et al*, 2007, 2010), we observed no significant difference in *P. berghei*-induced thrombocytopenia in VWF^{-/-} 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 ULVWF multimers. In addition, VWF^{-/-} mice are clearly protected against ECM through a platelet-independent mechanism.

VWF synthesis is essential for the normal formation of WPB within EC (Rondaij *et al*, 2006). Consequently, VWF^{-/-} mice are not only deficient in plasma and platelet VWF, but also do not possess WP bodies (Denis *et al*, 1998). In addition to VWF and VWF:pp, WPB also store other proteins including P-selectin, Ang-2 and osteoprotegerin. Whether the absence of WPB 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 (Suidan *et al*, 2013). Ang-2 is another component of WPB. In this study we have shown that plasma Ang-2 levels are also significantly increased from an early stage in murine ECM. This observation is consistent with previous data from children with severe *P. falciparum* malaria (Conroy *et al*, 2010; Erdman *et al*, 2011; Conroy *et al*, 2009; Lovegrove *et al*, 2009; Yeo *et al*, 2008). Interestingly, plasma Ang-2 is also elevated in VWF^{-/-} mice during infection with *P. berghei*. This increase in Ang-2 levels in VWF^{-/-} mice suggests protection is not mediated by the lack of WPB and analytes such as P-selectin and Ang-2.

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 (Noubade *et al*, 2008; Suidan *et al*, 2013). For example, significantly attenuated BBB permeability was observed in VWF^{-/-} mice compared to WT controls in a hypoxia/reoxygenation model, and also in an experimental model of status epilepticus (Suidan *et al*, 2013). 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^{-/-} mice compared to WT controls (Suidan *et al*, 2013). 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.

In conclusion, we demonstrate that early and consistent EC activation is a feature of the murine model of ECM similar to previous findings of children infected with *P. falciparum*. In addition, our findings show that marked elevation of VWF:Ag, and the appearance of pathological ULVWF multimers in the plasma, both represent hallmarks of human and murine severe malaria infection. Finally, we report that VWF^{-/-} mice exhibit significantly prolonged survival against CM, and that this effect is mediated through a platelet-independent mechanism. BBB disruption is delayed in the VWF^{-/-} mice which could contribute to the survival advantage we observed. Given the significant morbidity and mortality associated with CM, these novel data have direct translational significance.

4. Further investigating the role of VWF in the pathogenesis of ECM

4.1 Introduction

On the basis of the data presented in chapter 3, it is clear that elevated plasma VWF levels and/or the presence of ULVWF contribute to the pathogenesis of ECM. To further investigate the underlying molecular mechanisms, we have studied other genetically modified mice strains in which either the ADAMTS13, or the ASGPR gene have been knocked out.

4.1.1 ADAMTS13

Isolation of a plasma protease capable of cleaving VWF into smaller forms was accomplished in 1996 (Furlan *et al*, 1996; Tsai, 1996). Subsequently, ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13) a 190kDa glycosylated protein, was purified and characterised by two groups in 2001 (Fujikawa *et al*, 2001; Zheng *et al*, 2001). ADAMTS13 is a zinc-metalloprotease that employs divalent cations for its catalytic activity (Malemud, 2006) and is primarily synthesised in hepatic stellate cells. This metalloprotease circulates in the plasma at a concentration of 0.5-1µg/mL (Uemura *et al*, 2005; Zhou *et al*, 2005). In addition, constitutive secretion of ADAMTS13 from human umbilical vein endothelial cells (HUVECs) has also been described (Turner *et al*, 2009).

4.1.2 ADAMTS13 function

Regulation of VWF adhesive activity is crucial for maintaining homeostasis in the blood. ULVWF multimers can be as large as 20,000 kDa in size and represent the most hyperactive thrombogenic form of VWF (Sporn *et al*, 1987). These ULVWF are not present in blood under normal conditions. However, upon EC activation or injury, ULVWF is released from WPB. During this secretion, ULVWF initially remains

transiently bound to the EC surface where it undergoes rapid, partial proteolysis by ADAMTS13 into smaller VWF multimers with reduced thrombotic potential (Sadler, 2008). ADAMTS13 specifically cleaves the Y1605/M1606 scissile bond within the VWF A2 domain (Levy *et al*, 2005). In order for ADAMTS13 cleavage to occur, conformational extension of the A2 domain is required in order to expose the hidden cleavage site (Dong, 2005). Proteolysis of VWF by ADAMTS13 is blood-group dependent, such that Blood Group O is cleaved more readily than non-O VWF (Bowen, 2003). Additionally, VWF from individuals with the rare Bombay phenotype, who fail to express any ABH antigens, show increased proteolysis compared to Group O individuals (O'Donnell *et al*, 2005). Despite the fact that ADAMTS13 has been identified in platelet lysates (Suzuki *et al*, 2004), platelet-VWF multimers do not depict a triplet pattern after electrophoresis (Fernandez *et al*, 1982), suggesting that platelet-VWF does not undergo proteolysis by ADAMTS13 prior to its secretion into the plasma.

Several amino acid residues and exosites present in ADAMTS13 are vital for effective proteolytic function. The non-catalytic spacer domain of ADAMTS13 binds at the carboxy-terminal end of the VWF A2 domain (Gao *et al*, 2006). Additionally, three amino acid residues in the spacer domain of ADAMTS13 have been shown to be important for specific activity; Arg659, Arg660 and Tyr661 (Jin *et al*, 2010). Similarly, Zhou *et al* reported four cysteine residues (C1192, C1213, C1236 and C1254) which were essential for efficient secretion of ADAMTS13, and also for the efficient proteolysis of VWF (Zhou *et al*, 2011b). Other ADAMTS13 domains have been demonstrated to play roles in mediating interaction with the A2 domain of VWF including the first thrombospondin-1 repeat; the cysteine rich; and the spacer domain (Gao *et al*, 2008). Moreover, a crucial functional role for the ADAMTS13 disintegrin-like domain has also been shown (de Groot *et al*, 2009). Finally, sites within the metalloprotease domain are important for binding and cleavage of VWF (De Groot *et al*, 2010).

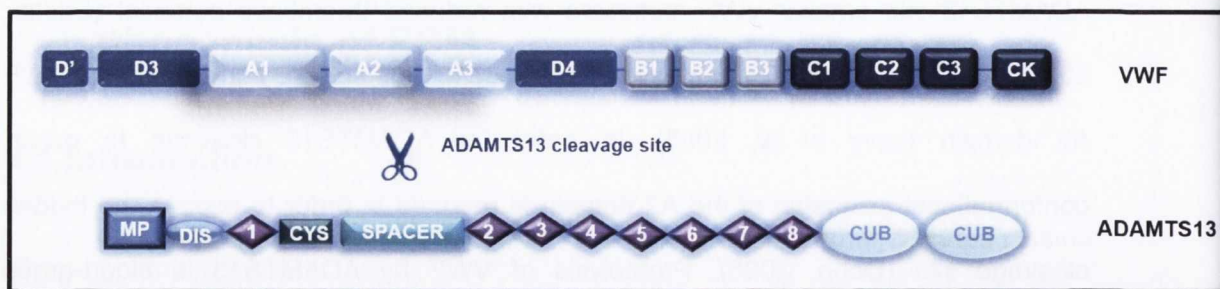


Figure 4. 1 ADAMTS13 cleaves VWF in the A2 domain

4.1.3 ADAMTS13 regulation

The mechanisms through which ADAMTS13 activity is regulated *in vivo* are not well defined. However, recent studies have identified putative cofactors (Vomund & Majerus, 2009; Cao *et al*, 2008) and inhibitors (Bernardo *et al*, 2004; Chung *et al*, 2008). For example, thrombospondin-1 (TSP-1) binding protects VWF from proteolysis by ADAMTS13 (Bonneyfoy *et al*, 2006). In addition, interleukin-6 and free haemoglobin have also been identified as inhibitors of ADAMTS13 *in vitro* (Bernardo *et al*, 2004; Studt *et al*, 2005). Finally, thrombin and plasmin can both cleave, inhibit and inactivate ADAMTS13, which may contribute to *in vivo* regulation of VWF proteolysis (Crawley *et al*, 2005). Oxidation of tyrosine and methionine residues in VWF by neutrophil oxidants (hydrogen peroxide and hypochlorous acid) also serves to inhibit ADAMTS13 proteolysis (Chen *et al*, 2010).

The importance of regulated VWF proteolysis by ADAMTS13 is demonstrated by two opposing haematological pathologies. Type 2A VWD can result from increased proteolysis of VWF by ADAMTS13, with loss of high molecular weight multimers and a consequent bleeding phenotype (Batlle *et al*, 1986). Conversely, deficiency of ADAMTS13 can lead to thrombotic thrombocytopenic purpura (TTP), which is

characterised by a thrombotic microangiopathy, thrombocytopenia, and an excess of circulating (Furlan, 1996). Inherited or acquired ADAMTS13 deficiency plays a critical role in the aetiology of TTP. Most cases can be attributed to low ADAMTS13 antigen levels or decreased ADAMTS13 activity (Luken *et al*, 2005).

4.1.4 VWF and ADAMT13 in disease and inflammation

Recent studies suggest that both VWF and ADAMTS13 may play important roles in regulating inflammatory responses. VWF has been shown to adhere to both polymorphonuclear cells and monocytes, and thus may be important in leukocyte tethering and rolling (Pendur *et al*, 2006). Previous studies have also reported reduced ADAMTS13 activity in patients with acute systemic inflammation or sepsis (Ono *et al*, 2006; Nguyen *et al*, 2007; Reiter *et al*, 2005). Proinflammatory cytokines such as Interleukin-6 and TNF- α released during inflammation have been demonstrated to effect EC release of ULVWF and its processing (Bernardo *et al*, 2004). The importance of reduced ADAMTS13 activity in plasma in these disease states or other chronic inflammatory conditions remains unknown. However, an inverse correlation has been observed between high levels of ULVWF and low levels of ADAMTS13 in systemic inflammation (Claus *et al*, 2009). In addition, high levels of ULVWF and low levels of ADAMTS13 have been found in patients with systemic inflammation resulting from cardiac surgery or severe sepsis (Bockmeyer *et al*, 2008).

As previously discussed, ADAMTS13 deficiency is associated with the rare clinical condition TTP. Interestingly, previous studies have also reported that plasma ADAMTS13 levels are significantly reduced in patients with severe *P. falciparum* compared to normal controls (Larkin *et al*, 2009; de Mast *et al*, 2009; Löwenberg *et al*, 2010). However, although ADAMTS13 activity is reduced in malaria, absolute plasma ADAMTS13 levels remain above 20%. Previous studies suggest that this residual level of ADAMTS13 should be sufficient to prevent the accumulation of ULVWF multimers (Dong *et al*, 2004). Nevertheless, circulatory ULVWF are present in both children with

CM and mice with ECM. Consequently, it seems likely that *in vivo* inhibition of plasma ADAMTS13 activity may also be occurring in children with severe *P. falciparum* (Figure 4.2).

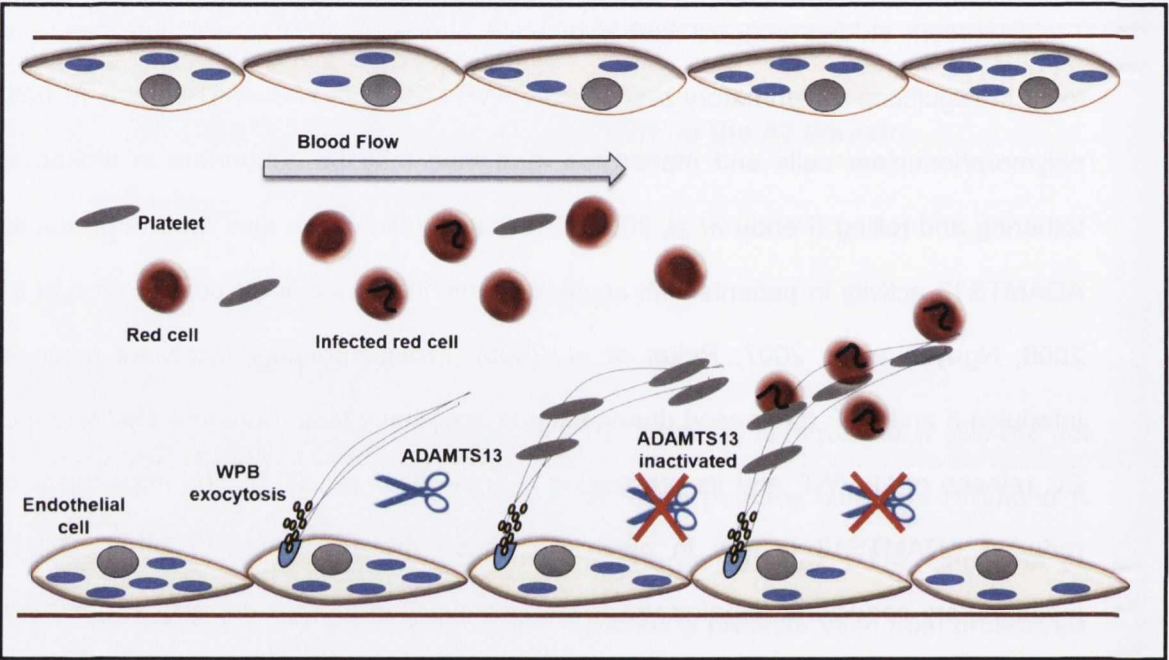


Figure 4. 2 ULVWF released from WPB is normally cleaved by ADAMTS13, however in malaria ADAMTS13 appears to have decreased activity and/or is also inhibited

To further explore the role of ADAMTS13 in normal haemostasis and the pathogenesis of TTP, ADAMTS13^{-/-} mice have been generated by several groups. Interestingly, total lack of ADAMTS13 is not lethal in mice and is well tolerated in most genetic backgrounds of mice (Motto *et al*, 2005). However in some strains of mice, notably CASA/Rk strain which exhibit approximately 5- to 10-fold higher VWF levels

than C57BL/6J mice (Motto *et al*, 2005), clinical development of spontaneous TTP was observed in ADAMTS13^{-/-} mice.

Previous studies have shown that recombinant ADAMTS13 (rADAMTS13) reduces vascular inflammation and subsequent atherosclerotic plaque formation (Gandhi *et al*, 2012a). Thus, ADAMTS13^{-/-} mice developed larger myocardial infarctions than WT mice (De Meyer *et al*, 2012). Similarly, De Meyer *et al* also showed that WT mice treated with rADAMTS13 developed smaller infarctions. A significant reduction in the number of neutrophils infiltrating ischemic myocardium after rADAMTS13 treatment has been implicated in the biology underlying this ADAMTS13 effect, suggesting a possible anti-inflammatory role (Chauhan *et al*, 2006). Consequently, ADAMTS13 may form an important link between thrombosis and inflammation through its direct effects on VWF down-regulating inflammation by preventing excessive leukocyte tethering, rolling and extravasation (Chauhan *et al*, 2008; Pendu *et al*, 2006; Bernardo *et al*, 2004). Since thrombosis and inflammation constitute an integral part of the pathogenesis of many diseases including stroke, ADAMTS13 could provide benefit as a therapeutic agent.

4.2 Murine plasma ADAMTS13 activity is not significantly altered in ECM

As described in the previous chapter, abnormal ULVWF multimers are a feature of human CM and ECM. In addition, previous studies have described modest reductions in plasma ADAMTS13 activity levels in children with *P. falciparum* infection (Larkin *et al*, 2009; de Mast *et al*, 2009; Löwenberg *et al*, 2010). ADAMTS13 therapy has been reported to reduce both thrombosis and inflammation in disease states including atherosclerosis (Chauhan *et al*, 2006, 2008; Gandhi *et al*, 2012a). Since thrombosis and inflammation constitute an integral part of the pathogenesis of CM, we further investigated ADAMTS13 levels in the murine model of ECM. In WT and VWF^{-/-} mice no change in ADAMTS13 activity was observed during the course of *P. berghei* infection (Figure 4.3) (n=3-6). Interestingly however, higher baseline ADAMTS13 activity levels were observed in VWF^{-/-} mice in keeping with previous reports of human patients with VWD (Mannucci *et al*, 2004).

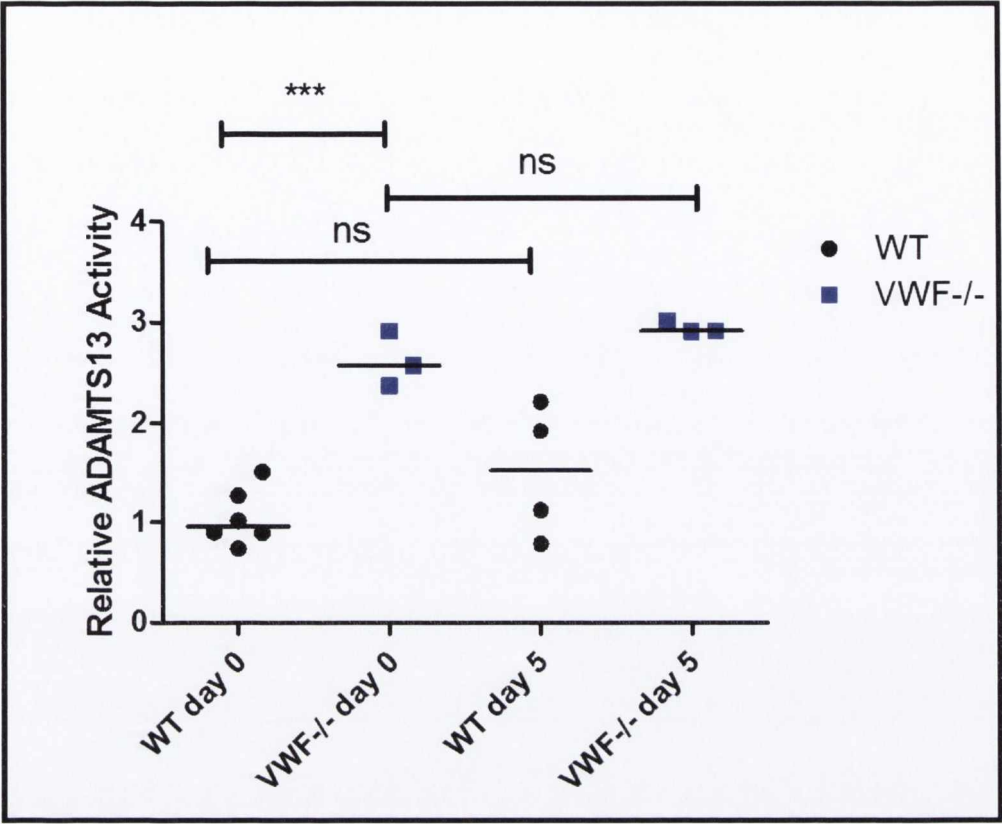


Figure 4. 3 Plasma ADAMTS13 levels in WT and VWF^{-/-} mice infected with *P. berghei*

4.3 Clinical progression of ECM in ADAMTS13^{-/-} mice

Previous studies have shown that specific types of infection associated with EC damage (e.g. Shiga toxin) can trigger TTP in ADAMTS13^{-/-} mice (Banno *et al*, 2006; Motto *et al*, 2005). Since *P. berghei* infection causes acute EC activation, significantly increased plasma VWF levels, and accumulation of pathological ULVWF multimers in plasma, we further investigated *P. berghei* infection in ADAMTS13^{-/-} mice. In particular, we investigated whether the pathogenesis of *P. berghei* might be affected in the absence of ADAMTS13. In addition, we also studied whether *P. berghei* inoculation might trigger acute TTP in these genetically modified mice. Interestingly, only a small increase in malaria severity was observed in the ADAMTS13^{-/-} mice compared to WT controls (WT n=12, ADAMTS13^{-/-} n=11).

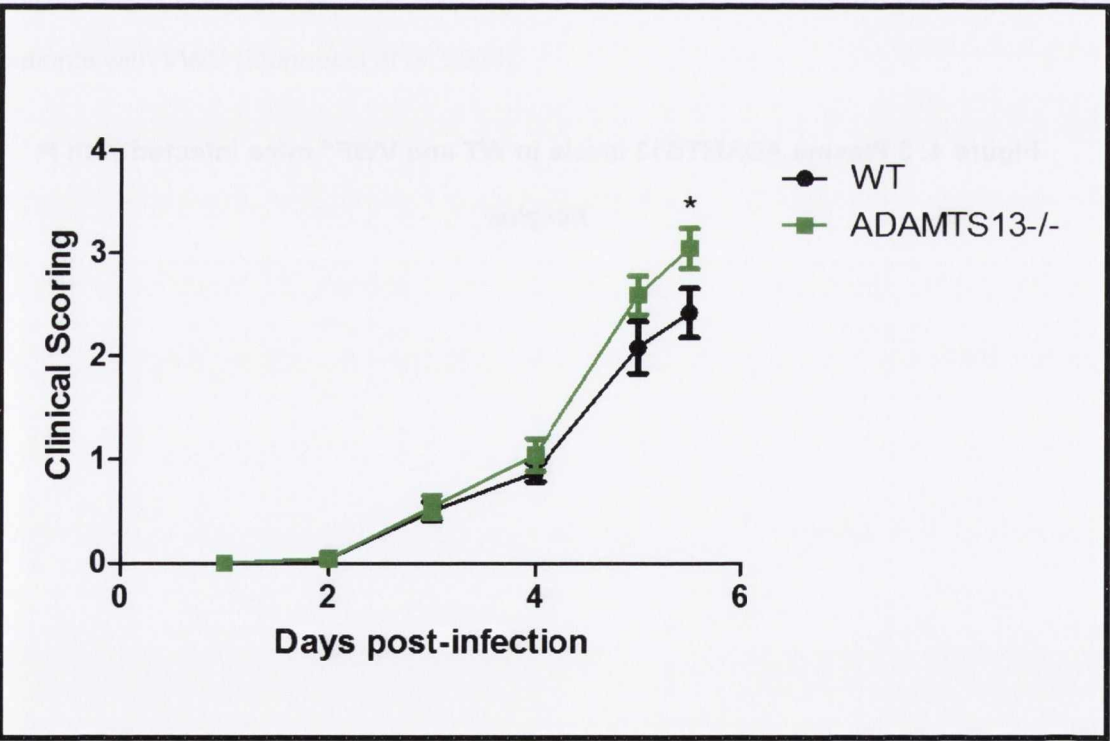


Figure 4. 4 Clinical progression of ECM in ADAMTS13^{-/-} mice compared to WT

4.4 Overall survival following *P. berghei* infection in ADAMTS13^{-/-} compared to WT

Following *P. berghei* inoculation, overall survival in ADAMTS13^{-/-} and WT mice was studied using the same methodology outlined in chapter 3. Although a moderate difference between the ADAMTS13^{-/-} and WT mice was observed using the clinical scoring algorithm, we observed no significant difference in overall survival (Figure 4.5, $P=0.14$) (WT $n=12$, ADAMTS13^{-/-} $n=11$).

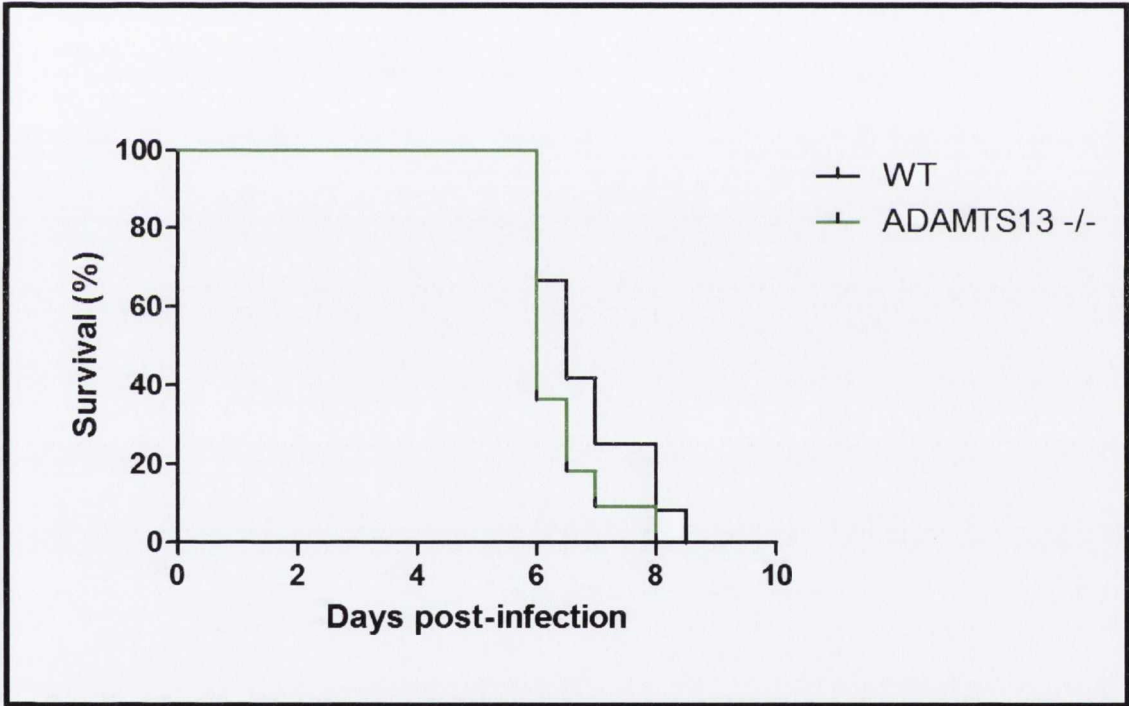


Figure 4. 5 Overall survival following *P. berghei* infection in ADAMTS13^{-/-} mice compared to WT mice

4.5 Weight change and parasitemia in ADAMTS13^{-/-} compared to WT mice

According to ethical guidelines, daily monitoring of weight change as well as general distress were recorded. There were no discrepancies in weight loss between the ADAMTS13^{-/-} and WT mice (Figure 4.6A). Peripheral blood parasitemia levels in WT and ADAMTS13^{-/-} mice were also assessed by standard Giemsa staining on a daily basis as previously described in chapter 3. Once again, there was no significant difference between ADAMTS13^{-/-} and WT mice (Figure 4.6B) (WT n=12, ADAMTS13^{-/-} n=11).



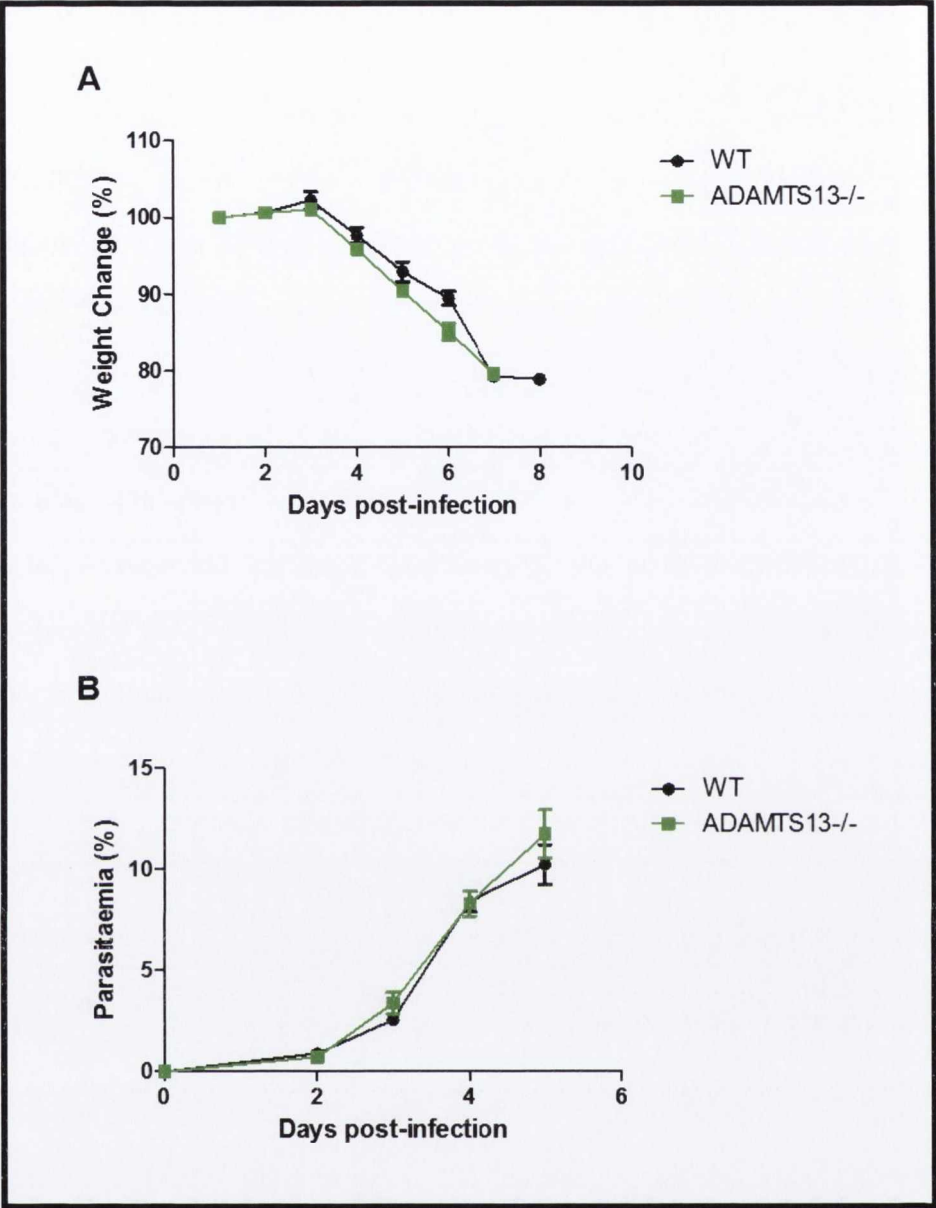


Figure 4. 6 (A) Weight change during the course of infection (B) Percentage parasitemia in ADAMTS13^{-/-} and WT mice following inoculation

4.6 VWF:Ag levels increase following *P. berghei* inoculation in ADAMTS13^{-/-} mice

No difference was observed in baseline VWF:Ag levels between WT and ADAMTS13^{-/-} mice ($P= 0.43$) (Figure 4.7A) ($n=7$). This is in keeping with previous reports of ADAMTS13 deficiency in this mouse background (Motto *et al*, 2005). Similar to previous findings in children with severe *P. falciparum* malaria (Hollestelle *et al*, 2006; Larkin *et al*, 2009) and the observations in WT C57BL/6J mice following *P. berghei* infection (section 3.5.1), we observed a significant increase in plasma VWF:Ag levels in ADAMTS13 deficient mice (Figure 4.7B) ($n=6-10$). For example, at Day +5 following inoculation, median plasma VWF:Ag levels were increased 2.5-fold (251.2% versus 98.6% at Day +5 and Day 0 respectively; $P\leq 0.0001$). This increase in VWF was similar to the magnitude of the increase in plasma VWF:Ag levels observed in children with CM (Hollestelle *et al*, 2006) and also comparable with WT mice.

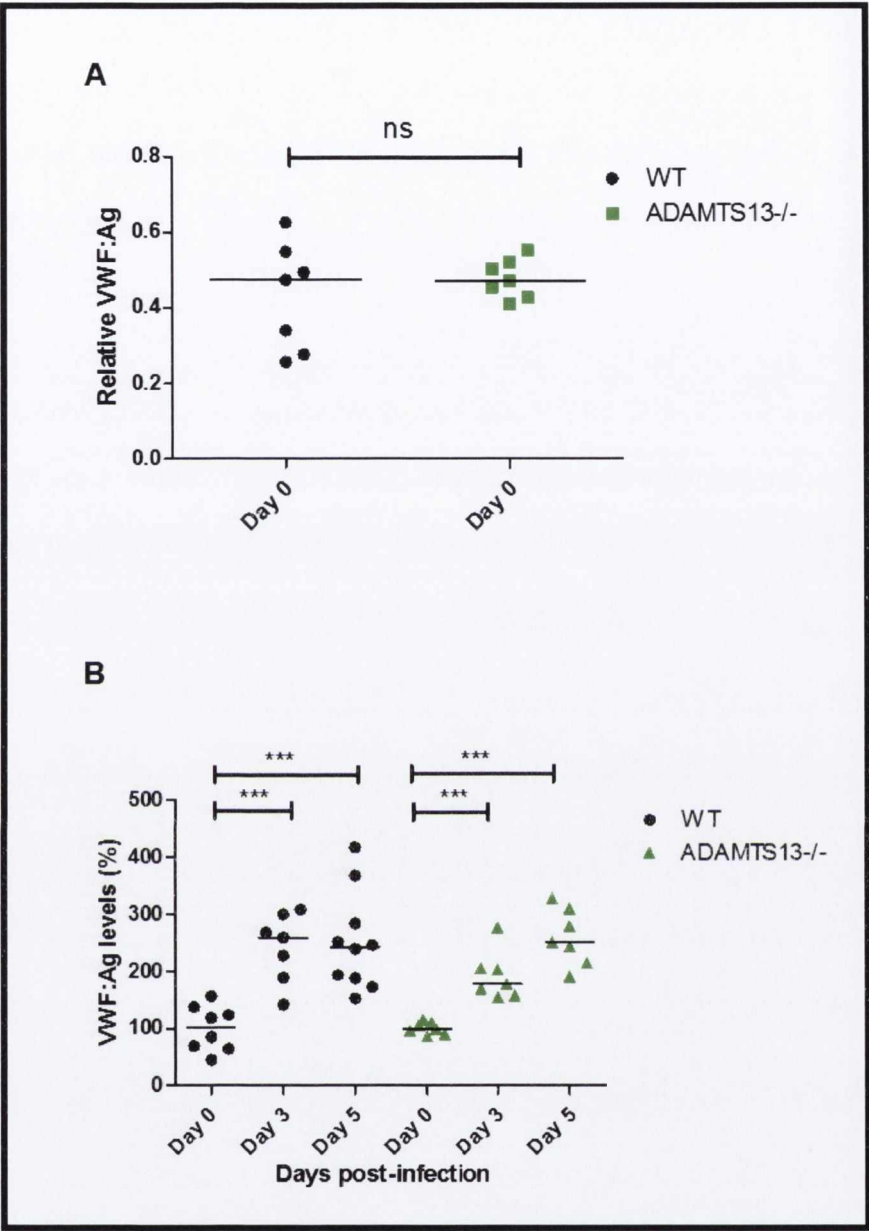


Figure 4. 7 (A) Baseline VWF:Ag levels do not differ between WT and ADAMTS13^{-/-} mice (B) VWF:Ag levels increase in ADAMTS13 deficient mice following *P. berghei* infection and correlate with disease progression

4.7 Plasma VWF:CB activity is elevated in ADAMTS13 deficient mice following *P. berghei* infection

VWF:CB is an assay which detects the ability of VWF to bind to collagen. VWF:CB is particularly sensitive to circulating high molecular weight multimer VWF (Larkin *et al*, 2009). No difference was observed in VWF:CB prior inoculation with *P. berghei* between WT and ADAMTS13^{-/-} mouse strains (P= 0.16) (Figure 4.8A) (n=4-6). In keeping with our previous findings in WT mice (section 3.5.2), and with previous studies in children infected with *P. falciparum* (Larkin *et al*, 2009), plasma VWF:CB levels were also significantly elevated in ADAMTS13^{-/-} C57BL/6J mice following *P. berghei* infection (106.8% versus 268.9% at Day 0 and Day +5 respectively; P≤0.01) (Figure 4.8B) (n=4-9).

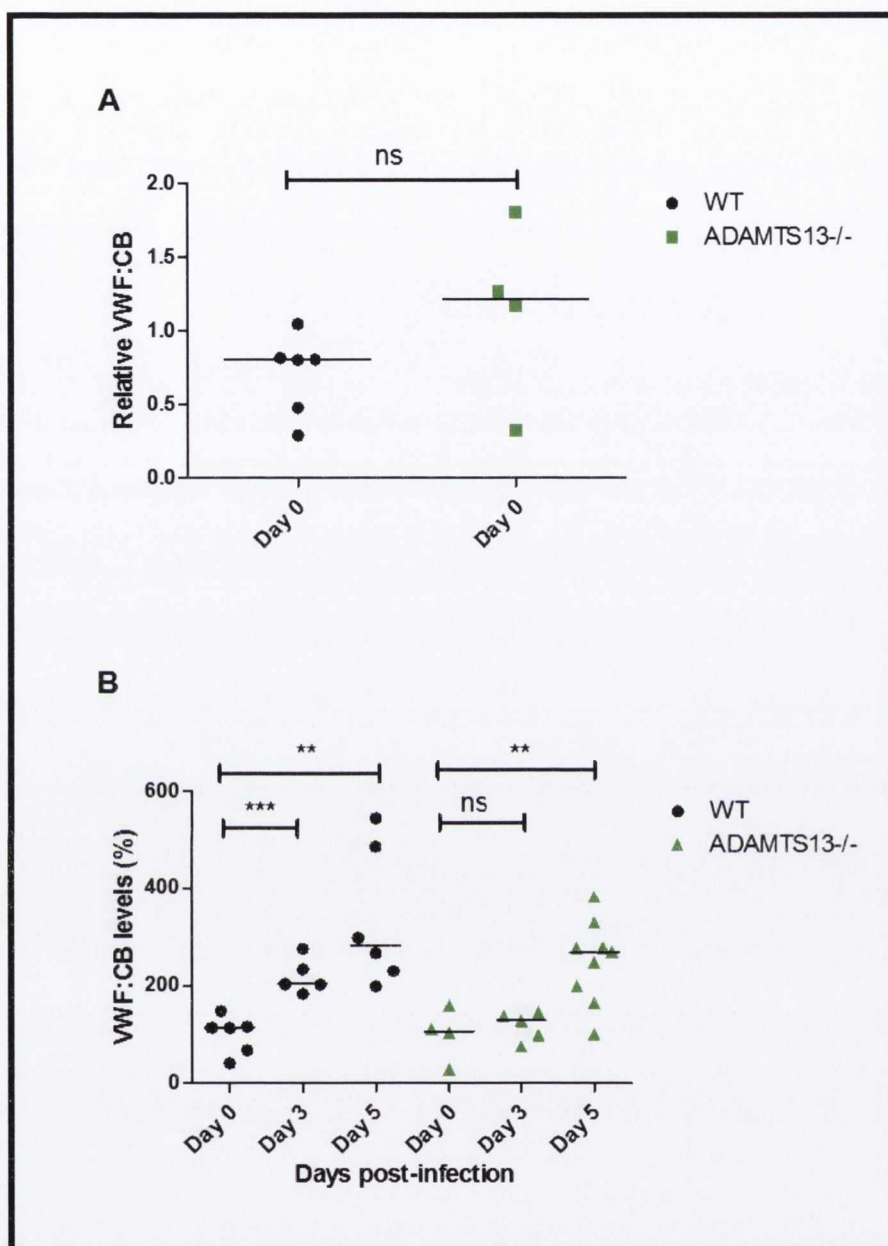
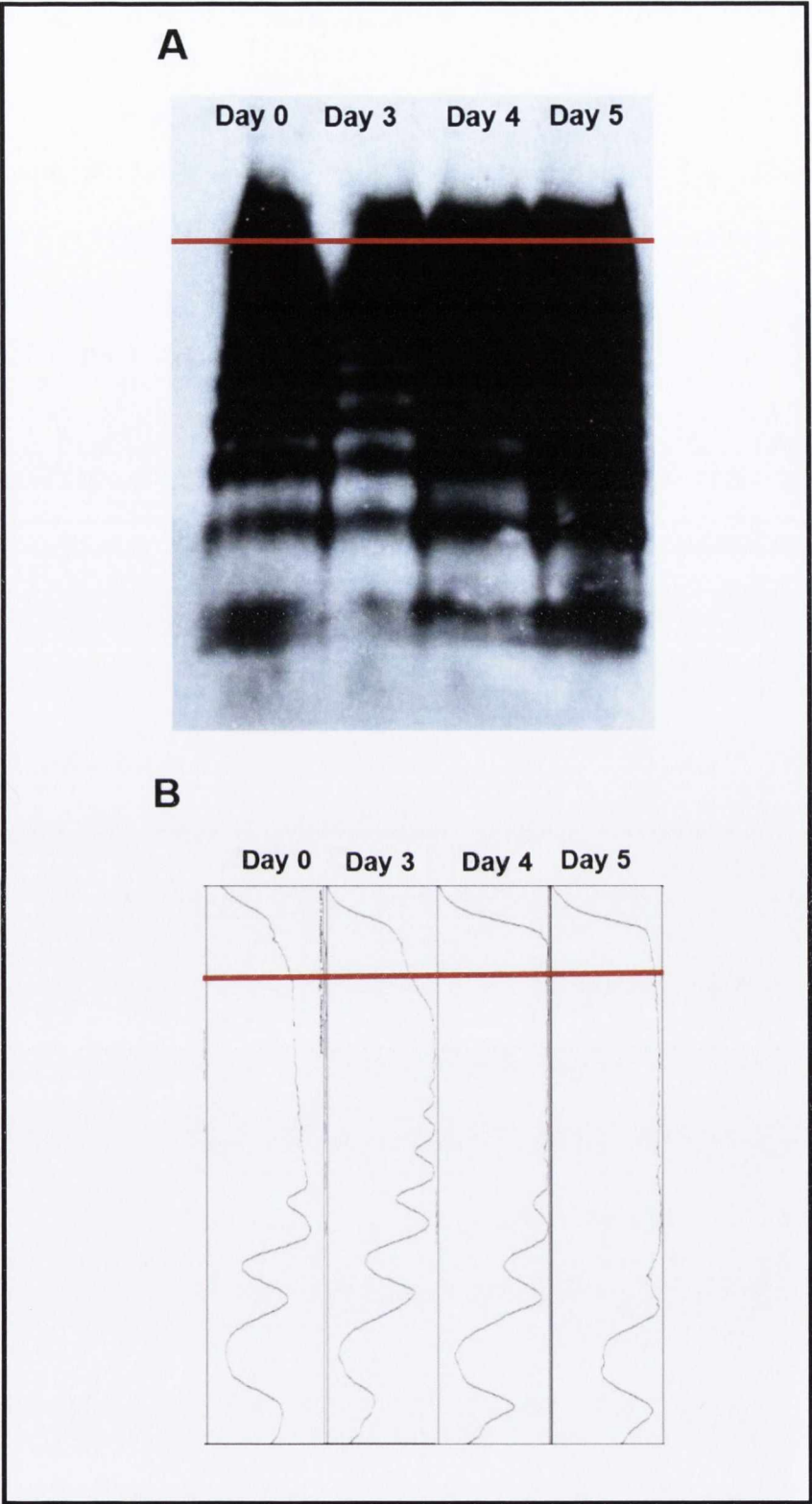


Figure 4. 8 (A) Baseline VWF:CB in WT versus ADAMTS13 deficient mice (B) VWF:CB activity in WT and ADAMTS13^{-/-} mice post inoculation with *P. berghei*

4.8 VWF multimer distribution not significantly altered in ADAMTS13^{-/-} mice

In the absence of inflammation, ULVWF multimers released from WPB are swiftly cleaved on EC surfaces by ADAMTS13. ULVWF multimers have significantly enhanced functional activity. Pathological ULVWF multimers have also been observed in the plasma of children with CM (Larkin *et al*, 2009). Since ADAMTS13 deficient mice do not have ADAMTS13 to regulate ULVWF, we investigated VWF multimer composition in ADAMTS13 mice following *P. berghei* infection. In contrast to WT mice (section 3.5.5), no significant shift in VWF multimer distribution was seen (Figure 4.9).



4.9 Thrombocytopenia is a consistent feature of ECM in ADAMTS13^{-/-} and WT mice

Thrombocytopenia is a common feature in patients with *P. falciparum* malaria (Cox & McConkey, 2010; Faille *et al*, 2009). This thrombocytopenia is predominantly due to increased platelet clearance. Daily platelet counts were performed in WT and ADAMTS13^{-/-} mice following inoculation with *P. berghei*. Significant thrombocytopenia was a consistent feature in both the WT and ADAMTS13^{-/-} mice. By Day +4 after *P. berghei* infection mean platelet count had fallen by approximately 70% in both strains (Figure 4.10) (n=3-7).

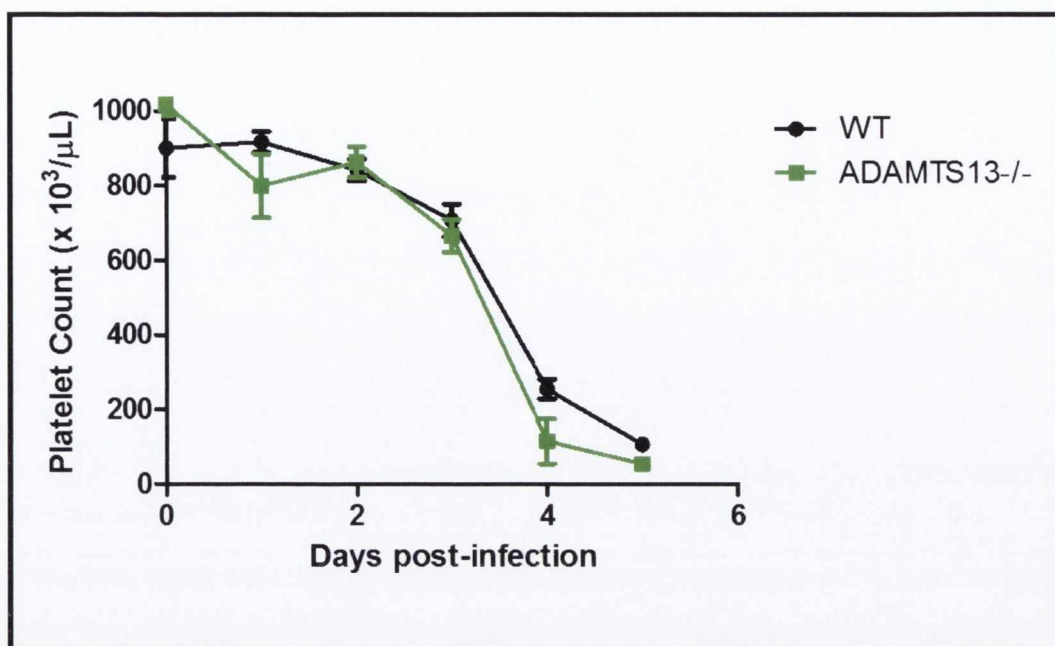


Figure 4. 10 Platelet count in WT and ADAMTS13^{-/-} mice following *P. berghei* infection

4.10 *P. berghei* does not precipitate acute TTP in ADAMTS13^{-/-} mice

Daily peripheral blood films were examined in ADAMTS13^{-/-} and WT mice following *P. berghei* inoculation to examine for signs of TTP. Typical Giemsa-stained peripheral blood films from a WT C57BL/6J mouse at Day +5 are shown in Figure 4.11A. Schistocytes (red cell fragments) and platelet clumping are common features of MAHA. No significant features of microangiopathic haemolytic anaemia (MAHA) were observed. Although, specific types of infection and inflammation have been shown to initiate TTP in ADAMTS13^{-/-} mice (Banno *et al*, 2006; Motto *et al*, 2005). *P. berghei* infection did not trigger TTP. (Figure 4.11B).

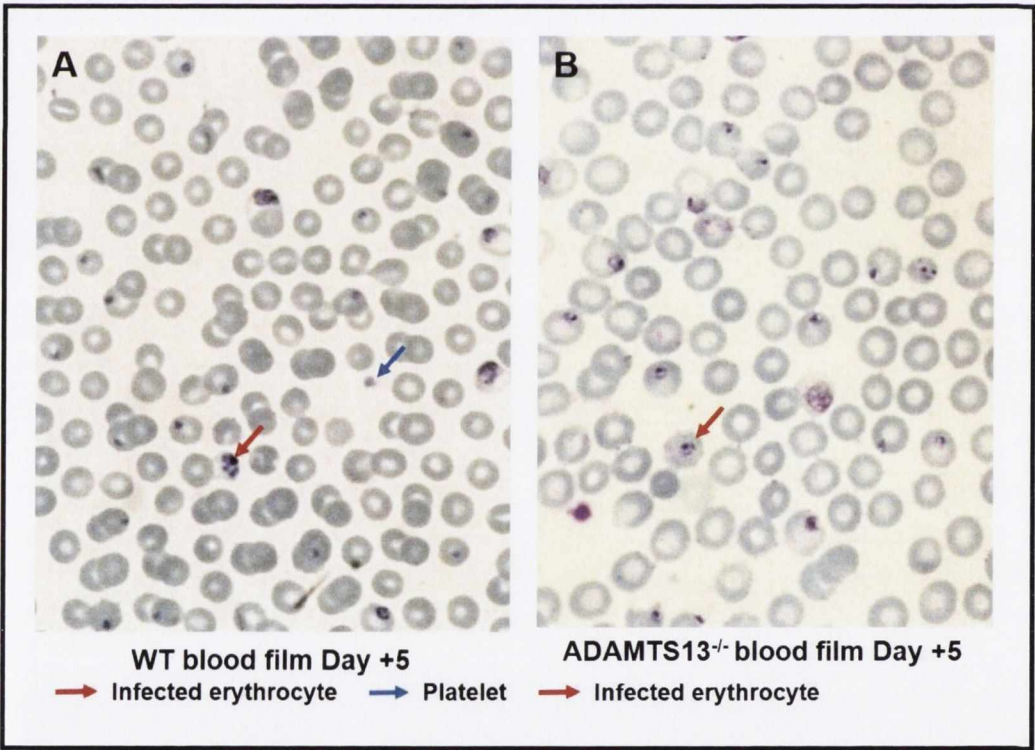


Figure 4. 11 (A) WT blood film at Day +5 post inoculation (B) ADAMTS13^{-/-} blood film Day +5 after infection

4.11 Asialoglycoprotein receptor

The first described VWF clearance receptor was the hepatic lectin asialoglycoprotein clearance receptor (ASGPR). ASGPR exists as a hetero-oligomeric receptor composed of two homologous transmembrane polypeptides (Spiess & Lodish, 1985) and is abundantly expressed on hepatocytes (Steer & Ashwell, 1980). Glycoproteins which are endocytosed, dissociate from the ASGPR intracellularly in a pH-dependent manner, and are then targeted for lysosomal degradation (Bridges *et al*, 1982). A high degree of ASGPR conservation suggests a critical role for this lectin in the clearance of abnormally glycosylated proteins. However to date only two endogenous ligands (VWF and platelets) have been described for ASGPR (Grewal *et al*, 2008). ASGPR was shown to bind and remove VWF from the circulation and thereby regulate normal VWF homeostasis. Mice with genetic inactivation of the ASGPR-1 subunit have a 1.5-fold increase in circulating VWF plasma levels (Grewal *et al*, 2008). However, increased plasma VWF levels were not associated with elevated levels of asialo-VWF in the circulation suggesting that the ASGPR also modulates clearance of normal sialylated VWF. Enzymatic removal of terminal sialic acid residues from VWF glycans markedly reduced plasma half-life when administered in rabbits (Sodetz *et al*, 1977). In keeping with the exposure of sub-terminal galactose sugars, the rapid removal of asialo-VWF was mediated *via* hepatic lectin receptor ASGPR. The importance of VWF sialylation in regulating plasma clearance is further demonstrated by the RIIS/J inbred mouse strain, where aberrant glycosylation of VWF results in enhanced clearance (Mohlke *et al*, 1999).

After observing VWF^{-/-} mice are clinically protected from *P. berghei* infection we sought to investigate whether higher endogenous VWF levels in mice might influence ECM. As mentioned, mice deficient in the ASGPR-1 subunit have increased VWF plasma levels (Grewal *et al*, 2008). Thus we proceeded to study *P. berghei* infection in ASGPR^{-/-} mice.

4.12 Clinical progression of ECM infection in ASGPR^{-/-} mice

ASGPR^{-/-} mice have previously been characterized (Braun *et al*, 1996; Grewal *et al*, 2008). Despite the fact that low levels of VWF were protective against ECM, we observed no significant difference in *P. berghei* malaria clinical progression in ASGPR^{-/-} mice with elevated plasma VWF levels compared to WT mice during the course of infection (Figure 4.12) (WT n=8, ASGPR^{-/-} n=8).

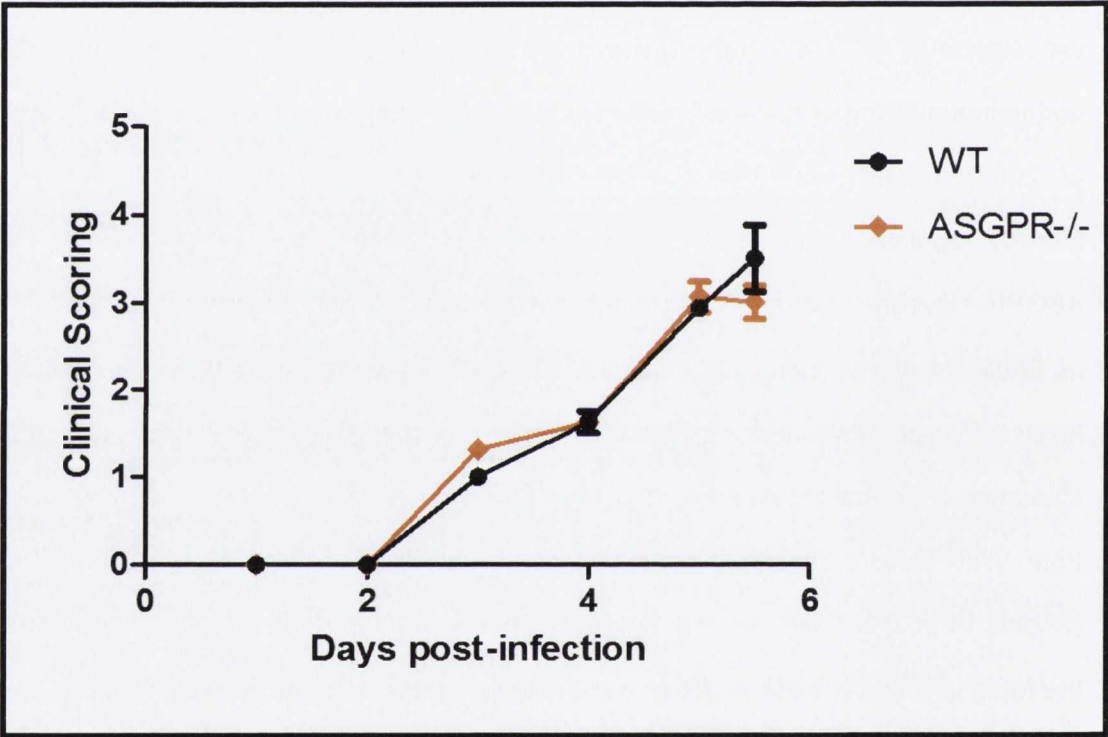


Figure 4. 12 Clinical progression following *P. berghei* infection in ASGPR^{-/-} mice compared to WT

4.13 Overall survival following *P. berghei* inoculation in ASGPR^{-/-} compared to WT mice

To elucidate a potential role for higher VWF levels in experimental CM, overall survival in ASGPR^{-/-} and WT mice infected with 2×10^6 *P. berghei* parasites was studied. Similar to ADAMTS13^{-/-} and in contrast to our findings in the VWF^{-/-} mice, no significant difference in overall survival was observed in ASGPR^{-/-} mice compared to WT mice (Figure 4.13, P= 0.27) (WT n=8, ASGPR^{-/-} n=8).

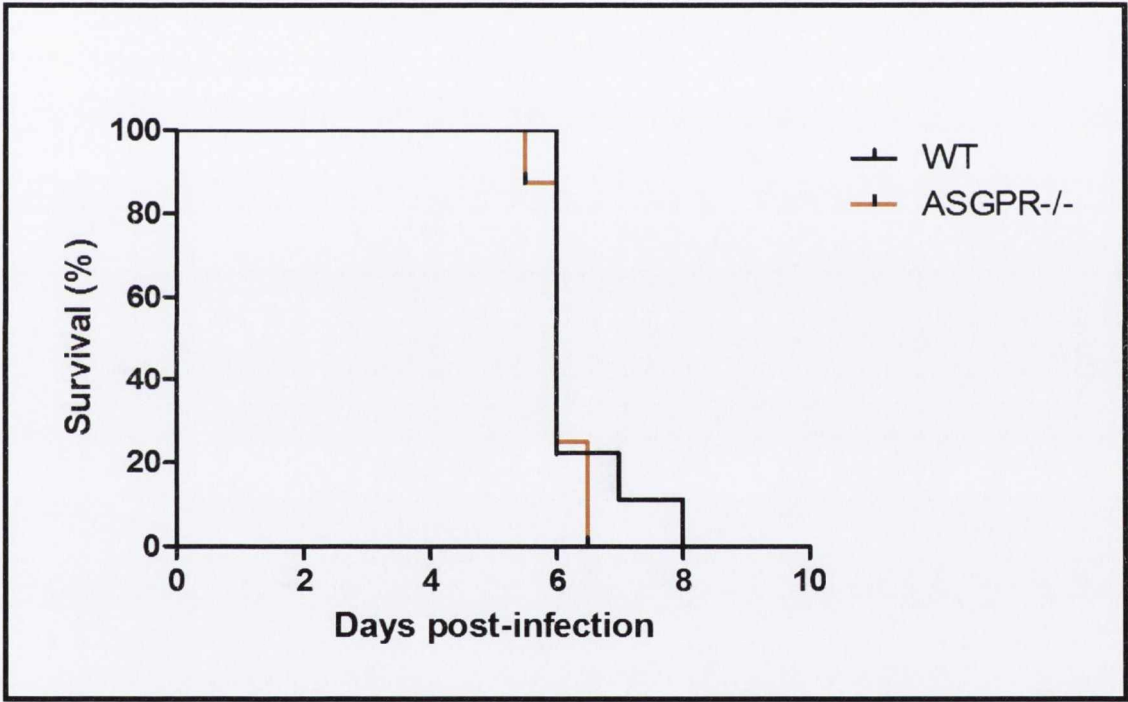


Figure 4. 13 Overall survival following *P. berghei* inoculation in ASGPR^{-/-} mice compared to WT

4.14 Weight and parasitemia in ASGPR^{-/-} compared to WT mice

Similar to other strains investigated in these studies, each mouse was monitored daily for weight loss as well as general distress as per ethical guidelines. There were no discrepancies in weight loss between ASGPR^{-/-} and WT mice (Figure 4.14A). Peripheral blood parasitemia in ASGPR^{-/-} and WT mice were assessed by standard Giemsa staining. We observed no significant differences in blood *P. berghei* parasitemia levels between ASGPR^{-/-} mice and WT mice during the course of infection (Figure 4.14B) (WT n=8, ASGPR^{-/-} n=8).

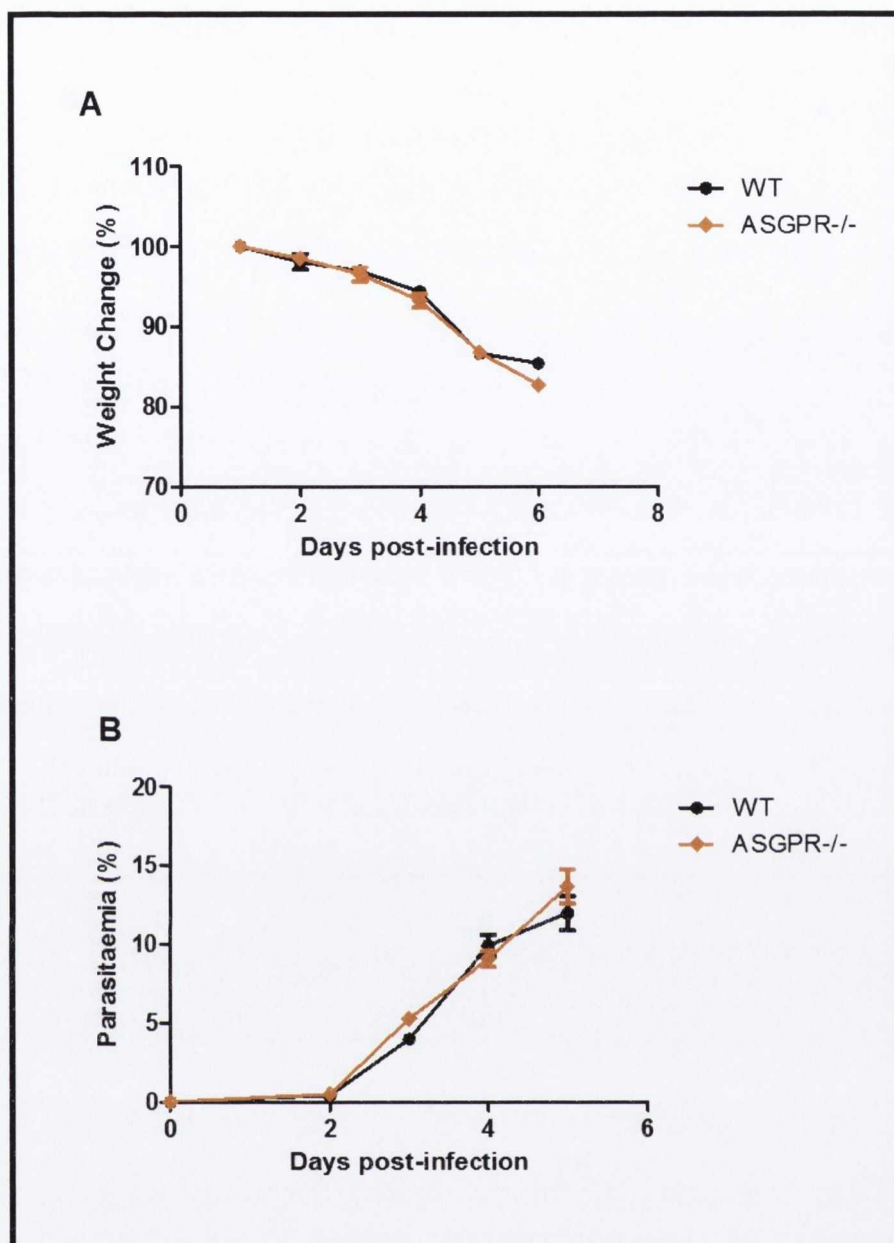


Figure 4. 14 (A) Weight loss and (B) Peripheral parasitemia in ASGPR^{-/-} compared to WT mice following *P. berghei* infection

4.15 Plasma VWF:Ag levels in ASGPR^{-/-} mice following *P. berghei* infection

Initial studies demonstrated that ASGPR^{-/-} mice had higher endogenous VWF plasma levels compared with WT C57BL/6J mice. Baseline VWF plasma levels were 1.6 fold higher in ASGPR^{-/-} mice than WT mice (0.67µg/mL versus 0.4µg/mL respectively) (Figure 4.15A) (n=6-8). This is consistent with previous reports of ASGPR^{-/-} mice having elevated plasma VWF levels due to increased VWF half-life (Grewal et al. 2008). Following inoculation with *P. berghei*, plasma VWF:Ag levels in ASGPR^{-/-} mice were markedly increased at Day +5 post infection ($P \leq 0.01$) (n=6-10). This increase is similar to that observed in WT mice. Although ASGPR-deficient mice have higher endogenous VWF levels and do get elevated plasma VWF levels after inoculation with parasites, the degree of VWF increase is in keeping with WT.

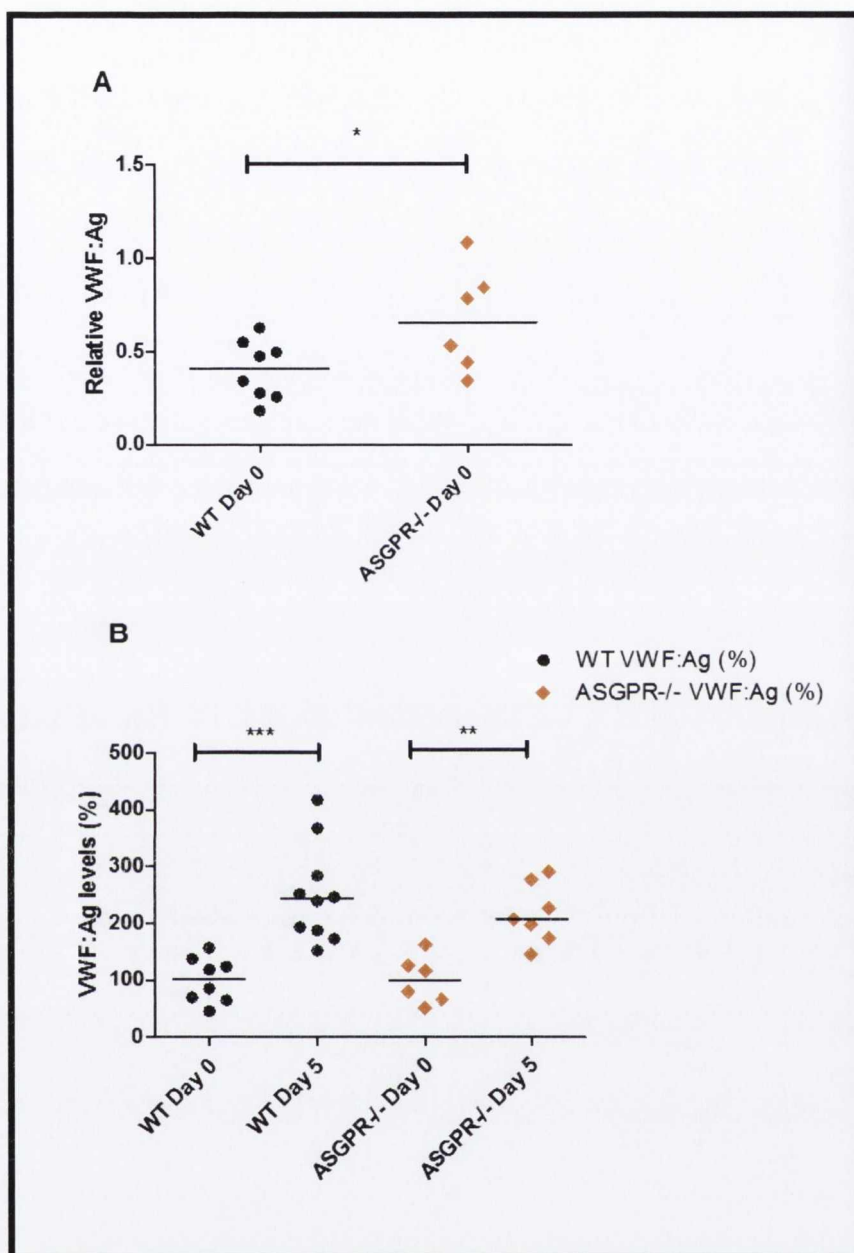


Figure 4. 15 (A) Basal plasma VWF:Ag levels in ASGPR^{-/-} compared to WT mice
 (B) Normalised plasma VWF:Ag levels in ASGPR^{-/-} compared to WT mice
 following *P. berghei* infection

4.16 Thrombocytopenia is more pronounced in ASGPR^{-/-} mice compared to WT

Thrombocytopenia is a hallmark of malaria infection. The ASGPR receptor has been shown to play a role in platelet clearance (Grewal *et al*, 2008). We therefore sought to investigate platelet counts in ASGPR^{-/-} after *P. berghei* infection. Interestingly, we observed a slightly increased degree of thrombocytopenia in ASGPR^{-/-} mice compared to WT in our studies at Day +3 of infection ($P \leq 0.05$) (n=3-8). Although ASGPR was not present in this strain of mice, thrombocytopenia was still a feature of infection and in fact more pronounced at Day +3 of infection when compared to WT mice.

Normal Platelet Counts	Day +3 WT	Day +3 ASGPR ^{-/-}
~1000 x10 ³ /μl	~707 x10 ³ /μl	~535 x10 ³ /μl

Table 4.1 Thrombocytopenia in WT versus ASGPR^{-/-} mice

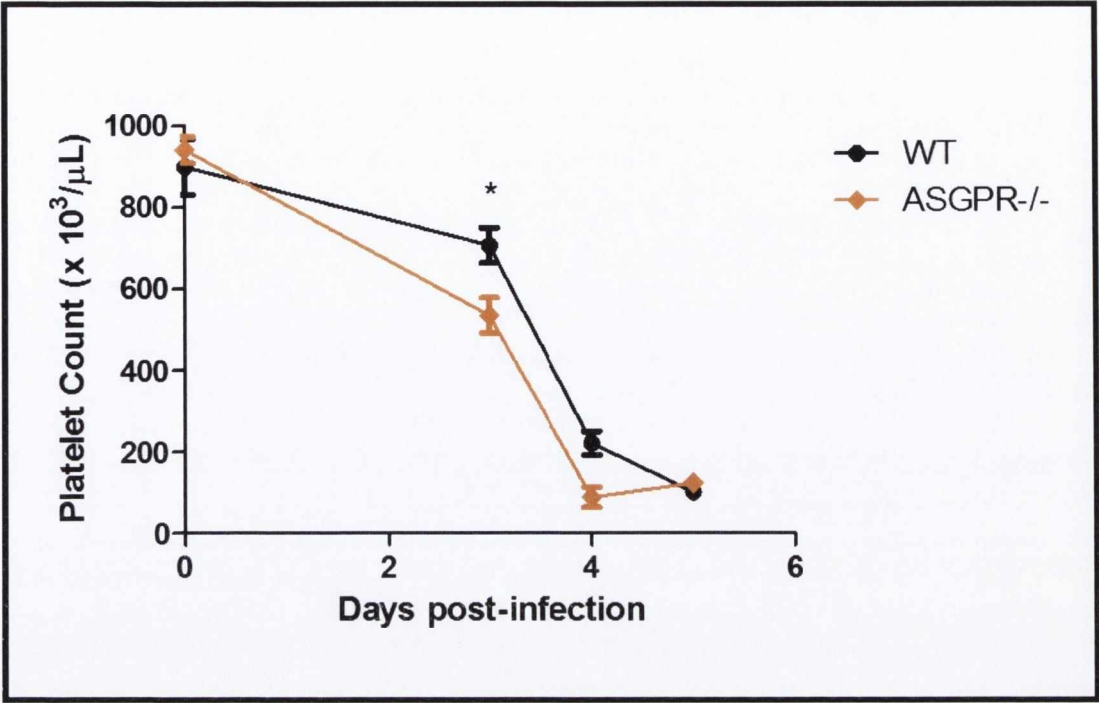


Figure 4. 16 Platelet counts in ASGPR^{-/-} compared to WT mice following *P. berghei* infection

4.17 Discussion

As discussed in Chapter 3, human and murine data suggest that elevated plasma VWF levels and/or the presence of ULVWF multimers may be important in the pathogenesis of CM in both children and mice. Our data suggest that this VWF effect may be modulated at least in part through alteration of the BBB. In this chapter, we sought to further understand the role of VWF in malaria biology using two previously established genetically modified murine models.

4.17.1 ADAMTS13 deficient mice

Pathological ULVWF multimers are a consistent feature of both human and murine CM. In normal plasma, accumulation of such ULVWF multimers is prevented by ADAMTS13 (Levy *et al*, 2005; Sadler, 2008). The explanation for the presence of these pathological multimers in association with CM remains unclear. Mild reductions in plasma ADAMTS13 antigen and activity levels have been reported in children with CM (Larkin *et al*, 2009; de Mast *et al*, 2009; Löwenberg *et al*, 2010). However absolute plasma ADAMTS13 activity levels have been reported to remain above 30% of normal. This residual ADAMTS13 in plasma should be entirely adequate to regulate plasma VWF multimer distribution (Dong *et al*, 2004). Consequently, previous studies have suggested that normal ADAMTS13 activity may also be specifically inhibited in malaria. Using ADAMTS13^{-/-} mice, we have further investigated ECM pathogenesis. Interestingly, although other types of infection have precipitated acute TTP in ADAMTS13^{-/-} mice, we observed no features of acute TTP following *P. berghei* inoculation. The overall increase in plasma VWF levels following *P. berghei* was similar to that observed in WT mice. In addition, the presence of ULVWF multimers was also observed in ADAMTS13^{-/-} mice. Consistent with these findings, the clinical progression and overall survival in ADAMTS13^{-/-} mice following *P. berghei* was not significantly different to that of WT mice.

Collectively, these findings suggest that the increase in VWF, and the appearance of ULVWF multimers is an ADAMTS13-independent phenomenon. Furthermore, the observation that *P. berghei* related thrombocytopenia is similar in both ADAMTS13^{-/-} and WT mice suggests that ULVWF does not contribute to the aetiology of the reduced platelet count. In summary therefore, although ADAMTS13 is critical in regulating VWF multimer distribution, and also important in modulating inflammatory responses, our data suggest that ADAMTS13 is not important in the pathogenesis underlying ECM.

4.17.2 ASGPR^{-/-} mice

ASGPR has previously been shown to mediate normal homeostatic clearance of VWF in a glycan dependent manner (Grewal *et al*, 2008). Mice deficient in ASGPR-1 have a 1.5-fold increase in circulating VWF plasma levels (Grewal *et al*, 2008). Upon investigation, we observed that ASGPR^{-/-} mice developed signs and symptoms of ECM similar to WT mice. In addition, we found no difference in survival between the two strains. Following infection with *P. berghei* ASGPR^{-/-} mice do have significantly increased VWF:Ag levels at Day +5 post inoculation. Although basal plasma levels were slightly elevated in ASGPR^{-/-} mice, the overall increase in plasma VWF levels following *P. berghei* was similar to that observed in WT mice.

Previously, platelets were examined during *P. berghei* infection in rats. Total sialic acid expression was reduced and was associated with reduced platelet survival and recovery (Inyang *et al*, 1995). Surprisingly, we observed a slightly increased thrombocytopenia in ASGPR^{-/-} mice compared to WT in our studies at Day +3 of infection. Furthermore, in contrast to previous reports that ASGPR-deficient mice infected with *S. pneumonia* (Grewal *et al*, 2008) demonstrated increased mortality, ASGPR^{-/-} mice were not more susceptible to *P. berghei* infection. Our data suggests

that other receptors are responsible for the thrombocytopenia observed in this strain of mice.

5. Marked elevation in plasma osteoprotegerin (OPG) constitutes an early and consistent feature of cerebral malaria

5.1 Introduction

P. falciparum malaria continues to be associated with significant morbidity and mortality (Lopez *et al*, 2006; Dondorp *et al*, 2008). Although the molecular mechanisms underlying the pathogenesis of severe *P. falciparum* remain poorly understood, previous studies have demonstrated a critical role for EC activation (Newton & Krishna, 1998; Turner *et al*, 1998). In particular, a number of specific EC surface receptors, including CD36, EPCR, E-selectin and ICAM-1, have been shown to regulate the adhesion and sequestration of *P. falciparum*-IE within the microvasculature (Craig & Scherf, 2001). Quantitative expression of these different EC surface receptors on EC surfaces is modified by inflammatory cytokines, such as interleukin-1 and TNF respectively (Grau *et al*, 1989). In addition to these IE surface receptors, EC also contain intracellular storage organelles known as WPB (Lenting PJ, Christophe OD, 2015). These WPB are rod shaped organelles that can be up to 5µm in length and are specific to EC. VWF synthesis within EC is a prerequisite for WPB formation (Nightingale & Cutler, 2013; Valentijn *et al*, 2011). In normal plasma, VWF circulates as a series of heterogeneous multimers. In contrast, VWF stored within WPB is enriched in HMWM that demonstrate enhanced binding affinities for both collagen and platelets (Lenting PJ, Christophe OD, 2015; Valentijn *et al*, 2011). In addition, a number of other EC proteins have also been identified as WPB constituents. These include VWF:pp, P-selectin, factor VIII, Ang-2, tissue-type plasminogen activator (tPA), interleukin-8, galectin-3 and osteoprotegerin (OPG) (Lenting PJ, Christophe OD, 2015; Valentijn *et al*, 2011). Following acute EC activation, WPB fuse with the EC surface membrane, and their contents are secreted directly into the plasma.

Previous studies from our laboratory and others have demonstrated that plasma levels of HMWM VWF and VWF:pp are both markedly elevated in patients with severe *P. falciparum* malaria (Conroy *et al*, 2010; de Mast *et al*, 2009; Erdman *et al*, 2011; Hollestelle *et al*, 2006; Larkin *et al*, 2009; Löwenberg *et al*, 2010; Phiri *et al*, 2011). Furthermore, plasma VWF:Ag and VWF:pp levels have also been shown to correlate with other biochemical prognostic markers in patients with severe malaria (Hollestelle *et al*, 2006; de Mast *et al*, 2010). Interestingly, in a study of healthy volunteers infected with *P. falciparum*, de Mast *et al* observed that the increase in plasma VWF:Ag and VWF:pp levels was evident from a very early stage following *P. falciparum* infection (de Mast *et al*, 2007). The molecular mechanism(s) underlying this early marked EC activation and WPB secretion have not been defined. Nevertheless, emerging data suggest that the early release of WPB contents, and in particular VWF, may play a role in malaria pathogenesis. We recently reported that platelet-decorated HMWM VWF strings on the surface of activated EC can directly adhere to trophozoite-stage *P. falciparum* IE in a shear-based assay (Bridges *et al*, 2010). Thus, the rapid release of HMWM VWF multimers from WPB which occurs at an early stage in the clinical course of *P. falciparum* infection may provide a novel mechanism through which IE can adhere to activated EC surfaces.

In keeping with the marked elevated VWF and VWF:pp levels observed in children with CM, plasma levels of Ang-2, another protein stored within WPB, are also significantly elevated (Conroy *et al*, 2010; Erdman *et al*, 2011; Conroy *et al*, 2009; Lovegrove *et al*, 2009). Moreover, higher plasma Ang-2 levels have also been associated with a worse clinical outcome (Conroy *et al*, 2009; Lovegrove *et al*, 2009; Yeo *et al*, 2008). OPG is another EC protein stored within WPB (Zannettino *et al*, 2005) and is a soluble member of the tumour necrosis factor receptor family that plays a critical role in bone remodelling (Takahashi *et al*, 1999; Yasuda *et al*, 1999). Full length OPG is composed of 401 amino acids and is synthesised as a monomer (60kDa) and assembled as a dimer (120kDa) within the cell (Yamaguchi *et al*, 1998; Simonet *et al*,

1997). The N-terminal region of OPG is cysteine-rich and is important for dimerization of the protein and osteoclastogenesis. The C-terminal contains death domains and a heparin binding domain (Yamaguchi *et al*, 1998).

OPG is expressed in heart, lung, liver, kidney and bone marrow stromal cells and dendritic cells amongst others (Simonet *et al*, 1997; Yun *et al*, 1998) and is produced by vascular EC and smooth muscle cells (Hofbauer *et al*, 2001). Additional ligands which have been defined for OPG include VWF and FVIII-VWF complex (Baud'huin *et al*, 2013). The recruitment of OPG into WPB has been shown to be result through the ability of OPG to bind directly to the A1 domain of VWF (Zannettino *et al*, 2005; Shahbazi *et al*, 2007) and OPG is also co-localised with VWF in platelet α -granules (Chollet *et al*, 2010). OPG binds to the A1 domain of VWF intracellularly (Figure 5.1) and can remain associated after release into circulation (Shahbazi *et al*, 2007). The presence of FVIII-VWF complex has been shown to enhance OPG binding to Receptor Activator of NF κ B Ligand (RANKL) (Baud'huin *et al*, 2009; Shahbazi *et al*, 2007).

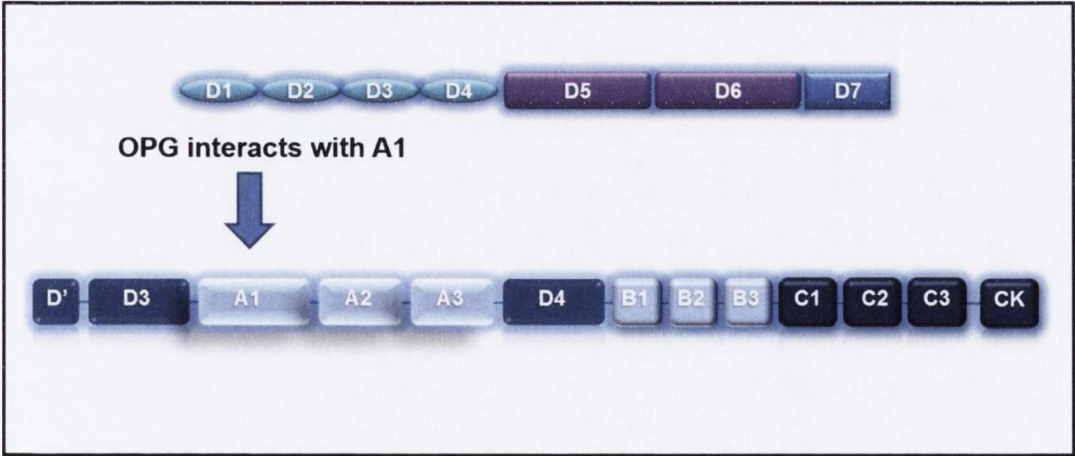


Figure 5. 1 OPG interacts with the A1 domain on VWF

OPG plays a key role in regulating bone remodelling by acting as a soluble decoy receptor for RANKL expressed on osteoblasts (Lacey *et al*, 1998). However, recent studies have also identified additional functional roles for OPG in modulating vascular biology (Baud'huin *et al*, 2013; Caidahl *et al*, 2010). In particular, OPG has been shown to upregulate expression of adhesion molecules on EC surfaces, and thereby facilitate leucocyte adhesion (Mangan *et al*, 2007; Zauli *et al*, 2007). Furthermore, OPG also influences EC apoptosis, and elevated OPG levels have been associated with vascular disease (Baud'huin *et al*, 2013; Caidahl *et al*, 2010). Recently, Djamiatun *et al* demonstrated that severe dengue fever is associated with exocytosis of WPB with markedly elevated VWF:Ag, VWF:pp and OPG plasma levels (Djamiatun *et al*, 2012). Dengue fever shares many of the key clinical features seen in malaria including EC activation and a pronounced thrombocytopenia. Given the key role of EC activation in the pathogenesis of severe *P. falciparum* malaria, we have investigated plasma OPG levels in children with cerebral malaria. To further understand the relationship between WPB secretion and malaria, we have further investigated OPG levels in an established murine model of experimental CM.

5.2 Patient demographics of samples from Malawi

Recruitment took place at the Queen Elizabeth Central Hospital, Blantyre, Malawi between 2010 and 2011 by Dr. Christopher Moxon. A total of 146 paediatric patients with severe *P. falciparum* malaria with cerebral symptoms, 52 with uncomplicated malarial infection, 48 paediatric patients with non-malarial febrile illness and 26 healthy controls were enrolled in this study. Clinical details were recorded for all patients at the time of recruitment. Baseline clinical characteristics are shown in Table 5.1. Healthy controls were well children admitted for elective surgery. Non-malarial febrile illness (NMFI) samples included children not requiring admission, who had a documented febrile illness ($>37.5^{\circ}\text{C}$) with no malaria parasites on a single thick blood film, a negative rapid diagnostic test for malarial antigens, and no history of having taken any antimalarial medication in the previous two weeks. Uncomplicated malaria (UM) cases were recruited from children with acute febrile illness without signs of organ compromise (Marsh *et al*, 1995). Children in this cohort with cerebral malaria (CM) had a characteristic febrile phenotype, thrombocytopenia and peripheral *P. falciparum* parasitemia. These recruited patients had an initial clinical diagnosis of CM, defined as a Blantyre Coma Score of 2 or less (Molyneux *et al*, 1989), no other recognisable cause of coma (Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster., 2000). All patients with malaria received standard supportive and anti-malarial treatment.

	Healthy	NMFI	UM	CM
Age (Years)	5.7 ± 0.4	4.1 ± 0.2	5.5 ± 0.3	4.2 ± 0.1
Sex, female (%)	10 (38%)	27 (52%)	22 (49%)	64 (47%)
Mortality (%)	n.a.	n.a.	n.a.	20 (15%)
Temperature (°C)	36.6 ± 0.1	38.4 ± 0.1	38.5 ± 0.1	38.6 ± 0.1
Parasitemia (x10 ³ /μl)	n.d	n.d	117 ± 30	200 ± 25
Haemoglobin (g/dl)	9.9 ± 1.27	10.6 ± 0.2	8.8 ± 0.3	7.1 ± 0.2
Platelets (x10 ³ /μl)	413.7 ± 102.9	332.5 ± 20.0	140.8 ± 12.8	102.8 ± 6.3
Lactate (mmol/l)	2.0 ± 0.1	1.8 ± 0.1	2.5 ± 0.1	6.9 ± 0.3
VWF:Ag (μg/ml)	8.2 ± 1.0	24.7 ± 1.6	15.4 ± 1.4	29.7 ± 1.2

Table 5. 1 Patient demographics

n.a.= not applicable, n.d.= not detected

5.3 Plasma VWF:Ag are markedly elevated in children with CM

At presentation, plasma VWF:Ag levels were significantly elevated in Malawian children with CM compared to healthy control children (means 29.7 versus 8.3µg/mL; $P\leq0.001$) (Figure 5.2). This ~3.6 fold increase is similar in magnitude to that previously reported in other cohorts of paediatric and adult patients with severe *P. falciparum* infection (de Mast *et al*, 2009; Hollestelle *et al*, 2006; Larkin *et al*, 2009; Löwenberg *et al*, 2010). Plasma VWF:Ag levels were also mildly increased in children with UM. However the elevation in plasma VWF:Ag observed in children with UM was similar to that observed in children with other NMFI.

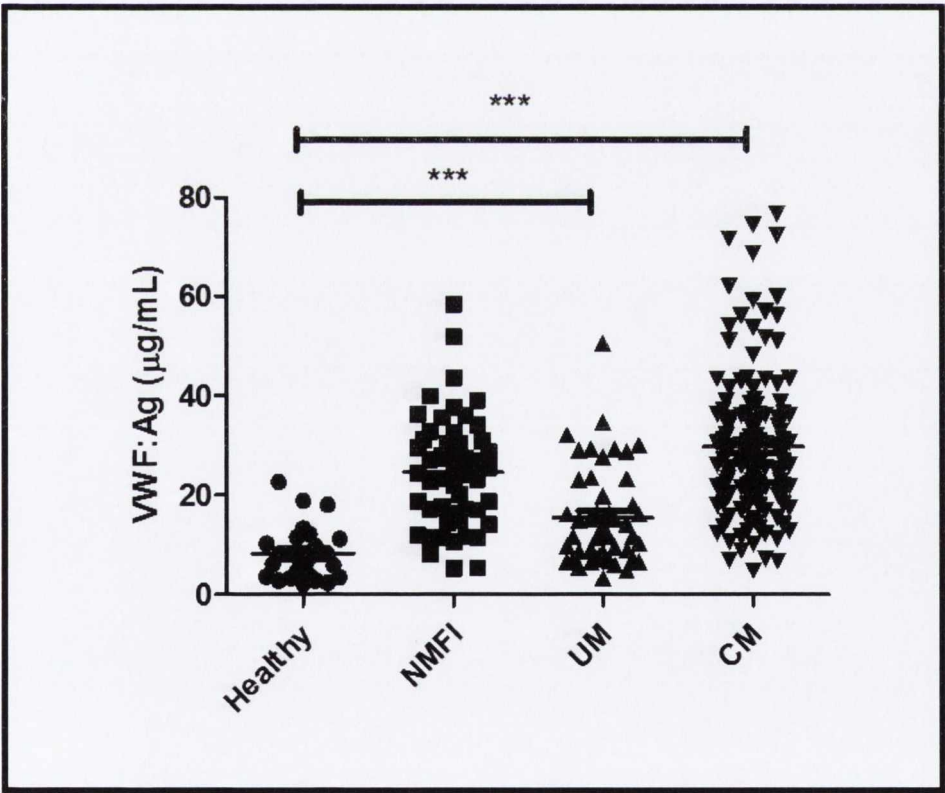


Figure 5. 2 VWF:Ag is markedly increased in children with *P. falciparum* infection

5.4 Plasma OPG levels are significantly increased in children with CM

Plasma OPG levels were also markedly elevated in children with CM compared to healthy controls (means 16.0 versus 0.79ng/mL; $P \leq 0.01$) (Figure 5.3). However in contrast to the 3.6 fold increase observed in VWF:Ag levels, plasma OPG levels were increased more than 20 fold. Plasma OPG levels were also significantly elevated in children with UM compared to healthy controls ($P \leq 0.05$). Although plasma VWF:Ag levels were significantly elevated in children with NMFI, plasma OPG levels were not significantly increased in these children. Thus, the marked increase in OPG seems to constitute a relatively malaria-sensitive phenomenon. Collectively, these findings support the hypothesis that acute EC activation and WPB secretion constitute characteristic features of severe *P. falciparum* malaria. In addition, these data further demonstrate for the first time that plasma OPG levels are markedly elevated in children with *P. falciparum*.

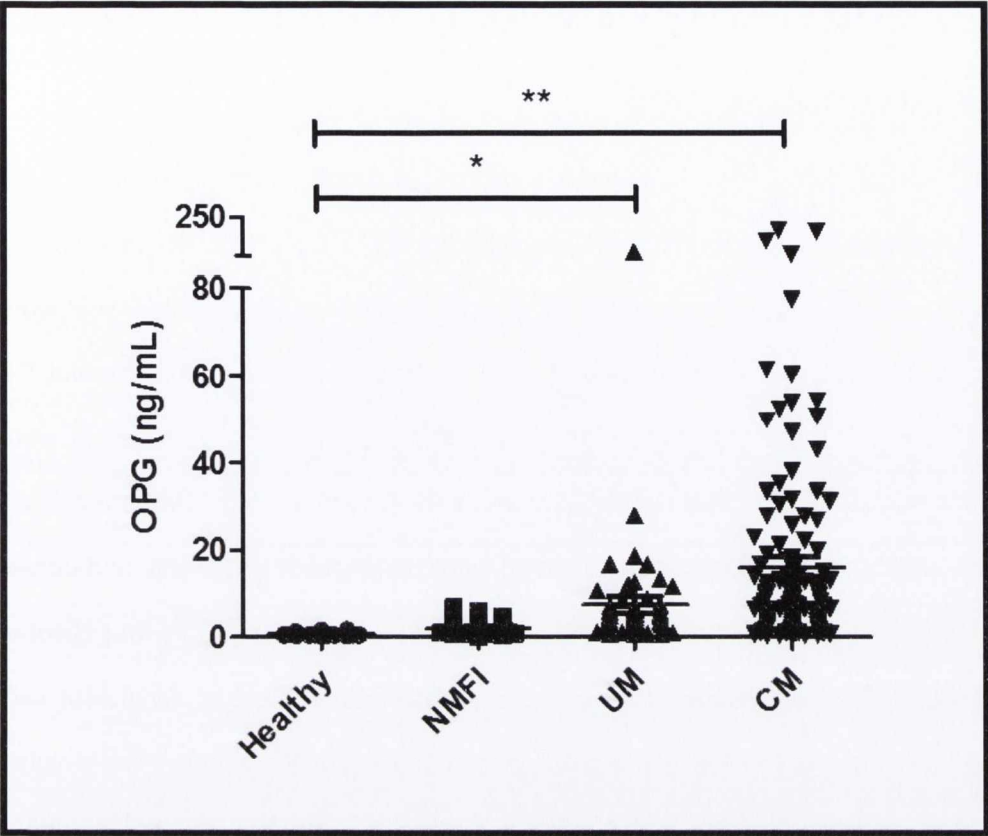


Figure 5. 3 OPG levels are markedly increased in children with cerebral malaria

5.5 Plasma lactate levels are markedly elevated in children with CM and correlate with OPG

In patients with severe *P. falciparum* malaria, it is well established that plasma lactate levels constitute a useful biochemical prognostic indicator (White *et al*, 1985; Molyneux *et al*, 1989; van Genderen *et al*). Unsurprisingly, plasma lactate levels were significantly elevated in children with CM compared to healthy controls (means 6.9 versus 2.0mmol/L; $P \leq 0.001$) (Figure 5.4A). In addition, plasma lactate levels were significantly higher in children with CM compared to those with UM respectively ($P \leq 0.001$). Among the 146 children with confirmed CM, plasma OPG levels and lactate levels were significantly correlated ($r=0.42$, $P \leq 0.001$) (Figure 5.4B). Eighty four children with CM demonstrated plasma lactate levels greater than 5mmol/L consistent with lactic acidosis. Plasma OPG levels in children with CM and lactic acidosis were higher compared to levels in children with plasma lactate levels less than 5mmol/L (means 19.6 versus 10.2; $P=0.06$).

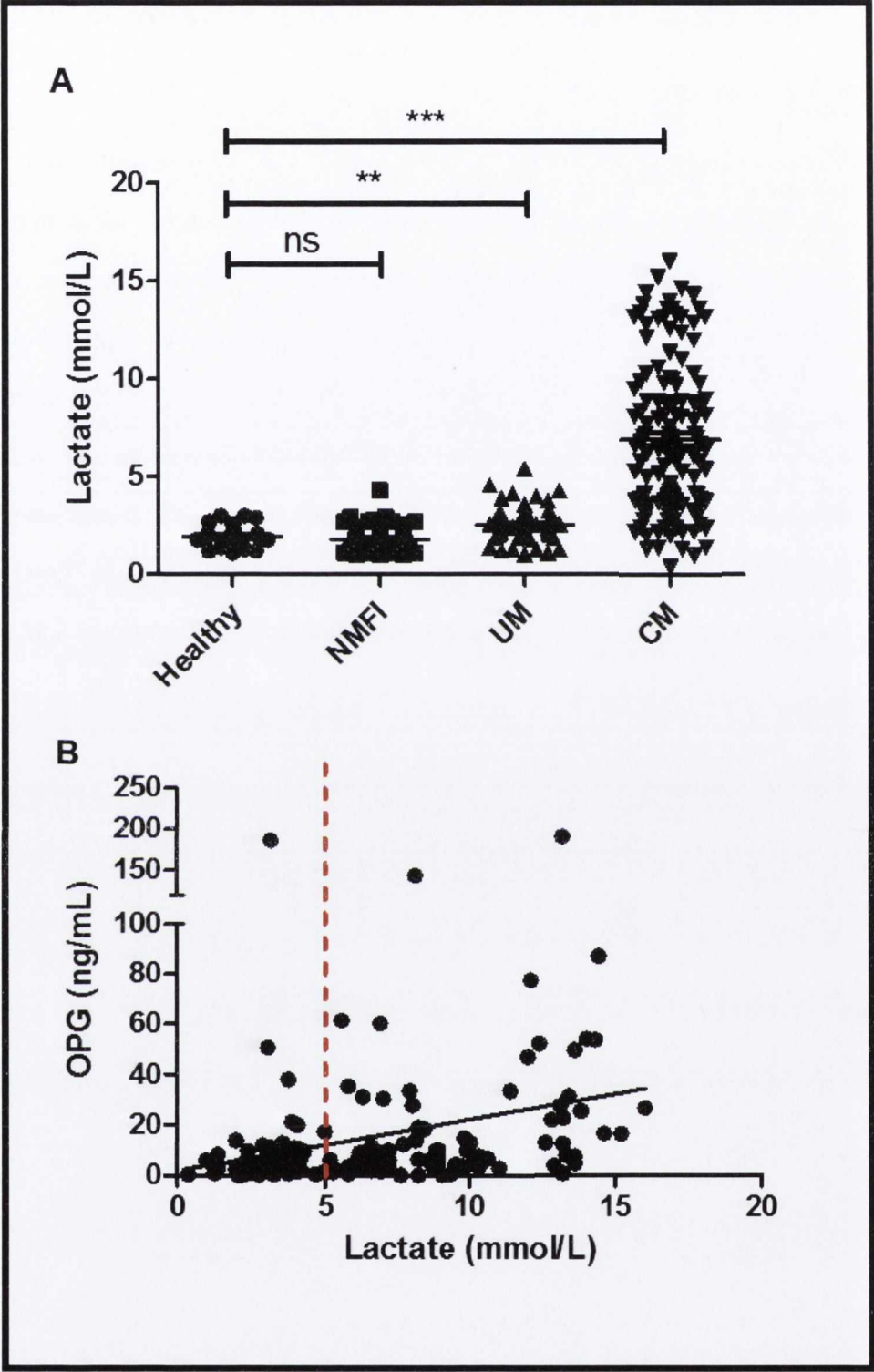


Figure 5. 4 (A) Highest lactate levels are seen in children with cerebral malaria
(B) Plasma OPG levels and lactate levels were significantly correlated in CM
(red line indicates plasma lactate levels consistent with lactic acidosis)

5.6 Plasma OPG levels relate to malarial severity in children with *P. falciparum* malaria

Parasite counts are a known marker of malaria disease severity (Cowman & Crabb, 2006). Platelet counts have also been previously defined as a marker of disease severity in patients with severe *P. falciparum*. In previous studies, our lab previously found no significant correlation between plasma VWF:pp levels and peripheral parasitemia levels (Hollestelle *et al*, 2006; Larkin *et al*, 2009). In contrast however, in children with CM we observed that OPG was significantly correlated with parasite count ($r=0.65$; $P\leq0.001$) (Figure 5.5A), and that OPG levels were also inversely correlated with platelet count ($r= -0.5$; $P\leq0.001$) (Figure 5.5B). OPG levels also correlate with VWF:Ag although this correlation is less significant than with lactate ($r= 0.2225$, $P\leq0.01$) (Figure 5.5C).



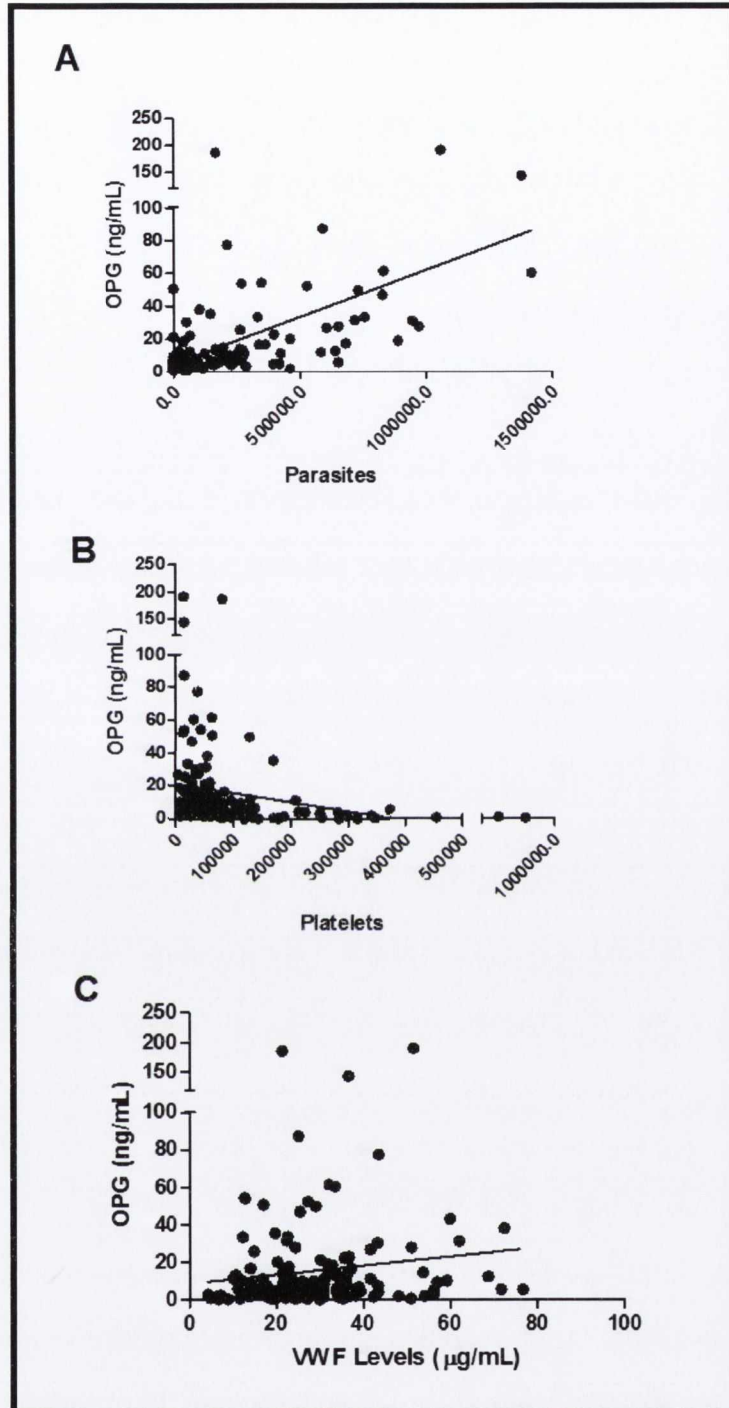


Figure 5. 5 Plasma OPG levels correlate with markers of malaria severity in children with *P. falciparum* malaria

(A) Peripheral blood parasitemia levels (B) Platelet counts (C) Plasma VWF:Ag levels

5.7 Plasma OPG levels are markedly elevated in experimental CM

To further investigate the relationship between severe malaria and OPG, we utilised an established murine model of experimental CM. In this model, C57BL/6J mice were infected with *P. berghei* ANKA. In keeping with our findings in children with CM, a significant increase in plasma OPG levels was also observed in wild type C57BL/6J mice following *P. berghei* infection (Figure 5.6A). For example, by Day +5 following inoculation, median murine plasma OPG levels were found to be increased approximately 18 fold (1.38ng/ml at Day 0 versus 25.5ng/ml at Day+5; $P \leq 0.001$). This increase in murine plasma OPG levels seen was thus strikingly similar in magnitude to that observed in children with *P. falciparum* CM.

Interestingly, a significant increase in plasma OPG levels was observed from a very early stage following *P. berghei* infection. Plasma OPG levels were already increased 4.6 fold (1.38 versus 6.4ng/ml; $P \leq 0.05$) within 24 hours following *P. berghei* inoculation. This early marked elevation in murine plasma OPG levels was established before the onset of any objective clinical signs were apparent (Figure 5.6B). Furthermore, the increase in plasma OPG levels also preceded the development of significant peripheral blood parasitemia levels (Figure 5.6C). As the mice became increasingly unwell, plasma OPG levels progressively increased. Finally, and again in keeping with our observations in children with CM, a correlation between plasma OPG levels and peripheral blood parasitemia levels was also observed in the murine ECM model ($r = -0.9$; $P = 0.08$). Collectively, these findings confirm that marked elevation in plasma OPG constitutes a consistent finding in both human and experimental murine CM. Moreover, the increase in plasma OPG occurs at an early stage during the pathogenesis of severe malaria infection.

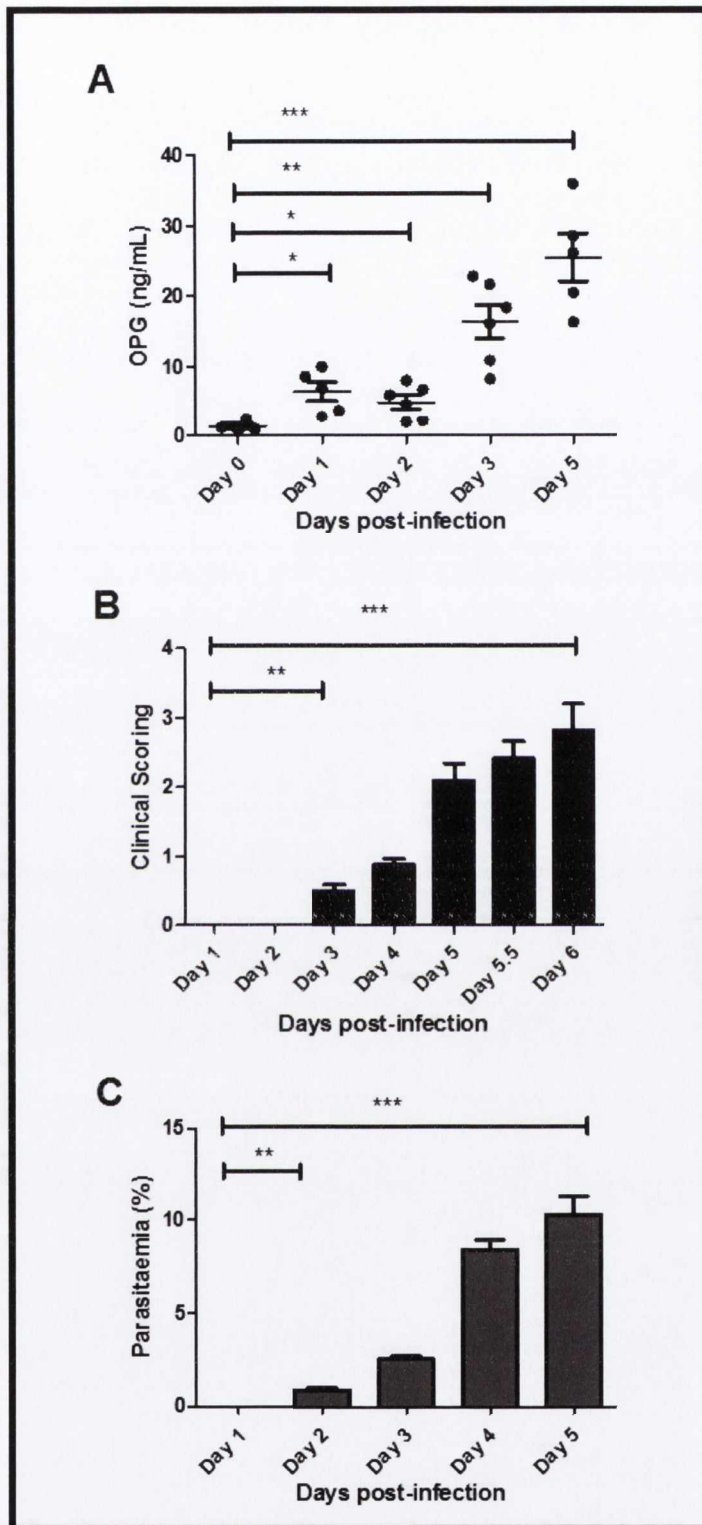


Figure 5. 6 (A) Plasma OPG levels are markedly increased in WT mice infected with *P. berghei* and correlate with disease progression (B) Clinical scoring (C) Peripheral blood parasitemia levels

5.8 OPG and VWF are physically associated in murine plasma

Previous studies have also demonstrated that OPG is able to bind directly to the A1 domain of VWF within EC prior to secretion (Zannettino *et al*, 2005). In contrast however, due to the ionic conditions existing in normal plasma, it seems unlikely that further OPG binding to VWF occurs in the peripheral circulation. Nevertheless, VWF-OPG complexes formed prior to secretion from WPB remain relatively stable in normal plasma. Immunoprecipitation experiments performed using WT and VWF^{-/-} mice infected with *P. berghei* demonstrated that the increased plasma OPG was circulating at least in part in complex with VWF (Figure 5.7).

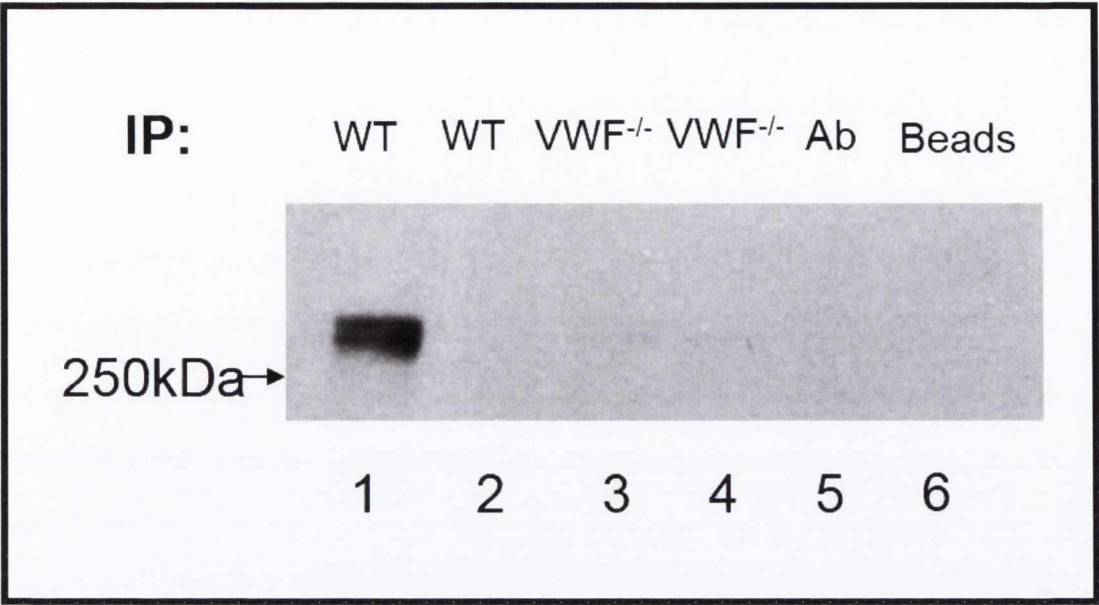


Figure 5. 7 OPG and VWF co- immunoprecipitated from murine peripheral blood

Murine plasma was incubated with the antibody against murine OPG and protein-G beads. Following incubation with an OPG antibody and subsequent to electrophoresis, membranes were probed for with a VWF polyclonal antibody. Using an OPG antibody and beads, VWF could be detected also bound to these beads.

- Lane 1: WT plasma and antibody
- Lane 2: WT plasma no antibody
- Lane 3: VWF^{-/-} plasma and antibody
- Lane 4: VWF^{-/-} no antibody
- Lane 5: Antibody alone
- Lane 6: Beads alone

5.9 Increased plasma OPG in ECM is predominantly monomeric and circulates in complex with VWF

In normal plasma, OPG circulates predominantly in the form of a 120kDa homodimer (Yamaguchi *et al*, 1998; Simonet *et al*, 1997). Furthermore, dimerization has been shown to significantly influence the functional activity of OPG. Consequently we investigated OPG in murine plasma before and after *P. berghei* infection by Western blot analysis. In normal uninfected C57BL/6J mice, almost all OPG circulates as a dimer. Interestingly however, we found that monomeric OPG accounted for the majority of the increased plasma OPG observed in *P. berghei* infected mice (Figure 5.8)

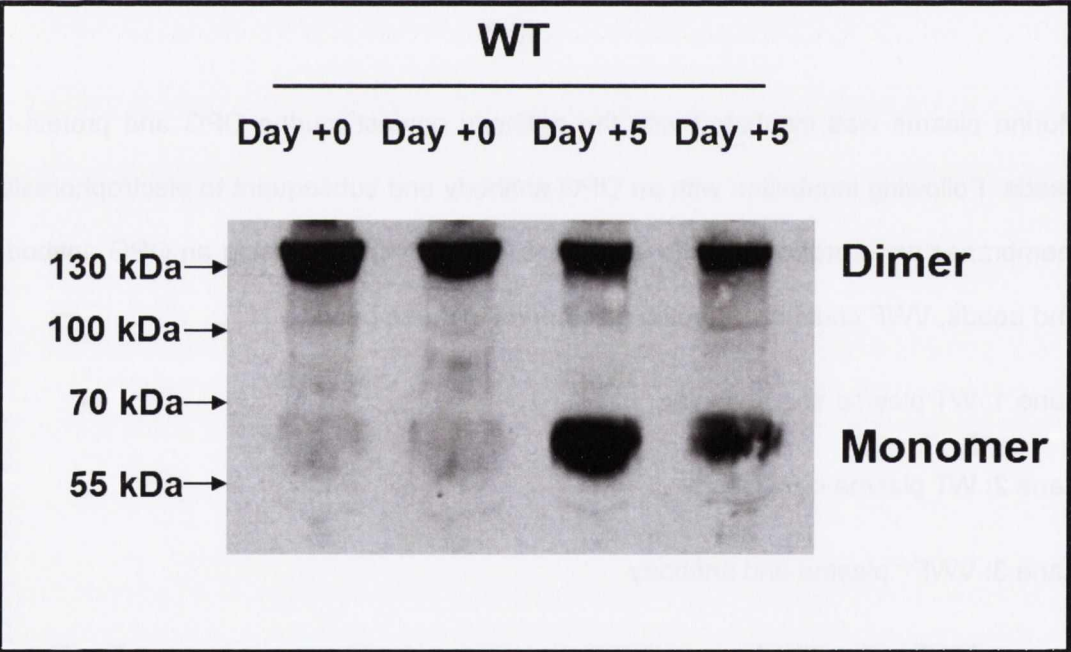


Figure 5. 8 Western blot of murine plasma OPG before and after *P. berghei* inoculation

5.9 In the absence of WPB, plasma OPG levels in ECM remain significantly increased

It is interesting that the VWF and OPG data demonstrate important differences. The extent of the VWF response in malaria and non-malaria is similar. In contrast, plasma OPG levels are significantly increased in malaria and correlate with disease severity, indicating that OPG may be a more malaria-sensitive marker than VWF. VWF is essential for WPB formation (Denis *et al*, 1998). Consequently, VWF^{-/-} mice lack WPB. We used this knock out model to further investigate the relationship between ECM and OPG. Despite the absence of WPB, we observed that *P. berghei* infection in VWF^{-/-} mice was also associated with a marked increase in plasma OPG levels ($P \leq 0.01$; Figure 5.9A). Moreover, the magnitude of the OPG increase was not significantly different to that observed in WT mice. Basal plasma OPG levels were higher in VWF^{-/-} mice compared to WT controls (Figure 5.9B; $P = 0.0509$). Since VWF^{-/-} mice lack WPB, this increase in OPG levels may reflect inadequate storage of OPG. Previous *in vitro* studies have indicated that smooth muscle cells can produce large quantities of OPG (approximately 30 times more than EC) (Olesen *et al*, 2005). OPG could be coming from this source in the absence of WPB.

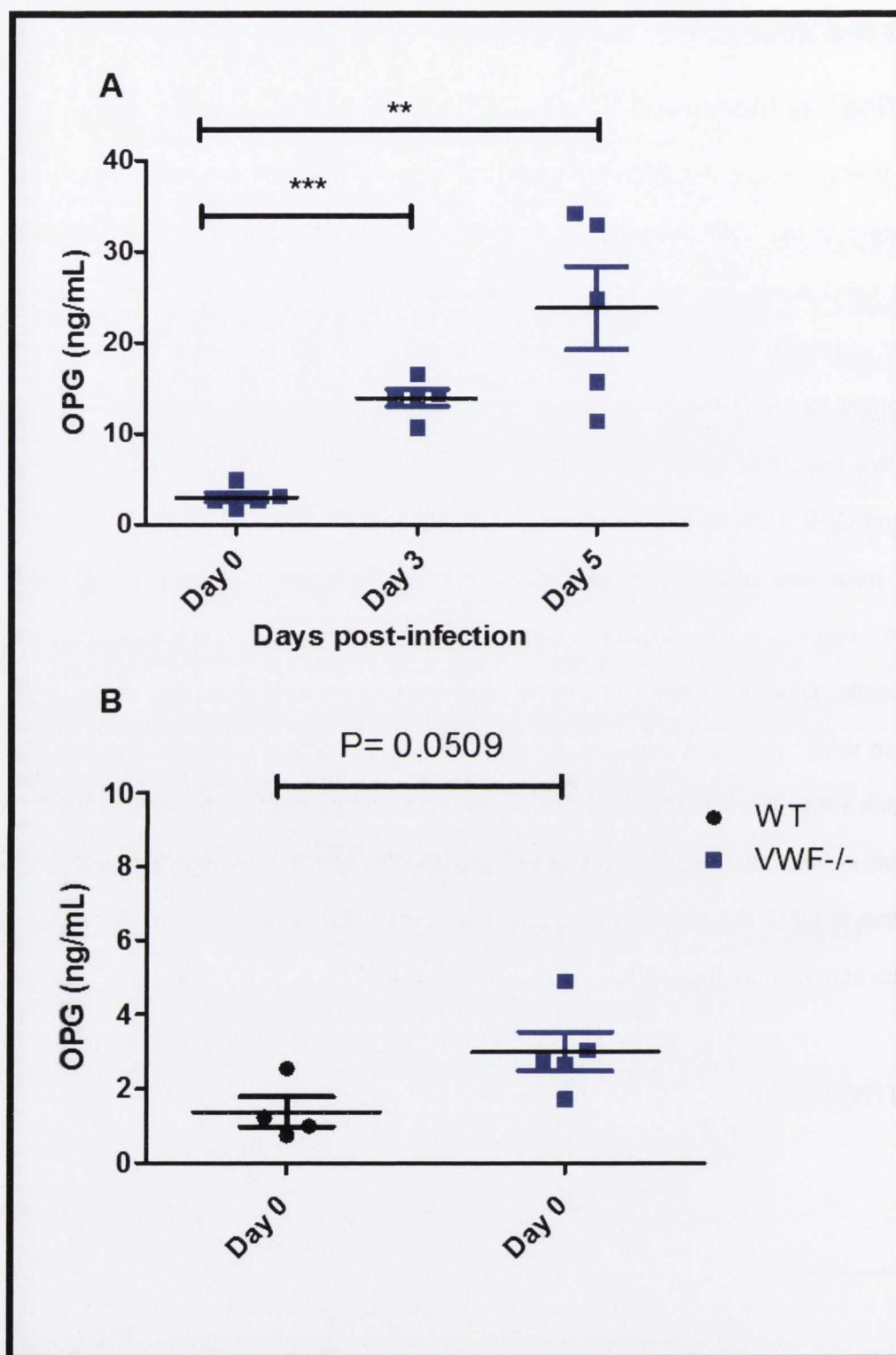


Figure 5. 9 (A) OPG levels are significantly increased in VWF^{-/-} mice during *P. berghei* infection (B) Basal OPG levels are increased in VWF^{-/-} mice

5.10 Discussion

EC activation plays a critical role in modulating the pathogenesis underlying severe *P. falciparum* malaria (Newton & Krishna, 1998; Turner *et al*, 1998). This EC activation triggers the secretion of WPB contents into the plasma. Previous studies have consistently shown that plasma levels of WPB-stored HMW VWF are significantly increased in patients with both *P. falciparum* and *P. vivax* malaria (de Mast *et al*, 2009; Hollestelle *et al*, 2006; Larkin *et al*, 2009; Löwenberg *et al*, 2010). Furthermore, significant elevations in the plasma levels of other WPB components (including VWF:pp and Ang-2) have also been previously reported in patients with malaria infection (Erdman *et al*, 2011; Hollestelle *et al*, 2006; Conroy *et al*, 2009). In this study, we demonstrate for the first time that severe *P. falciparum* malaria is also associated with a marked increase in plasma OPG levels. Although the biological mechanism(s) responsible for this increase in OPG have not been defined, it seems likely that fulminant acute EC activation is at least in part responsible. Interestingly, we observed a similar marked increase in plasma OPG levels in a murine model of ECM. In addition, using this murine model we further demonstrated that the increase in plasma OPG develops at an early stage following malaria infection. Indeed, plasma OPG levels were significantly increased prior to the presence of detectable levels of parasitemia, and well before the onset of any clinical signs. This finding is consistent with previous studies in which elevations in plasma VWF levels were observed at a surprisingly early stage in both human volunteers infected with *P. falciparum*, and in mice infected with *P. berghei* (de Mast *et al*, 2007).

The early and marked increase in plasma OPG levels associated with malaria may represent an epiphenomenon, consistent with acute EC activation and WPB secretion. Alternatively, it is possible that OPG may have a role in the pathogenesis of severe malaria. Although OPG has an established role in regulating osteoclast differentiation, more recent studies have identified additional novel biological functions.

Of particular relevance, OPG has been shown to influence the expression of adhesion molecules on EC surface, and to modulate EC apoptosis (Caidahl *et al*, 2010; Mangan *et al*, 2007; Zauli *et al*, 2007). For example, *in vitro* studies have shown that OPG increases expression of Ang-2, VCAM-1, and ICAM1 on HUVEC (Mangan *et al*, 2007). Interestingly, in this study we found significant correlations between plasma OPG levels and several other prognostic markers (including plasma lactate levels, parasitemia and thrombocytopenia) in children with CM. Further studies will be required to determine whether OPG plays any direct role in contributing to malaria pathogenesis. To investigate whether plasma OPG levels with infection was dependent on the presence of VWF, we investigated a VWF^{-/-} mouse model. Interestingly, OPG levels increase in VWF^{-/-} mice during the course of ECM. The increase in OPG during *P. berghei* infection in the VWF^{-/-} mice is not WPB derived. The cellular origin of the OPG therefore remains unclear. Previous *in vitro* studies have indicated that smooth muscle cells can produce large quantities of OPG (approximately 30 times more than EC) (Olesen *et al*, 2005).

Notwithstanding any putative role of OPG in malaria pathogenesis, our combined human and animal data support the hypothesis that plasma OPG levels may constitute a novel biomarker with prognostic significance in malaria. In this context, it is interesting that although both VWF and OPG are stored together within WPB, the increase observed in plasma OPG levels in children with CM was much more marked compared to that in plasma VWF levels. This discrepancy may relate to different relative concentrations of VWF and OPG being stored within WPB. Alternatively, it is not known whether significant differences in plasma half-life may exist between OPG and VWF. Finally as previously described, significant OPG synthesis in other non-EC tissues has been reported (Simonet *et al*, 1997).

The combined increases in plasma levels of both VWF and OPG observed in severe malaria are of further interest in that previous studies have demonstrated that OPG can bind directly to the A1 domain of VWF (Zannettino *et al*, 2005; Shahbazi *et al*,

2007). This OPG-VWF binding occurs within EC prior to secretion from WPB (Shahbazi *et al*, 2007). Due to the ionic conditions existing in normal plasma, it seems unlikely that any further OPG binding to VWF takes place happens in the peripheral circulation. Nevertheless, VWF-OPG complexes formed prior to secretion from WPB have been shown to remain relatively stable in normal plasma (Shahbazi *et al*, 2007). Further studies will be required to determine whether increased levels of this VWF-OPG complex are circulating in patients with severe malaria. Moreover, it remains unclear whether the formation of this complex has any effect upon the normal functional properties of either VWF and/or OPG respectively.

In conclusion, we have shown that plasma OPG levels are elevated approximately 20 fold in both children with CM, and in a murine model of ECM. This marked increase in plasma OPG is evident from an early stage following malaria infection. Furthermore, plasma OPG level increase progressively through the course of infection, and correlate with other established markers of outcome in patients with severe malaria. Collectively, these findings suggest that OPG may have a role to play as a novel biomarker in patients with severe malaria infection, and also raise the intriguing possibility that OPG may have a role to play in modulating malaria pathogenesis. These data presented here warrant further investigation and could potentially suggest the basis of where a clinical trial could be implemented.

6. Coagulation activation and the Protein C pathway in ECM

6.1 Introduction

6.1.1 Primary haemostasis

Primary haemostasis describes platelets adhering to sub-endothelium to form aggregates following vascular injury (Furie & Furie, 2008). Platelet accumulation at the site of damage is initiated by tethered VWF. Upon exposure to the sub endothelial surface, VWF binds collagen of the vessel wall *via* its A3 domain. Under the shear forces of the vasculature, VWF bound to this collagen unwinds to form strings revealing platelet binding sites (GPIb). This process enables platelet adherence via the GPIb/IX/V complex and platelet “rolling” along the VWF strings (Brass *et al*, 2005). Tethering to VWF strings facilitates the subsequent activation of platelets by thrombin. Platelet activation leads to conformational changes in platelet integrins, greatly enhancing affinity for VWF, fibrinogen and collagen (Nieswandt & Watson, 2003; Jung & Moroi, 2000). Activated platelets also express soluble messengers such as ADP which recruit further platelets to the site of damage. Inter-platelet bridges are formed through integrins and adhesive proteins such as fibrin and VWF (Brass *et al*, 2005). Platelet activation induces transport of negatively-charged phospholipids to the platelet surface, providing a catalytic surface for enzyme complexes of the coagulation cascade.

6.1.2 The Coagulation Cascade

In addition to triggering primary haemostasis and formation of the platelet plug, breakdown of the endothelial barrier concomitantly exposes sub-endothelial tissue factor (TF), the trigger of the coagulation cascade. Upon exposure to blood, TF acts as

the initiator of coagulation *via* formation of a complex with activated vitamin K-dependent serine protease factor VII (FVIIa), which catalyses the activation of vitamin K-dependent zymogens factor X (FX) and factor IX (FIX) to FXa and FIXa respectively (Mackman *et al*, 2007). TF binds and activates FVII (Dickinson & Ruf, 1997) and once activated, FVIIa remains associated with TF to form a ternary complex with either FX or FIX, converting them to FXa and FIXa respectively (Mann *et al*, 2003). FXa generated by the ternary complex activates small amounts of prothrombin to thrombin. Thrombin produced in this initial phase is unable to support thrombus formation but is adequate to activate procoagulant cofactors factor V (FV) and factor VIII (FVIII) (Pieters *et al*, 1989). FVa and FVIIIa accelerate the enzymatic activity of the prothrombinase and intrinsic tenase complexes respectively and their generation results in rapid amplification of thrombin generation (Brummel *et al*, 2002). FVIIIa associates with FIXa on the surface of negatively-charged phospholipids in the presence of Ca^{2+} to form the intrinsic tenase complex, which converts zymogen FX to FXa. During the propagation phase of coagulation, the intrinsic tenase complex is the primary activator of FX, doing so 50-fold more proficiently than the extrinsic tenase (Ahmad *et al*, 1992).

FVa associates with FXa on the negatively charged phospholipid surface of activated platelets in the presence of Ca^{2+} to form the prothrombinase complex. The prothrombinase complex generates thrombin quickly (Rosing *et al*, 1980). Thrombin converts fibrinogen monomers to fibrin. The integrity of the fibrin mesh is stabilized by activated factor XIII (FXIIIa), increasing fibrin clot resistance to proteolytic degradation. Primary haemostasis in conjunction with fibrin initiate the development of a haemostatic plug and these elaborate pathways are depicted in Figure 6.1.

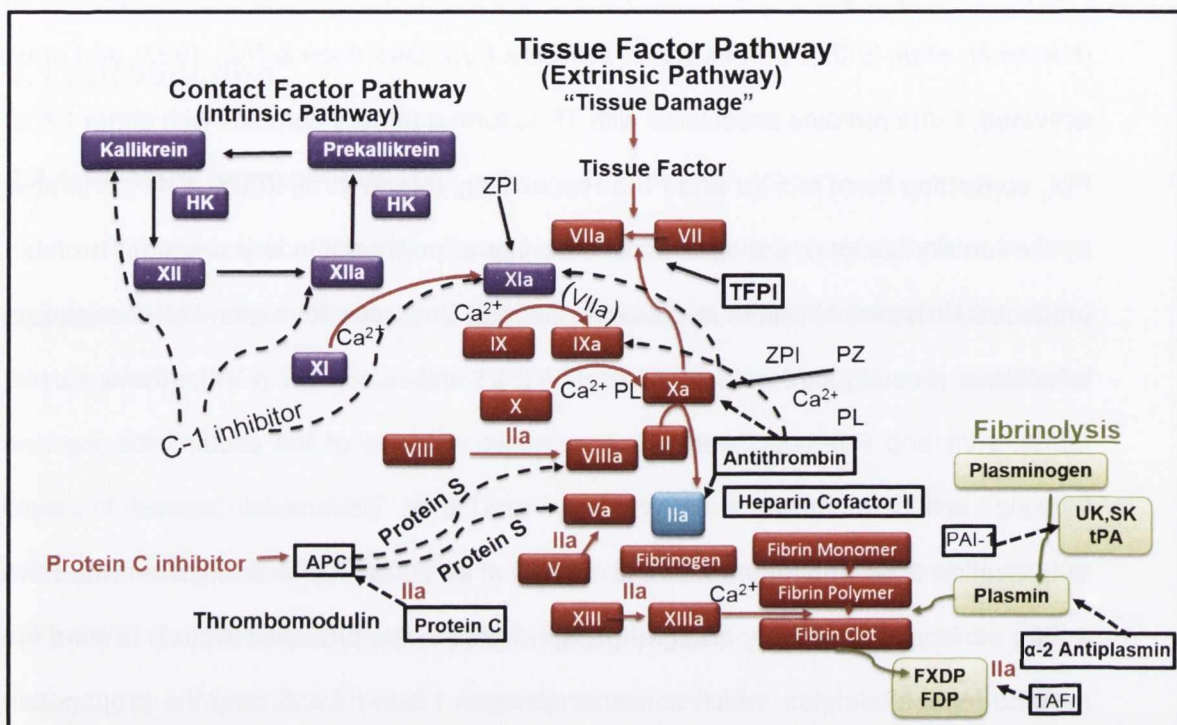


Figure 6. 1 The coagulation cascade

6.1.3 Regulation of coagulation

Prevention of pathogenic thrombosis in the absence of vascular injury is vital to maintain the circulatory system. Unregulated haemostasis can result in thromboembolic disease, vessel occlusion and ischaemic tissue injury. Consequently, a delicate balance exists between pro-thrombotic and anti-thrombotic clotting factors *in vivo*. Importantly, the coagulation cascade is regulated by three major endogenous anticoagulant pathways; antithrombin (AT) (Pike *et al*, 2005), tissue factor pathway

inhibitor (TFPI) (Sandset, 1996) and the protein C (PC) pathway (Dahlbäck & Villoutreix, 2005b).

6.1.4 Protein C anticoagulant pathway

PC is a plasma vitamin K-dependent serine protease which circulates in plasma as a zymogen (Dahlbäck & Villoutreix, 2005b) and was first identified in 1976 (Stenflo, 1976). PC is synthesised in the liver and circulates in plasma at a concentration of 4–6 µg/mL. Mature PC is comprised of a 41kDa heavy chain, containing the activation peptide and serine protease domain, and a 21kDa light chain containing the signal peptide, the Gla domain and 2 EGF-like domains (Beckmann *et al*, 1985). PC undergoes extensive posttranslational modification. Uniquely among vitamin K-dependent proteins, endogenous PC exists in 3 different glycoforms: ~70% of PC circulates fully glycosylated (α -PC), ~25% missing a glycan chain at Asn-329 (β -PC) and the remaining ~5% is missing glycans at both Asn-329 and Asn-248 (γ -PC) (Miletich & Broze, 1990). The molecular basis for partial glycosylation of PC is currently unknown.

Zymogen PC is converted to activated PC (APC) by thrombin cleavage, which releases a 6kDa activation peptide (Kisiel, 1979). The activation of PC by thrombin is greatly enhanced by association of thrombin with the endothelial cell glycoprotein receptor thrombomodulin (Tm) (Figure 6.2) (Esmon, 1995). Once thrombin is bound to Tm, it loses its procoagulant properties and instead triggers the PC anticoagulant pathway (Dahlbäck & Villoutreix, 2005a). Endothelial protein C receptor (EPCR) is a multi-ligand high-affinity EC surface receptor and is an essential component of PC pathway (Fukudome & Esmon, 1994). EPCR-PC complex formation on EC accelerates APC production by the thrombin–Tm complex (Stearns-Kurosawa *et al*, 1996; Gleeson *et al*, 2012). APC functions to attenuate coagulation by proteolytic inactivation of FVa and FVIIIa (Walker *et al*, 1979; Fay *et al*, 1991b). The anticoagulant function of APC in plasma is dependent upon interaction with its cofactor protein S (Saller *et al*, 2005).

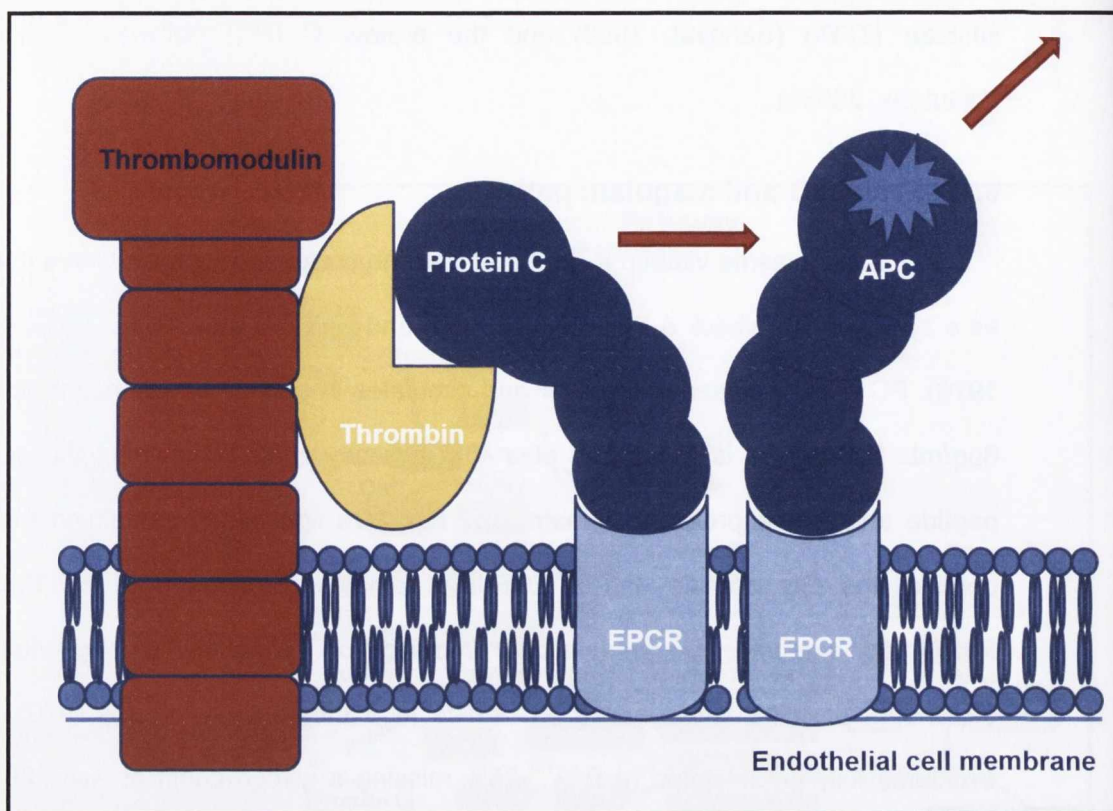


Figure 6. 2 Activation of Protein C on endothelial cells

As well as its critical anticoagulant function, APC has been shown to play a key role in modulating inflammation on different cells through activation of PAR1-dependent and independent signalling (Bouwens *et al*, 2013). Through initiation of intracellular signalling, APC can induce anti-inflammatory, anti-apoptotic and endothelial barrier stabilising activities. Administration of APC to patients with severe sepsis and in murine models of inflammatory diseases has proved to be beneficial. APC also possesses anti-inflammatory and anti-apoptotic signalling properties on other cell types, including monocytes (White *et al*, 2000; Hancock *et al*, 1995; Brueckmann *et al*, 2004), macrophages (Cao *et al*, 2010), and dendritic cells (Kerschen *et al*, 2010). APC provides cytoprotective signalling on EC through EPCR-dependent activation of PAR1

(Riewald *et al*, 2002; Gleeson *et al*, 2012). Unlike thrombin-PAR1 signalling, APC activation of PAR1 enhances integrity of the EC barrier and protects against thrombin disruption (Feistritzer & Riewald, 2005). Additionally, APC prevents EC surface adhesion molecule expression and apoptosis (Joyce *et al*, 2001; Cheng *et al*, 2003).

6.1.5 APC as a therapeutic

The hypothesis that APC may be of therapeutic benefit in severe sepsis was first suggested after inhibition of PC activation was linked with increased mortality in a baboon model of *E.coli*-induced sepsis. Furthermore, APC infusion significantly improved overall survival in the same sepsis model (Taylor *et al*, 1987). Murine models of LPS-induced endotoxaemia demonstrated that APC treatment reduced white cell apoptosis, vascular permeability and mortality in WT, but not PAR1^{-/-} mice, confirming an essential role for PAR1 and its signalling (Kerschen *et al*, 2007). Additionally, the beneficial effect of APC was significantly decreased in mice with reduced EPCR levels, suggesting a vital role for this receptor. The comparative contributions of APC signalling on immune and vascular cells eliciting the beneficial therapeutic effects *in vivo* are yet unknown.

Plasma PC levels are reduced in up to 80% of patients with severe sepsis and are inversely correlated with mortality (Bernard, 2001). The PROWESS trial, carried out in 2001, reported that administration of recombinant APC reduced mortality risk by 19.4% in patients with severe sepsis, leading to its approval by the FDA (Bernard, 2001). However, the subsequent ADDRESS trial, investigating the use of recombinant APC in patients, found no reduction in mortality between APC and placebo groups (Abraham, 2005). More recently, the PROWESS-SHOCK trial failed to identify any survival benefit associated the use of APC in severe sepsis, leading to its withdrawal from the market (Ranieri *et al*, 2012). Variations in inclusion criteria and dosing strategy of treatment may account for the discrepant outcomes of different clinical trials. In an effort to

improve APC therapy in severe sepsis, a number of different APC variants with altered anticoagulant and cytoprotective signalling functions have been engineered (Mosnier *et al*, 2004; Kerschen *et al*, 2007).

6.1.6 Coagulopathy and Malaria

Infection with *P. falciparum* is associated with thrombocytopenia, decreased levels of anticoagulant clotting factors, and thus generation of thrombin. Whether coagulopathy is involved in the pathogenesis of malaria still remains unknown (Kelton *et al*, 1983; Pukrittayakamee *et al*). Although fulminant disseminated intravascular coagulation (DIC) is not present (Dennis *et al*, 1967; Pukrittayakamee *et al*) nevertheless, coagulation activation has been described.

Several studies have described elevated levels of circulating thrombin-antithrombin III (TAT) complexes, fibrinopeptide A and soluble thrombomodulin (sTm) in patients with malaria. In addition, reduced levels of antithrombin, Protein S and C have also been reported (Angchaisuksiri, 2014; Hemmer *et al*, 1991; Clemens *et al*, 1994; Mohanty *et al*, 1997; Vogetseder *et al*, 2004). IE have been reported to promote a procoagulant endothelial surface through induction of TF (Francischetti *et al*, 2007). Recently, a novel role for EPCR has been reported in the pathogenesis of *P. falciparum* malaria (Moxon *et al*, 2013; Turner *et al*, 2013; Petersen *et al*, 2015). Interestingly, post-mortem samples from African children with CM have shown marked fibrin deposition localised to sites of IE sequestration. Since the brain demonstrates constitutively low expression of both TM and EPCR, these data suggest that the cerebral microvasculature may be particularly vulnerable to CM coagulopathy (Moxon *et al*, 2013).

The molecular mechanisms by which coagulopathy and the PC pathway contribute to CM remain unknown. We sought to examine if the mouse model of CM could be used to study malarial-induced coagulopathy, and whether the PC pathway was important in this context.

6.2 Macroscopic Haemorrhages during experimental CM

Petechial lesions observed in fatal cases of CM has led to the suggestion of an involvement of coagulation in CM pathogenesis (White *et al*, 2009; Boonpucknavig *et al*, 1990; Moxon *et al*, 2009). Small haemorrhages seen in post-mortem of the brain and the retina are an established feature (Dorovini-Zis *et al*, 2011; Thomas, 1971).

To determine whether the characteristic petechial lesions observed post-mortem in the brains of children with CM are also present in murine ECM we took uninfected controls and mice at Day +5 post-infection and cardiacally perfused them with 40mL of 0.9% saline once deeply anaesthetised. The brains were subsequently removed and compared to one another. On gross examination we observed the presence of petechial haemorrhages widely distributed in mice with CM (Figure 6.3A + B) (n=3). These lesions are consistent with those in human CM, and are likely to be mediated in part by thrombosis and haemorrhage and altered coagulation.

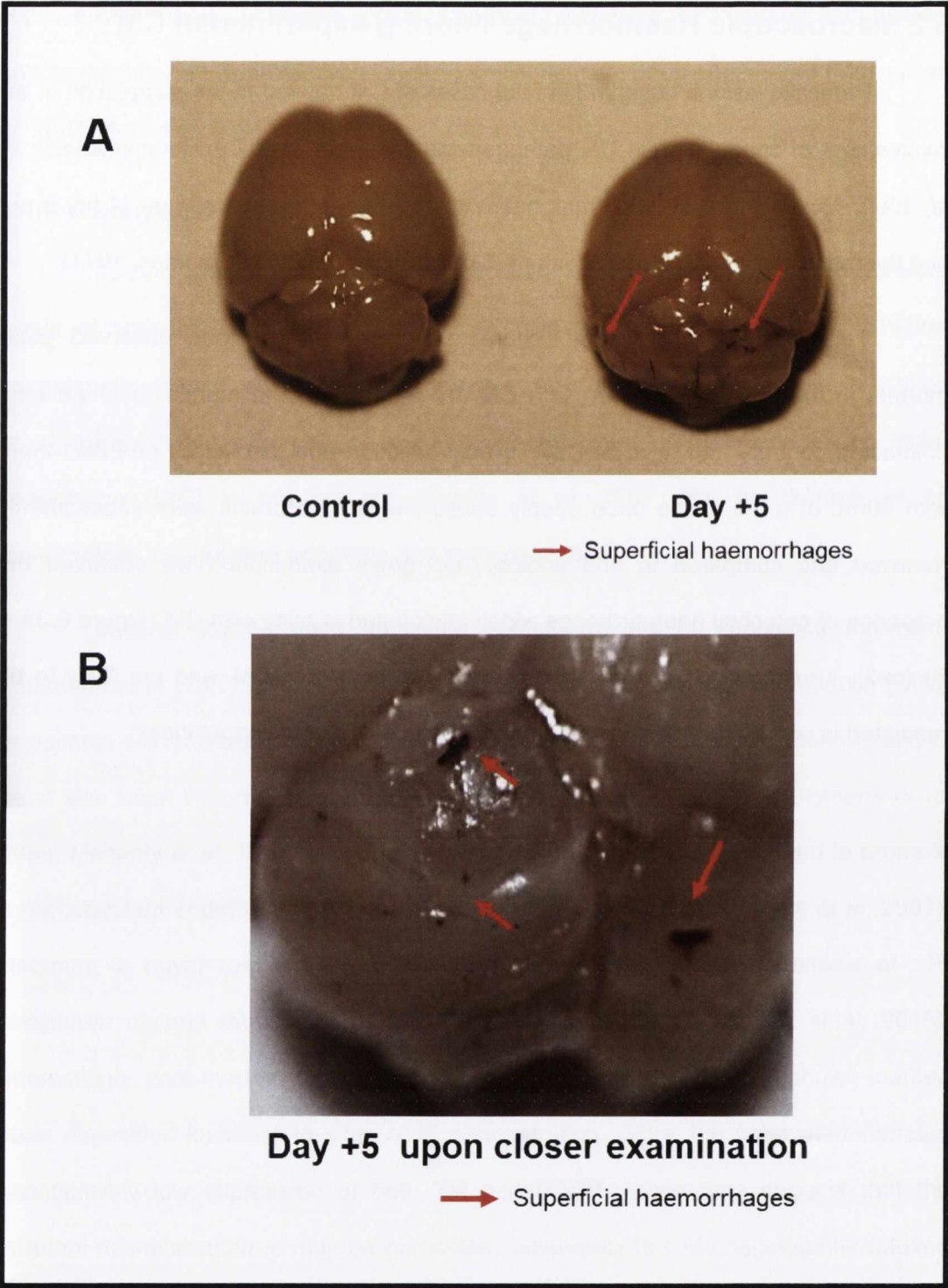


Figure 6. 3 (A) Gross petechial lesions in experimental CM are readily seen in Day +5 brains but not in uninfected controls (B) Closer inspection of Day +5 brain

6.3 Activated Prothrombin Time during *P. berghei* infection

Activated Partial Thromboplastin Time (APTT) measures the activity of the intrinsic and common pathways of coagulation. The APTT is the time taken from the addition of calcium to the formation of a fibrin clot. The APTT has previously been reported to be normal in *P. vivax* infection but significantly prolonged in *P. falciparum* infection (Rojanasthien *et al*, 1992; Prasad *et al*, 2009). To investigate coagulopathy in the experimental model of CM we assessed APTT in WT mice following *P. berghei* infection. By Day +5 following infection when mice develop CM symptoms we observed that the APTT was significantly prolonged ($P \leq 0.001$) ($n=8-13$) which is consistent with altered coagulation in these mice.

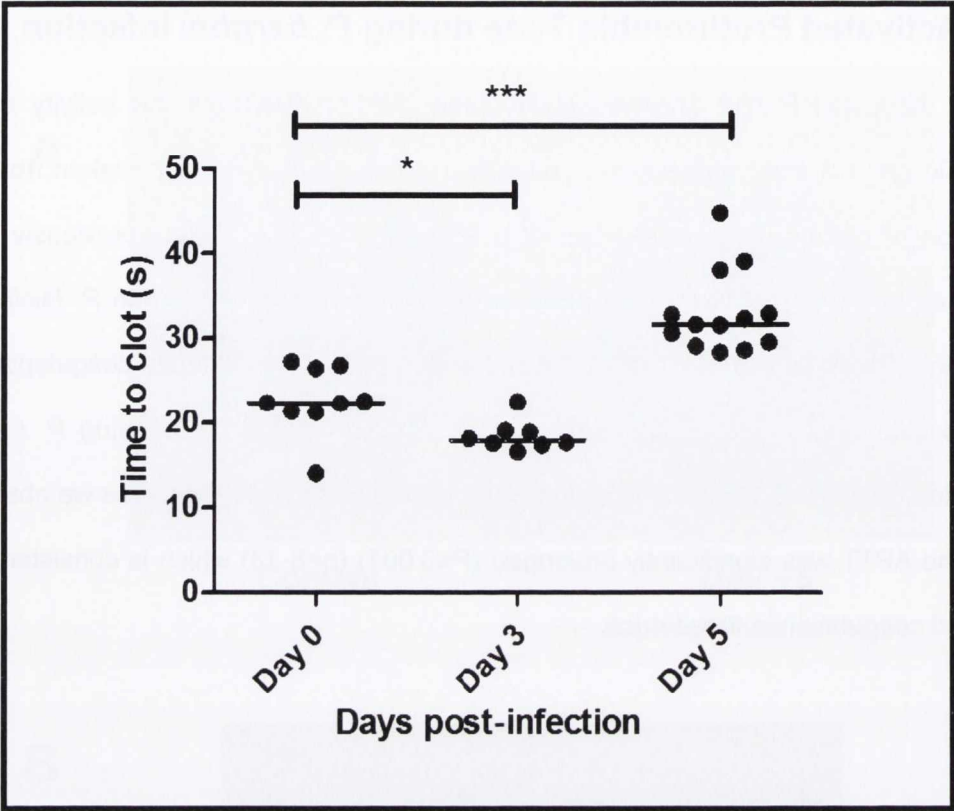


Figure 6. 4 APTT in WT mice following *P. berghei* infection

6.4 Prothrombin Time in experimental CM

The Prothrombin Time (PT) measures the activity of the extrinsic and common pathways of coagulation and therefore, is reliant on the functional activity of factors VII, X, V, II and fibrinogen. Previously PT measurements in patients with severe *P. falciparum* infection have shown different results (Dennis *et al*, 1967; Prasad *et al*, 2009; Akingbola *et al*, 2006). When the PT was examined in our murine model, we found by Day +5 of infection the PT was modestly reduced ($P \leq 0.05$) ($n=8-13$), suggesting some coagulation activation. Importantly however, although the murine PT and APTT were affected following *P. berghei* infection the absolute magnitude of the effects were small. In particular, the PT and APTT did not suggest acute DIC, which is entirely in keeping with previous human studies.

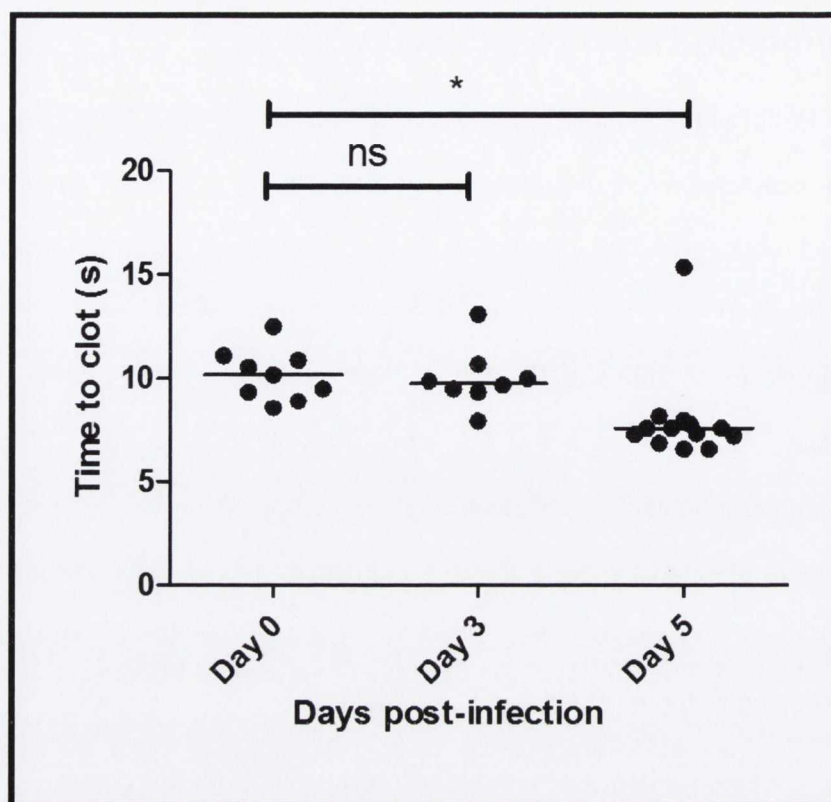


Figure 6. 5 PT is modestly decreased at Day +5 post infection with murine malaria

6.5 Fibrinogen levels in *P. berghei* malaria

Fibrinogen is a soluble plasma protein that is converted to polymeric fibrin in response to damage to the vascular system. Previously, elevated levels of fibrinogen have been demonstrated in patients with malaria (Stauga *et al*, 2013). We employed a radio-immunoassay to investigate levels at Day 0, Day +3 and Day +5 of *P. berghei* infection in WT C57Bl/6J mice. Fibrinogen levels increased significantly by Day +3 following inoculation ($P \leq 0.01$) ($n=3-7$). However fibrinogen levels had returned to normal by Day +5 following infection. However, once again, the magnitude of these effects on plasma fibrinogen levels was small.

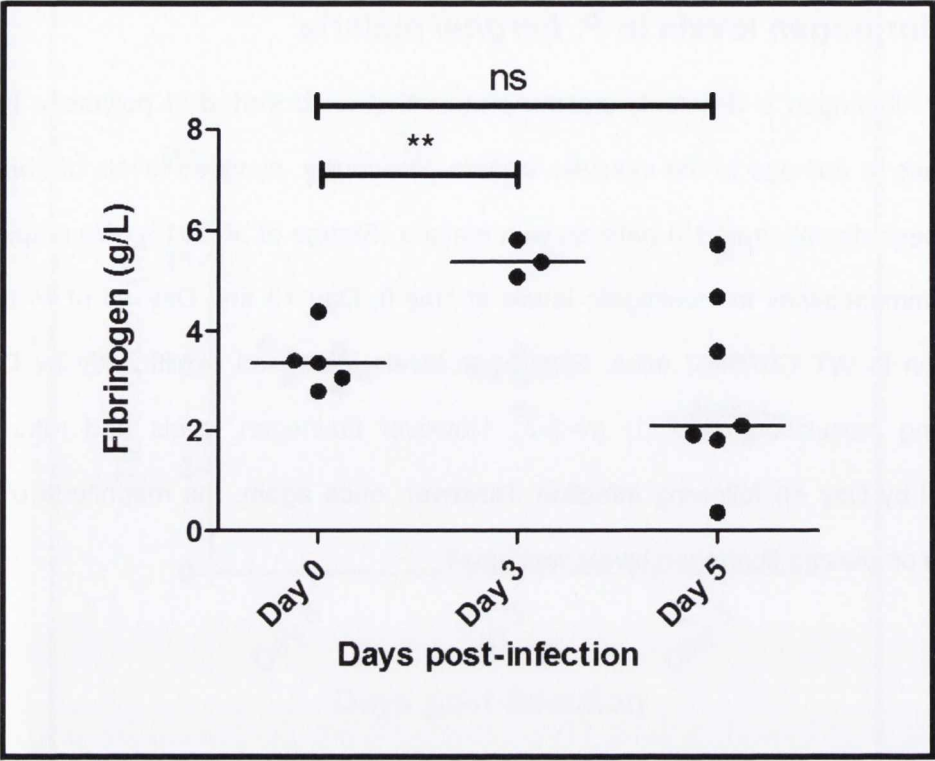


Figure 6. 6 Fibrinogen levels are increased early in experimental malaria

6.6 Thrombin Anti Thrombin complexes

Recent studies suggest that thrombin generation may be important in the pathogenesis of CM (Moxon *et al*, 2013). Thrombin Anti Thrombin (TAT) complexes are formed following the neutralisation of thrombin by antithrombin, and have been widely used as a surrogate marker to assess endogenous thrombin generation. In addition, TAT complexes have been previously shown to be elevated in patients with severe malaria (Hemmer *et al*, 1991; Clemens *et al*, 1994; Moxon *et al*, 2009). In keeping with these previous reports in humans, we found that murine TAT complexes were also significantly increased in mice by Day +5 after inoculation of *P. berghei* ($P \leq 0.01$) ($n=4-5$).

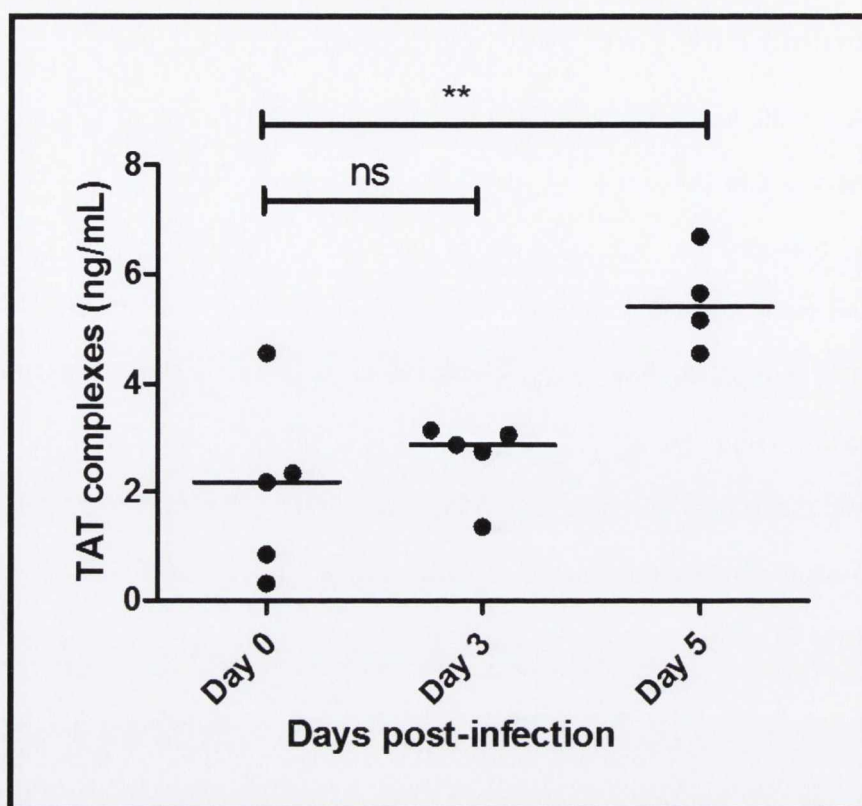


Figure 6. 7 Plasma TAT complex levels in WT mice following *P. berghei* infection

6.7 Antithrombin

The serine protease inhibitor Antithrombin (AT) is an important endogenous anticoagulant and anti-inflammatory mediator (Roemisch *et al*, 2002) and is a major inhibitor of thrombin activity. Previous studies have reported reduction in plasma AT levels in patients with severe *P. falciparum* infection (Vogetseder *et al*, 2004; Mohanty *et al*, 1997). Consequently we measured murine AT levels following *P. berghei* infection. No major effect on plasma AT levels was observed (n=5-7).

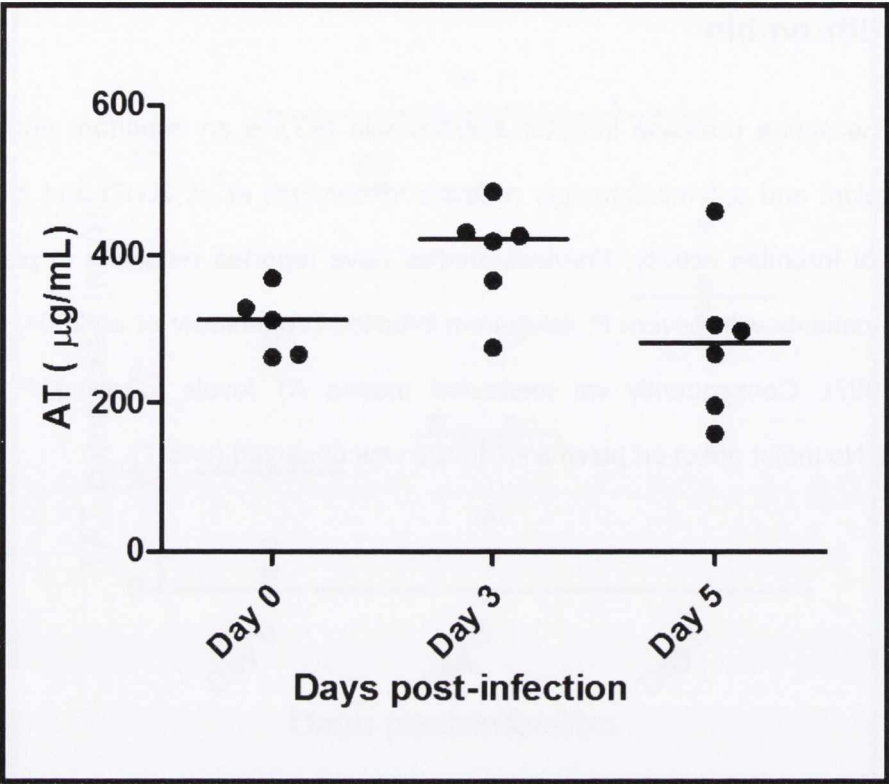


Figure 6. 8 Plasma antithrombin levels in mice following *P. berghei* infection

6.8 Soluble Thrombomodulin

The protein C pathway is a key modulator of both the inflammatory as well as haemostatic systems. There are three main components of this pathway; protein C, protein S and thrombomodulin (TM). These constituents have been demonstrated to be affected in *P. falciparum* infection. TM is an integral membrane glycoprotein expressed on nearly all EC although notably only at low levels in the brain (Wang *et al*, 1998) and is essential in PC activation (Emeis *et al*, 2007; Weiler H., Isermann, 2003). Circulating increased TM levels have previously been reported in patients with malaria (Hemmer *et al*, 1994; Moxon *et al*, 2009). We determined sTm levels in the mouse model of experimental CM and in keeping with the increase observed in patients, we found sTm levels increased from Day +5 following infection with *P. berghei* ($P \leq 0.01$) ($n=3-6$).

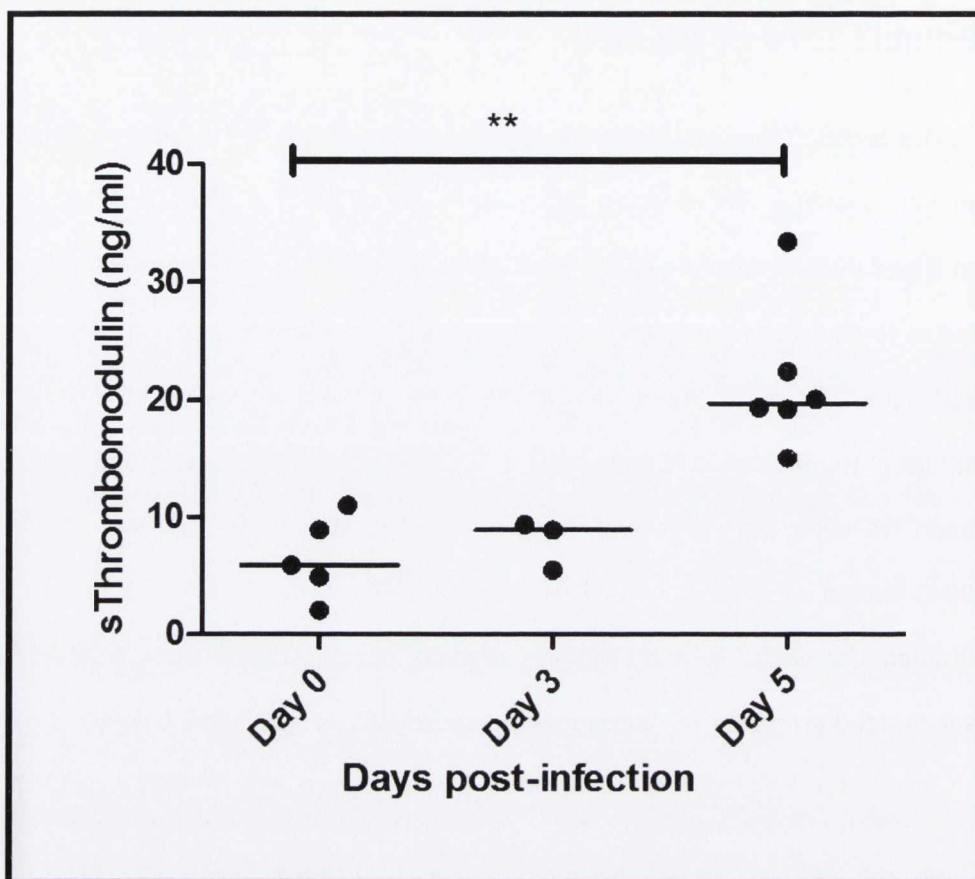


Figure 6. 9 Plasma soluble thrombomodulin levels in mice following infection with *P. berghei*

6.9 Soluble EPCR

Soluble EPCR (sEPCR) is cleaved from the endothelial surface by metalloproteinase ADAM17 dependent shedding (Xu *et al*, 2000; Qu *et al*, 2007) and is detectable in plasma. sEPCR retains the ability to bind PC and APC similar to membrane-bound EPCR (Liaw *et al*, 2000). However, unlike the membrane bound form, sEPCR acts to inhibit APC anticoagulant function and generation (Liaw *et al*, 2000). Increased levels of sEPCR have been reported in patients with systemic inflammatory diseases associated with increased thrombin production (Kurosawa *et al*, 1998).

Due to decreased EPCR expression in the brains of post-mortem children who died of CM, sEPCR levels were previously measured in blood and CSF to see if this decrease was accounted for by increased receptor shedding. Plasma levels remained unchanged however increased sEPCR was detected in CSF (Moxon *et al*, 2013). In contrast, in murine ECM we found that sEPCR levels in plasma were significantly increased in mice with CM compared to uninfected controls (n=4-6).

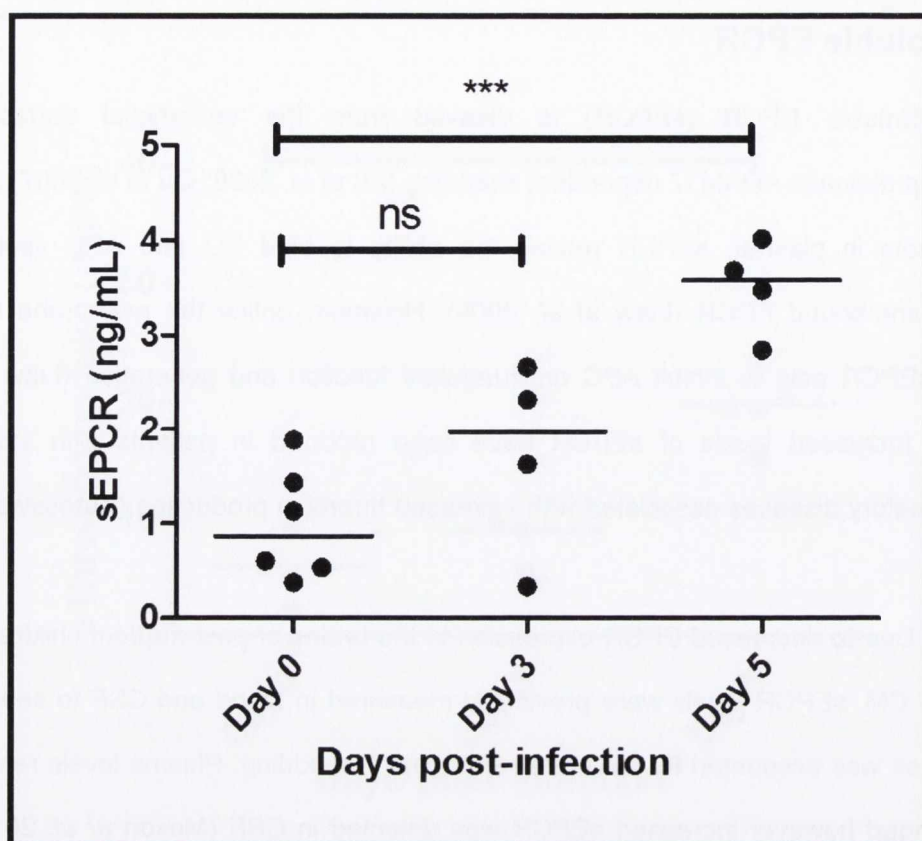


Figure 6. 10 Plasma soluble EPCR levels in mice following *P. berghei* infection

6.10.1 Treatment with EPCR blocking antibodies

RCR-16 is a function blocking monoclonal antibody against EPCR. This anti-EPCR antibody prevents interaction between PC or APC and EPCR. RCR-16 also strongly inhibits Protein C activation on endothelium (Centelles *et al*, 2010) (Figure 6.11A). To determine if EPCR plays a role in *P. berghei* induced CM we used this EPCR blocking antibody versus an isotype control (Rat IgG2A) Immediately before infection with 2×10^6 parasites, mice were I.V. injected either with 50 μ g of RCR-16 (dialyzed into PBS) or with isotype control antibody.

Interestingly, we observed that treatment with RCR-16 to inhibit the generation of APC was associated with significantly shortened survival following *P. berghei* infection ($P \leq 0.05$) (RCR-16 Ab $n=12$, Isotype Ab $n=6$). This finding clearly supports the hypothesis that the protein C pathway may be important in the pathogenesis of CM.

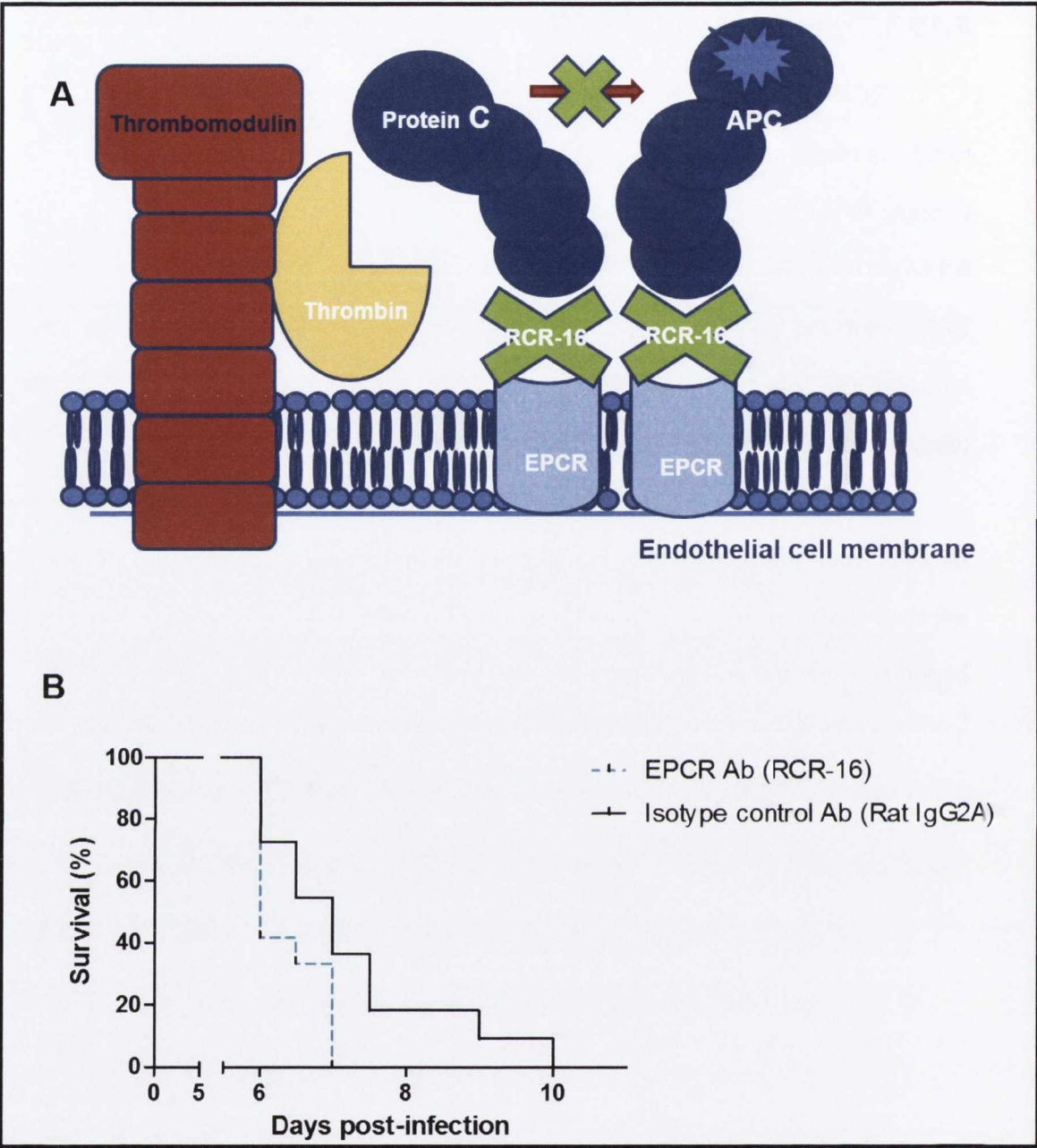


Figure 6. 11 (A) Schematic of RCR-16 blocking antibody inhibiting activation of PC on EC (B) Treatment with EPCR-blocking antibody RCR-16 is associated with decreased survival following *P. berghei* infection

6.10.2 Weight change and parasite counts after treatment with RCR-16

Each animal was monitored daily for weight loss as well as general distress as per ethical guidelines. There were no discrepancies in weight loss between mice treated with anti-EPCR or the isotype control (Figure 6.12A). Percentage parasitemia in mice was also assessed by standard Giemsa staining as before. Interestingly, in keeping with the reduced survival in RCR-16 treated mice, we observed that *P. berghei* parasitemia levels were also slightly increased in these mice compared to mice treated with isotype control (Figure 6.12B) (RCR-16 Ab n=12, Isotype Ab n=6).

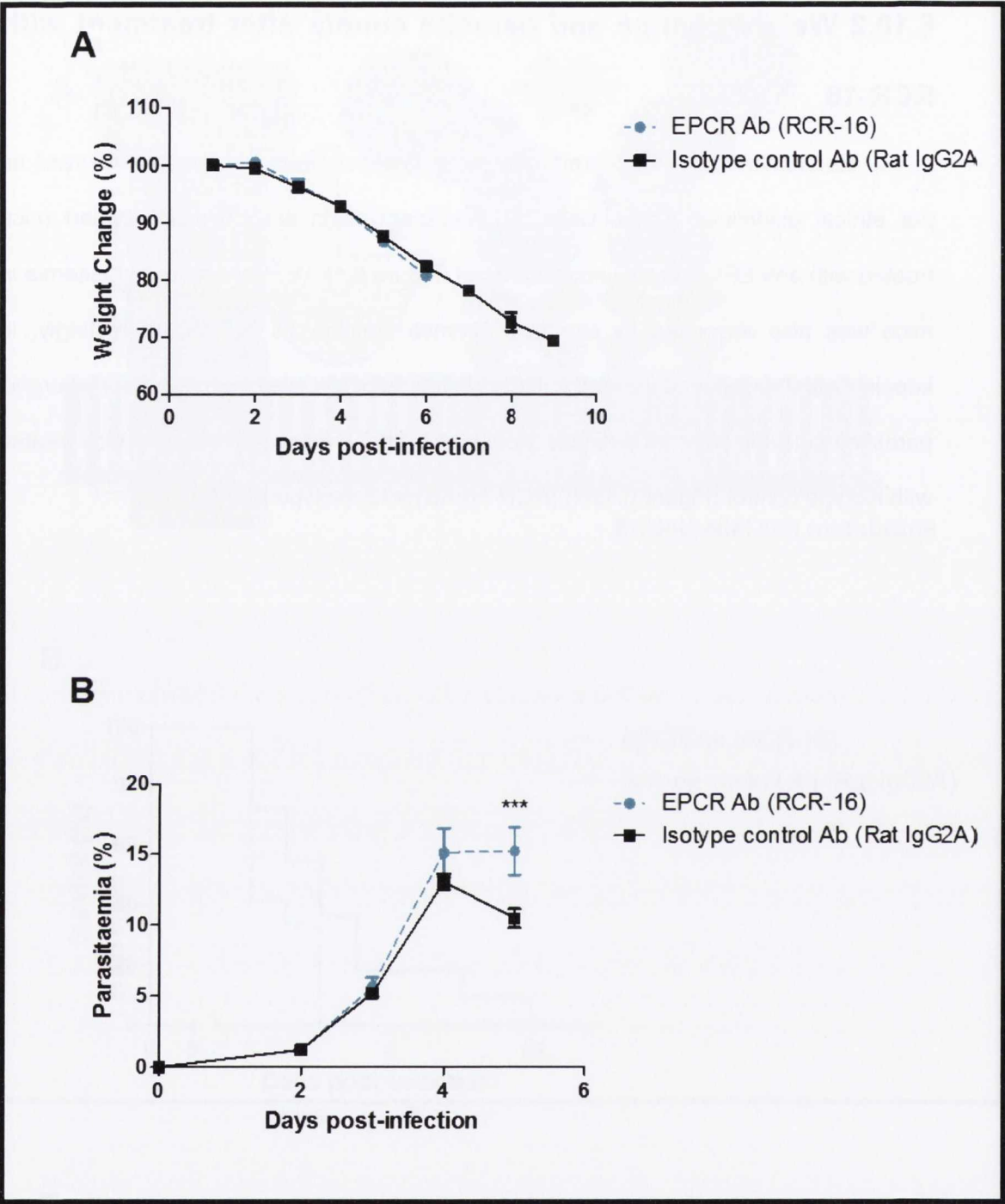


Figure 6. 12 Weight change (A) and peripheral parasitemia (B) in mice following treatment with EPCR-blocking antibody or isotype control

6.11 Targeting Protein C pathway as a novel treatment for experimental CM

6.11.1 Clinical scoring and overall survival in WT mice after APC administration

In murine models of LPS-induced endotoxaemia, APC treatment was associated with reduced mortality (Kerschen *et al*, 2007). Since the EPCR blocking antibody studies suggest a role for the PC pathway in the pathogenesis of CM, we further investigated whether administration of recombinant murine APC (mAPC) influenced the progression of ECM. In brief, 10µg of mAPC was administered I.V. into WT mice preceding infection with *P. berghei*. Subsequently, another dose of 10µg of mAPC I.V. was given four hours after infection. Clinical progression was monitored using the same clinical scoring system as previously described. Importantly, a marked difference in the clinical progression of ECM was observed following treatment with mAPC (Figure 6.13A, $P \leq 0.001$) (WT $n=13$, APC $n=11$). Furthermore mAPC treatment was also associated with a significant increase in overall survival (Figure 6.13B). Interestingly, the mice pre-treated with APC did not develop CM, but rather died at three weeks from severe anaemia. The parasitic burden increasing during infection causes a profound anaemia and this can result in death.

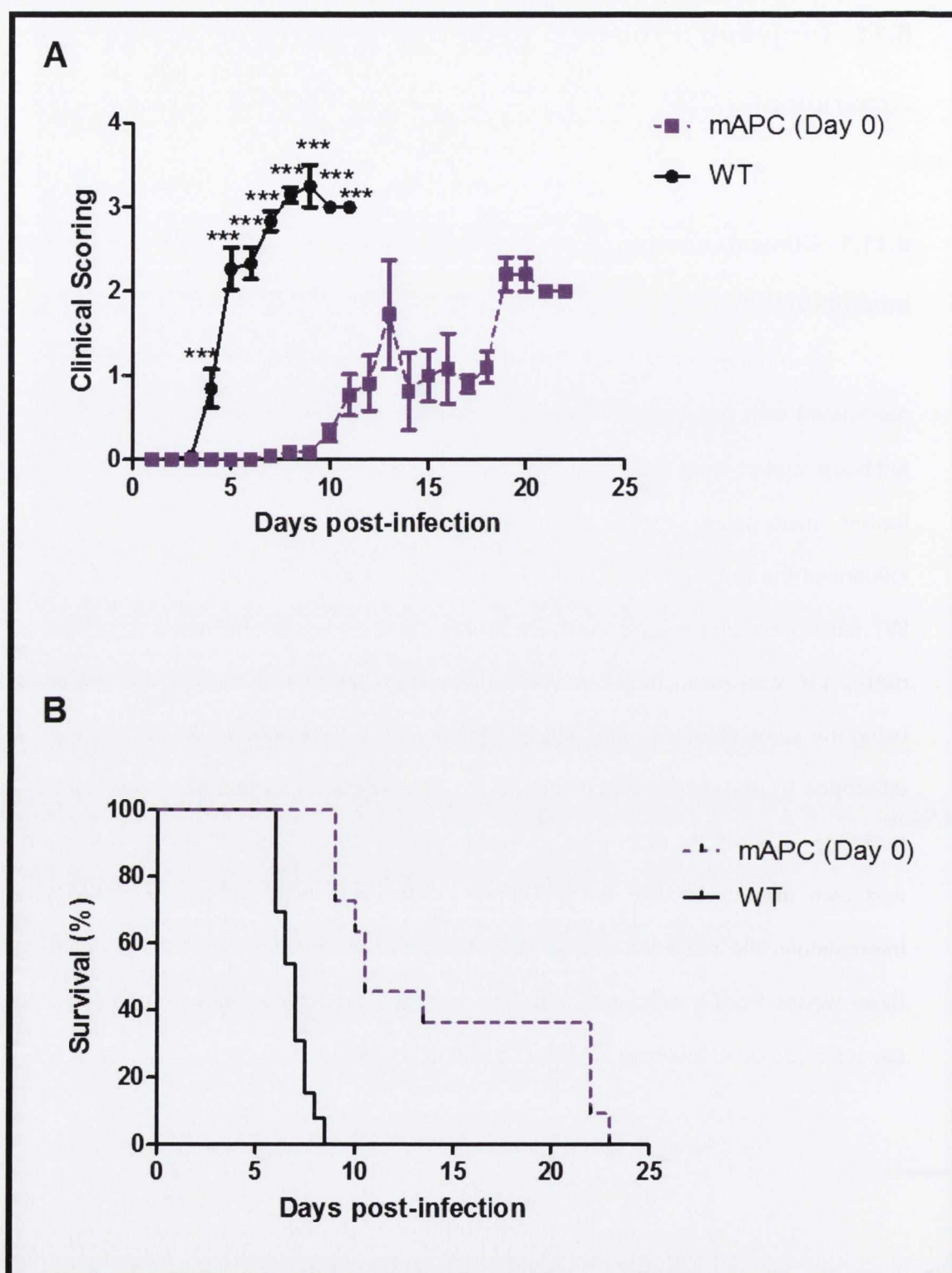


Figure 6. 13 Clinical progression (A) and overall survival (B) in mice treated with APC prior to inoculation with *P. berghei* compared to WT controls

6.11.2 Weight change and parasite counts after treatment with APC

Each animal was monitored daily for weight loss as well as general distress as per ethical guidelines. Weight loss was significantly delayed in mice that had been pre-treated with mAPC. (Figure 6.14A, $P \leq 0.001$) Percentage parasitemia in WT mice was assessed by standard Giemsa staining. Under normal conditions, a positive parasitemia could be seen from Day +2 post inoculation with *P. berghei*. Interestingly, parasite growth was delayed in mice treated with APC at Day +4 of infection (Figure 6.14B, $P \leq 0.001$) (WT $n=13$, APC $n=11$). This suggests APC may affect parasite growth, delaying the onset of disease.

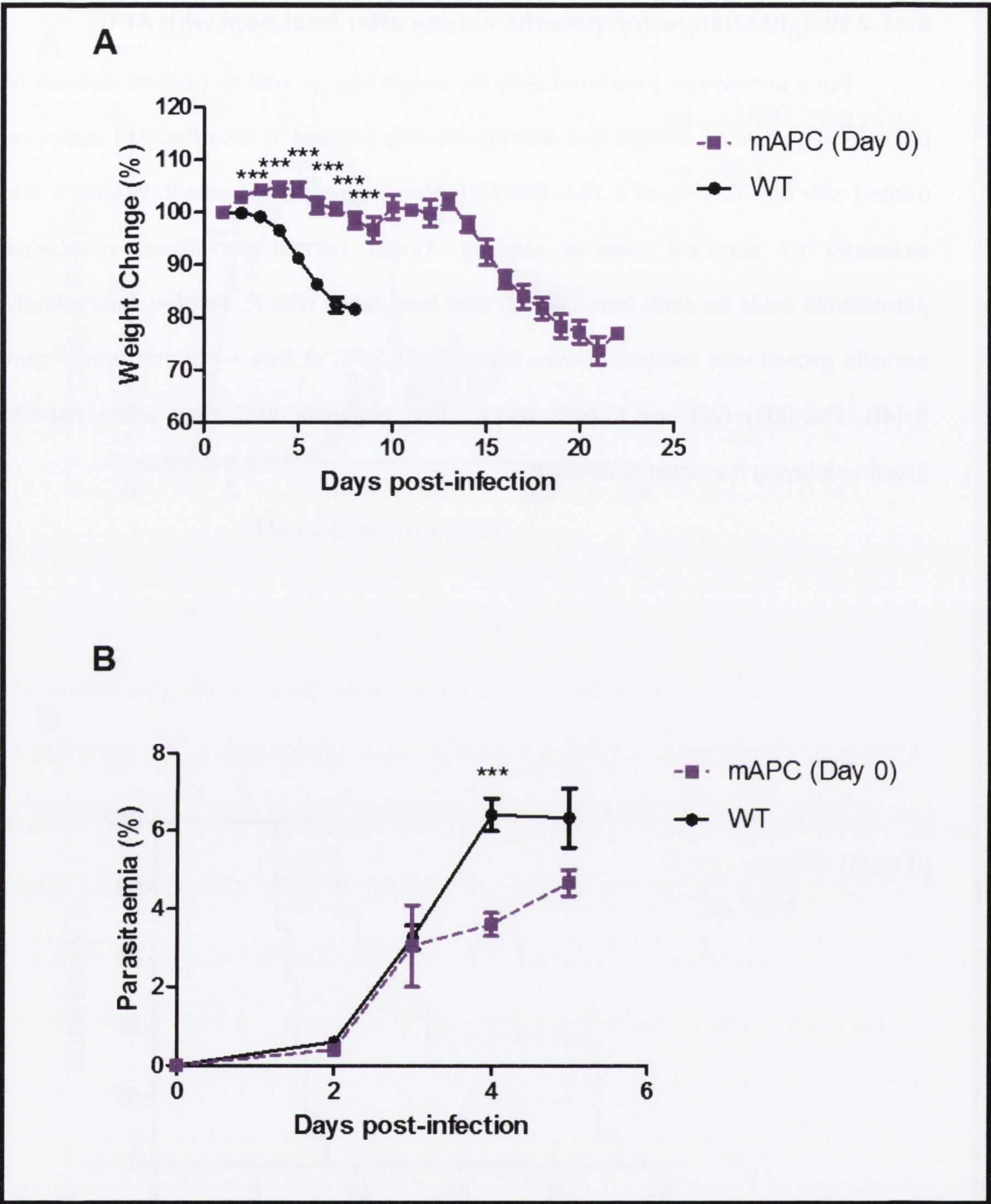


Figure 6. 14 (A) Weight loss and (B) parasitemia growth in mice treated with APC prior to inoculation with *P. berghei* compared to WT controls

6.11.3 Effect of APC administration at Day +3 following *P. berghei* infection

We sought to further investigate the potential use of mAPC as a therapeutic in severe malaria by examining the effects of later APC administration in mice following *P. berghei* infection. As discussed in Chapter 3 thrombocytopenia is a common feature in both patients with *P. falciparum* and in mice with *P. berghei*. By Day +4 following inoculation with parasites, mean platelet count had fallen in mice infected with *P. berghei* by approximately 70%. In order to reduce bleeding risk, we therefore elected to study the effect of treating *P. berghei* infected mice at Day +3. Mice received 10µg of mAPC on Day +3, after another 4 hours they received another 10µg dose of mAPC I.V.

Once again, the clinical progression (Figure 6.15A) (WT n=10, APC n=4) and overall survival (Figure 6.15B) were significantly improved in mice treated with mAPC. Importantly however, the magnitude of this effect was much less than the effect was much less than that observed in mice treated with mAPC on Day 0 prior to *P. berghei* inoculation. In addition, unlike mice treated with mAPC at Day 0, mice treated Day +3 still developed overt clinical features of CM.

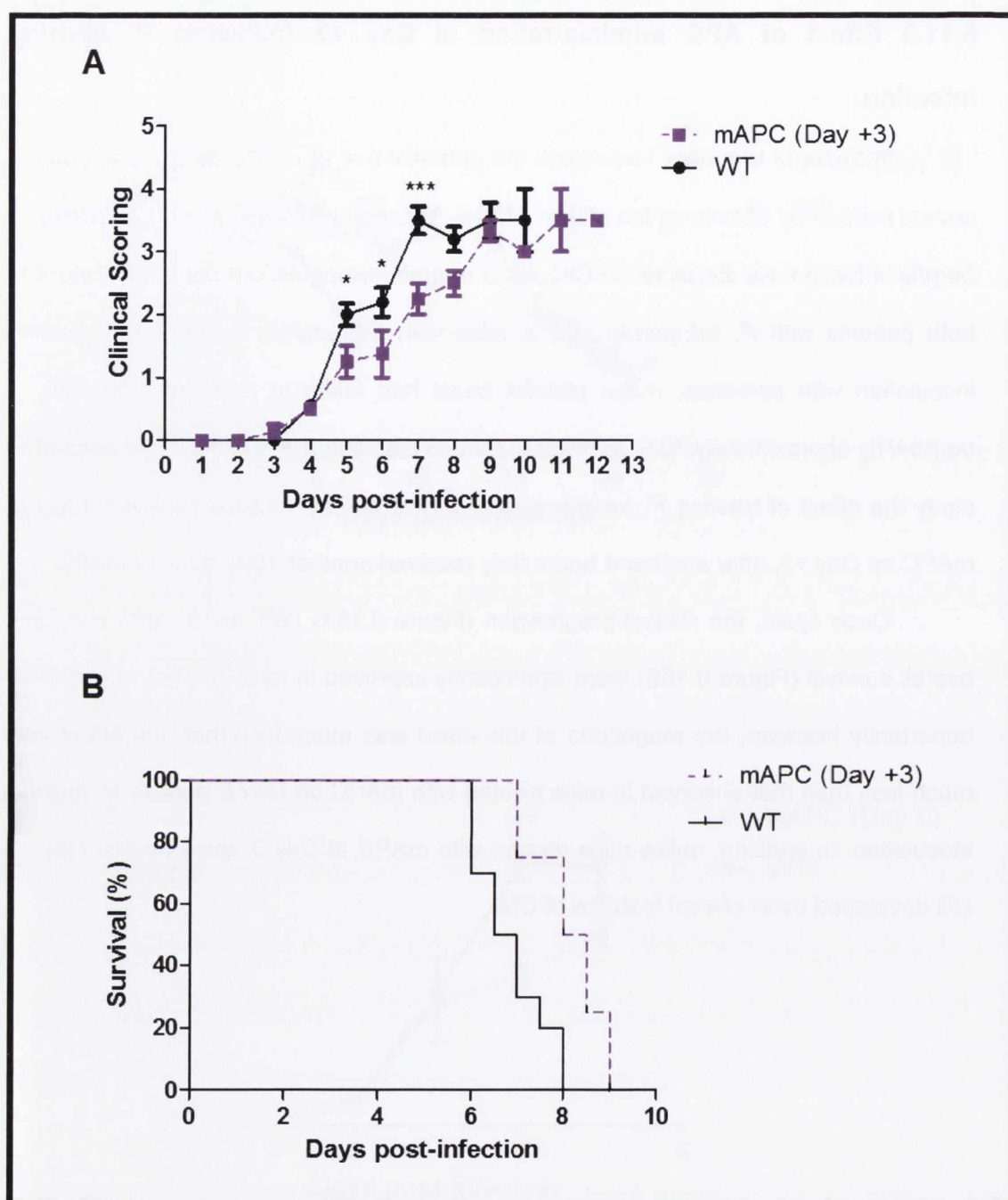


Figure 6. 15 Clinical progression (A) and overall survival (B) in mice treated with APC at Day +3 following *P. berghei* infection compared to WT controls

6.12 Discussion

Recently published data indicates EPCR plays a role in *P. falciparum* malaria (Moxon *et al*, 2013; Turner *et al*, 2013). The brain has a constitutively low expression of both TM and EPCR and might therefore be particularly vulnerable to coagulopathy in CM. However, the molecular mechanisms by which coagulopathy and the PC pathway contribute to CM remain unknown. It has been reported in Gram-negative sepsis that down regulation of both PC and Tm receptors on the endothelium are linked with microvascular thrombus formation in children with meningococcal disease (Faust *et al*, 2001). In addition, plasma PC levels are reduced in patients with severe sepsis and are inversely correlated with mortality (Bernard, 2001). Down regulation of Tm in sepsis could potentially explain the decreased activation of PC in *P. falciparum* infection. Moxon *et al* suggest coagulopathy of CM could potentially be caused by localized rather than a systemic mechanism (Moxon *et al*, 2013). Interestingly, APC has been shown to reduce mortality in a large trial of adults with severe bacterial sepsis. These studies are only adjunctive therapy in management of sepsis with proven efficacy (Bernard *et al*, 2001). Sepsis and malaria share some key similarities including increased plasma VWF levels and reduced ADAMTS13 activity (Ono *et al*, 2006; Löwenberg *et al*, 2010). APC could be of particular interest in the treatment of malaria. As well as roles as an anticoagulant and anti-inflammatory roles, APC is also anti-apoptotic (Joyce *et al*, 2001) and can protect endothelial barrier function (Dömötör *et al*, 2003). Furthermore its functions may be protective against some of the underlying mechanisms associated with CM pathogenesis. We sought to examine if the mouse model of CM could be used to study malarial coagulopathy.

Small haemorrhages seen in post-mortem of the human brain and the retina are a specific feature observed in patients with CM (Dorovini-Zis *et al*, 2011; Thomas, 1971). We observed the presence of macroscopic petechial haemorrhages widely distributed in mice with CM. By Day +5 of infection with *P. berghei* the APTT was

significantly prolonged. This would suggest coagulation factor consumption during experimental infection as seen in sepsis. Although murine APTT and PT were altered in *P. berghei* infection, the extent of the effects were relatively small. These data did not suggest DIC in the murine model of malaria similar to previous findings in human studies. Fibrinogen levels were increased early in infection similar to previous reports in human patients. TAT complexes were significantly elevated during experimental infection. Circulating increased sTm levels have previously been reported in patients with malaria (Moxon *et al*, 2009). We determined sTm levels in the mouse model of experimental CM and found levels increased in similar magnitude to that reported in patients with *P. falciparum*. Shedding of Tm from the endothelium has been shown *in vitro* using serum of *P. falciparum* infected patients (Hemmer *et al*, 1994).

These data indicate that the coagulopathy in experimental CM is similar to that in patients with malaria infection. Further, they strongly implicate the protein C pathway in the pathogenesis of experimental CM. Concurrent loss of TM and EPCR in IE-containing cerebral vessels is suggested by increased sTm and sEPCR in murine malaria. These findings are similar to those in patients with CM (Moxon *et al*, 2013) and the loss of both receptors has also been reported in other diseases (Faust *et al*, 2001; Scaldaferri *et al*, 2007). By diminishing the capacity to activate protein C in this way, there is a shift in the balance of the thrombin-protein C axis toward a proinflammatory and procoagulant state. The reduction in EPCR and TM may thus lead to functional loss and localised coagulopathy. Therefore, these findings suggest that the *P. berghei* experimental CM model may be useful to determine the roles of thrombin generation and the PC pathway in the pathogenesis of malaria.

APC administration has been shown to elicit beneficial effects in other murine disease models. Treatment with APC is effective post transient brain ischaemia (Thiyagarajan *et al*, 2008). Currently the adjunctive use of a recombinant variant of APC with tPA in stroke in humans is currently being assessed in Phase II clinical trials.

Since *P. falciparum* infection causes EC activation and in the release of pro-inflammatory cytokines (Turner *et al*, 1994; Grau *et al*, 1989), APC may offer clinical benefit in severe malaria (Hemmer *et al*, 2011). The therapeutic benefit of APC has not been systematically investigated in malaria. However, case reports of patients with severe *P. falciparum* malaria treated with APC have yielded some promising results (Kendrick *et al*, 2006; Rankin & Austin, 2007). Further larger clinical studies are warranted to prove safety of this treatment.

Our findings have demonstrated that injection of an EPCR function blocking antibody significantly shortened the survival of *P. berghei* infected mice. Furthermore, there was a difference in parasite growth between isotype treated and anti-EPCR treated mice. Moreover, when APC was pre-administered to WT mice they demonstrated significantly enhanced survival. These novel data suggest a key role for the protein C pathway in malaria infection. The huge effect of APC is somewhat surprising considering that the *in vivo* half-life of mAPC is only 15 minutes (Fernandez *et al*, 2009). It is not yet known how the APC is causing survival benefit. Further studies would be warranted to elucidate the molecular mechanisms through which APC is having a beneficial effect. The APC being given prior to infection could be priming the immune system so that it responds more efficiently to challenge with infection. Since APC is an anticoagulant, clearly any potential therapeutic use of APC in children with severe malaria will be associated with a potential bleeding risk. However, a number of different APC variants with altered anticoagulant and cytoprotective signalling functions have already been engineered (Mosnier *et al*, 2004; Kerschen *et al*, 2007).

7. Conclusions

7.1 A novel role for VWF in the pathogenesis of ECM

P. falciparum malaria infection results in early marked increase in plasma VWF levels, together with a pathological accumulation of ULVWF multimers. Moreover elevated VWF:Ag levels inversely correlate with clinical outcomes (Hollestelle *et al*, 2006; Larkin *et al*, 2009). This increase in plasma VWF raises the possibility that VWF may play a direct role in modulating malaria pathogenesis. Furthermore, a study of 14 healthy volunteers infected with *P. falciparum* showed that increased plasma VWF:Ag and VWF propeptide levels develop shortly after the onset of blood stage infection (de Mast *et al*, 2007). Consequently, acute EC activation constitutes an early and consistent feature of *P. falciparum* infection. Using a previously validated murine model of CM, we demonstrate that *P. berghei* infection is associated with significant increases in plasma VWF:Ag and VWF:CB levels, similar to those previously observed in African children with severe *P. falciparum* malaria (Hollestelle *et al*, 2006; Larkin *et al*, 2009; Conroy *et al*, 2010; Erdman *et al*, 2011; Phiri *et al*, 2011). In addition, and in keeping with previous data from human volunteers infected with *P. falciparum* (de Mast *et al*, 2007), these increased plasma VWF levels were observed from a very early stage following *P. berghei* infection. Thus, these data demonstrate that early and consistent EC activation is a feature of the murine CM similar to what has been reported in children infected with *P. falciparum*.

Moreover, we demonstrate that VWF^{-/-} mice are significantly protected against *P. berghei* infection compared to WT controls. This survival difference was not explained by a difference in *P. berghei* parasitemia levels, which were similar in both VWF^{-/-} and WT mice. Significant thrombocytopenia is a common feature of both human and murine malaria (Cox & McConkey, 2010; Faille *et al*, 2009). Interestingly, although previous studies suggested that the ULVWF might be important in mediating this

malaria-associated thrombocytopenia (de Mast *et al*, 2007, 2010), no significant differences in platelet counts were observed between VWF^{-/-} and WT mice at any time point. These findings suggest that VWF^{-/-} mice are protected against CM 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 (Noubade *et al*, 2008; Suidan *et al*, 2013). In this study, we observed that *P. berghei* infection was associated with a significant increase in BBB permeability in both WT and VWF^{-/-} mice. Interestingly however, BBB disruption is delayed in the VWF^{-/-} mice which could contribute to the survival advantage we observed. Given the significant morbidity and mortality associated with CM, these novel data have direct translational significance.

7.2 ADAMTS13 and ASGPR do not modulate susceptibility to ECM

To further investigate the role of VWF in pathogenesis of CM *P. berghei* was assessed in other genetically modified mice strains in which either the ADAMTS13, or the ASGPR gene have been knocked out. The novel data from these studies indicate that the increase in VWF, and the appearance of ULVWF multimers occur in an ADAMTS13-independent mechanism. Furthermore, the observation that *P. berghei* related thrombocytopenia is similar in both ADAMTS13^{-/-} and WT mice suggests that ULVWF does not contribute to the aetiology of the reduced platelet count. Although ADAMTS13 is critical in regulating VWF multimer distribution, and also important in modulating inflammatory responses our data suggest that ADAMTS13 is not important in the pathogenesis underlying ECM. Following infection in ASGPR-deficient mice with higher endogenous levels of VWF:Ag, we observed similar survival outcomes to WT

mice. These results suggest that small increases in basal plasma VWF levels are not important in modulating the effect(s) of VWF on malaria pathogenesis.

7.3 Plasma OPG increases progressively through the course of malarial infection

In keeping with the marked elevation in plasma VWF and VWF:pp levels observed in children with CM, plasma levels of Ang-2, another protein stored within WPB, were also significantly elevated (Conroy *et al*, 2010; Erdman *et al*, 2011; Conroy *et al*, 2009; Lovegrove *et al*, 2009). Moreover, higher plasma Ang-2 levels have also been associated with a worse clinical outcome (Conroy *et al*, 2009; Lovegrove *et al*, 2009; Yeo *et al*, 2008). OPG is another EC protein stored within WPB (Zannettino *et al*, 2005) and is a soluble member of the tumour necrosis factor receptor family that plays a critical role in bone remodelling (Takahashi *et al*, 1999; Yasuda *et al*, 1999). The recruitment of OPG into WPB has been shown to be result through the ability of OPG to bind directly to the A1 domain of VWF (Zannettino *et al*, 2005; Shahbazi *et al*, 2007). OPG binds to the A1 domain of VWF intracellularly and can remain associated after release into the circulation (Shahbazi *et al*, 2007). Djamiatun *et al* demonstrated that severe dengue fever is associated with exocytosis of WPB and consequently markedly elevated VWF:Ag, VWF:pp and OPG plasma levels (Djamiatun *et al*, 2012). Dengue fever shares many of the key clinical features seen in malaria including EC activation and a pronounced thrombocytopenia. Given the key role of EC activation in the pathogenesis of severe *P. falciparum* malaria, we investigated plasma OPG levels in children with cerebral malaria. To further understand the relationship between WPB secretion and malaria, we have further investigated OPG levels in an established murine model of experimental CM.

This data has shown that plasma OPG levels are elevated approximately 20 fold in both children with CM, and in a murine model of ECM. In addition, using this murine model we further demonstrated that the increase in plasma OPG developed at

an early stage following malaria infection. Furthermore, plasma OPG level increased progressively through the course of infection, and correlated with other established markers of outcome in patients with severe malaria. In conclusion, these findings suggest that OPG may have a role to play as a novel biomarker in patients with severe malaria infection, and also raise the intriguing possibility that OPG may have a role to play in modulating malaria pathogenesis.

7.4 Coagulation activation and the role of APC as a potential therapeutic

Infection with *P. falciparum* is associated with thrombocytopenia, decreased levels of anticoagulant clotting factors, and generation of thrombin. Whether coagulopathy is involved in the pathogenesis of malaria remains unknown (Kelton *et al*, 1983; Pukrittayakamee *et al*). Previously, APC has been shown to reduce mortality in trials of adult patients with severe bacterial sepsis (Bernard *et al*, 2001). Sepsis and malaria share some key similarities including increased plasma VWF levels and reduced ADAMTS13 activity (Ono *et al*, 2006; Löwenberg *et al*, 2010). The molecular mechanisms by which coagulopathy and the protein C pathway contribute to CM remain unknown. We sought to examine if the mouse model of CM could be used to study malaria-induced coagulopathy, and whether the PC pathway was important in this context.

Our novel data demonstrate that the coagulopathy of experimental CM is similar to patients with malaria infection. Furthermore, treatment with an EPCR function blocking antibody significantly shortened the survival of *P. berghei* infected mice. Moreover, when APC was pre-administered to WT mice, they demonstrated significantly enhanced survival. In conclusion, these data strongly implicate the protein C pathway in the pathogenesis of experimental CM.

8. Future directions

8.1 Further defining a role for VWF in modulating the pathogenesis of experimental CM using hydrodynamic injection strategy

On the basis of published data along with recent *in vitro* studies performed in our laboratory (Larkin *et al*, 2009; Bridges *et al*, 2010), it seems likely that ULVWF multimers released into plasma subsequent to acute EC activation are important in the progression of severe malaria. This hypothesis is supported by data demonstrated in these studies whereby VWF^{-/-} mice are significantly protected against a lethal dose of *P. berghei* malaria. VWF synthesized within EC is either directly released into the plasma through a constitutive secretory pathway, or stored in WPB (Sadler, 1998). Previous studies have shown that WPB formation within EC occurs only in the presence of VWF (Denis *et al*, 1998). It therefore remains unclear whether the malaria-resistant phenotype observed in VWF^{-/-} mice is due to the absence of plasma VWF, or is primarily attributable to a secondary absence of WPB.

- I. To definitively address whether plasma VWF plays a role in modulating the pathogenesis of experimental CM, we will replace plasma VWF in the VWF^{-/-} mice using a hydrodynamic injection strategy. Murine VWF cDNA has been cloned into a specific vector enabling high and continual levels of hepatic expression (pSC11-ET-mVWF-bGHpA; kind gift Prof David Lillicrap, Kingston, Canada). This plasmid DNA will be diluted in a volume of saline (0.9% NaCl) equivalent to 10% of the bodyweight (i.e., 2 mL for a 20 g mouse) and then injected into the mouse tail vein within 7 seconds as previously described (Lenting *et al*, 2007). Following hydrodynamic injection and the normalisation of plasma VWF levels in

the VWF^{-/-} mice, we will assess disease progression using the clinical scoring system and measure relevant plasma biomarkers as before.

II. VWF^{+/-} mice also have significantly reduced plasma VWF:Ag and VWF:pp levels. We will investigate *P. berghei* infection in there heterozygous to determine if there is any effect on survival. VWF heterozygosity has previously shown partial effects compared to full knock out animals in an ischemia model (Zhao *et al*, 2009).

III. *P. chabaudi* is another species of murine-specific malaria. Although *P. chabaudi* is also lethal in C57BL/6 wild type mice, the clinical phenotype is quite distinct to that observed with *P. berghei*. Notably, C57BL/6 mice infected with *P. chabaudi* do not develop encephalopathy, but rather die after 3-4 weeks of infection due to severe anaemia and hyperparasitemia (Hunt & Grau, 2003). Consequently, to gain further insight into the role of VWF in mediating *in vivo* malaria pathogenesis, we will also determine whether VWF^{-/-} and VWF^{+/-} mice exhibit any difference in susceptibility to *P. chabaudi* infection compared to matched WT controls.

8.2 Elucidating the mechanism responsible for the persistence of ULVWF in the presence of unaltered ADAMTS13 activity

ADAMTS13 cleaves the Y1605/M1606 bond in the VWF A2 domain, thus preventing the accumulation of ULVWF multimers in normal plasma, and therefore regulates the functional activity of VWF (Levy *et al*, 2001). Nevertheless, it remains undefined how VWF proteolysis by ADAMTS13 is regulated *in vivo*. ADAMTS13 activity as assessed by FRETS VWF73 assay remained unchanged during malaria infection. Residual ADAMTS13 activity above 10% has been suggested to be adequate

in maintaining normal plasma VWF multimer distribution in patients (Dong *et al*, 2004). From our novel data, it appears that *in vivo* inhibition of plasma ADAMTS13 activity may play a role in ECM contributing to ULVWF. There are many proteins suggested to regulate the ability of ADAMTS13 to cleave VWF *in vivo* including interleukin 6 (IL-6) (Bernardo *et al*, 2004), thrombospondin-1 (TSP-1) (Bonney *et al*, 2006), free plasma haemoglobin (Stoldt *et al*, 2005), and reduced factor VIII (FVIII) levels (Cao *et al*, 2008).

- I. We will further examine if the presence of ULVWF is attributable to one of these possible inhibitors. To investigate this we will measure these potential inhibitors to ascertain if any of these are contributing to the inhibition of ADAMTS13 activity in murine ECM.
- II. Previous studies have demonstrated that recombinant human ADAMTS13 treatment is beneficial in diseases such as stroke, ischemia and atherosclerosis (Zhao *et al*, 2009; Gandhi *et al*, 2012b; De Meyer *et al*, 2012). ADAMTS13 could potentially dismantle existing thrombi formed during ischemia, and prevent new thrombi from assembling by cleaving the VWF multimers. Administration of recombinant ADAMTS13 could therefore potentially represent a novel therapeutic in CM. To examine this we will administer recombinant ADAMTS13 prior to inoculation with *P. berghei* and at Day +3 of infection and assess clinical outcome.

8.3 Histological analysis

To further investigate the mechanism through which VWF^{-/-} mice are protected from ECM, histological studies will be performed.

- I. Brains will be harvested from sacrificed WT and VWF^{-/-} mice for this histological analysis. These histo-pathological studies will be performed as part of an ongoing collaboration with Prof. Georges Grau (University of Sydney, Australia). In brief, mice will be anaesthetised and perfused with 40mL saline. Fresh tissue samples will be frozen with Isopentane and liquid nitrogen immediately after harvesting. 3µM sections will be stained with hematoxylin and eosin. In addition, immunohistochemistry will be performed on frozen brain sections as previously described (Rogerson *et al*; Chang-Ling *et al*, 1992).
- II. To investigate coagulopathy in *P. berghei* infection mice will be infected with parasites and subsequently brains will be harvested for histological investigation of haemorrhages, lesions fibrin deposition and EPCR expression. In brief, control mice or mice inoculated with *P. berghei* (Day +5) will be anaesthetised with TBE and perfused with 40mL 0.9% saline. Fresh tissue samples will be frozen with Isopentane and liquid nitrogen immediately after harvesting. Immunohistochemistry investigating the EPCR will be performed on brain sections as previously described (Rogerson *et al*; Chang-Ling *et al*, 1992).

8.4 APC variants in the treatment of experimental CM

The efficacy of APC as a potential therapeutic in CM has not been systematically investigated. Previously, case reports of patients with severe *P. falciparum* malaria infection improving following treatment with APC have been reported (Rankin & Austin, 2007; Kendrick *et al*, 2006). From our findings, mAPC significantly prolongs survival when administered to mice either prior to, or subsequent to, infection with *P. berghei*. In an effort to improve APC therapy in severe sepsis, a

number of different APC variants with altered anticoagulant and cytoprotective signalling functions have been engineered (Mosnier *et al*, 2004; Kerschen *et al*, 2007).

- I. To investigate the effectiveness of variant mAPC in treatment of malaria, we will engineer different variants of APC. These variants will have different properties including enhanced cytoprotective variants and abolished anticoagulant properties. These variants will be administered at different time points of infection.

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Appendix I: Abstracts leading to poster presentations

VON WILLEBRAND FACTOR PLAYS A ROLE IN THE PATHOGENESIS OF MURINE CEREBRAL MALARIA

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Cerebral malaria (CM) is a major cause of death of children in sub-Saharan Africa. Previous studies demonstrated plasma VWF:Ag levels markedly elevated in children with falciparum malaria; these levels inversely correlate with clinical outcomes. Ultra-large VWF (ULVWF) multimers have also been observed in children with malaria. Platelet-decorated ULVWF strings have been shown to recruit infected red cells to endothelial cell (EC) surfaces. These emerging data suggest VWF may play a novel role in the pathogenesis of CM.

Wild type (WT) mice were inoculated with 2×10^6 *P. berghei* ANKA. CM symptoms were monitored using a clinical scoring algorithm. Blood was collected at different time points of infection. *In vivo* markers for EC activation were evaluated using ELISAs.

Similar to children with malaria, acute EC activation was also a feature in the murine model of CM. VWF:Ag levels were significantly elevated. Angiopoietin-2 and osteoprotegerin levels were both significantly elevated from Day+3. Despite murine plasma ADAMTS13 activity levels were not significantly reduced, ULVWF were observed following *P. berghei* inoculation.

To determine whether VWF plays a direct role in modulating the pathogenesis of CM, we investigated *P. berghei* infection in VWF^{-/-} mice. Overall survival was significantly prolonged in VWF^{-/-} mice compared to WT mice. A significant delay in clinical progression in VWF^{-/-} mice was also observed. Recent studies demonstrated platelets play a direct role in modulating malaria parasite killing. In keeping with observations in human patients infected with *P. falciparum*, we show significant thrombocytopenia was also a consistent feature in the murine model. Interestingly however, thrombocytopenia was observed in VWF^{-/-} mice. Despite significant differences in overall survival no difference in platelet counts were observed. These findings suggest VWF^{-/-} mice are protected through a platelet-independent mechanism.

We demonstrate EC activation is a feature of the murine CM similar to previous findings of children infected with *P. falciparum*. Our findings show marked elevation of VWF:Ag, and the appearance of pathological ULVWF multimers in plasma, both represent hallmarks of human and murine CM. We report that VWF^{-/-} mice exhibit significantly prolonged survival against CM, and this is mediated through a platelet-independent mechanism. Given the significant mortality associated with CM, these novel data have direct translational significance.

17th Institute of Molecular Medicine Annual Meeting, 2014

VON WILLEBRAND FACTOR PLAYS A ROLE IN THE PATHOGENESIS OF MURINE CEREBRAL MALARIA

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To determine whether VWF plays a direct role in modulating the pathogenesis of CM, we investigated *P.berghei* infection in VWF^{-/-} mice. Overall survival was significantly prolonged in VWF^{-/-} mice compared to WT mice. A significant delay in clinical progression in VWF^{-/-} mice was also observed. Recent studies demonstrated platelets play a direct role in modulating malaria parasite killing. In keeping with observations in human patients infected with *P.falciparum*, we show significant thrombocytopenia was also a consistent feature in the murine model. Interestingly however, thrombocytopenia was observed in VWF^{-/-} mice. Despite significant differences in overall survival no difference in platelet counts were observed. These findings suggest VWF^{-/-} mice are protected through a platelet-independent mechanism.

We demonstrate EC activation is a feature of the murine CM similar to previous findings of children infected with *P.falciparum*. Our findings show marked elevation of VWF:Ag, and the appearance of pathological ULVWF multimers in plasma, both represent hallmarks of human and murine CM. We report that VWF^{-/-} mice exhibit significantly prolonged survival against CM, and this is mediated through a platelet-independent mechanism. Given the significant mortality associated with CM, these novel data have direct translational significance.

3rd Irish Thrombosis and Vascular Biology meeting 2014

COAGULOPATHY IN *PLASMODIUM BERGHEI* MALARIA

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Background

Numerous studies have suggested the presence of coagulopathy in *P. falciparum* malaria due to elevated levels of circulating thrombin-antithrombin III complexes (TAT), fibrinopeptide A and soluble thrombomodulin (TM). More recently, the anti-coagulant and anti-inflammatory endothelial protein C receptor (EPCR) has been shown to play a role in *P. falciparum* malaria. The brain has a constitutively low expression of both TM and EPCR and might therefore be particularly vulnerable to coagulopathy in cerebral malaria. However, the molecular mechanisms by which coagulopathy and EPCR contribute to cerebral malaria remain unknown. We sought to investigate if the *P. berghei* cerebral malaria mouse model may be used to study malarial coagulopathy. We further investigated whether the EPCR plays a role in *P. berghei* induced cerebral malaria

Methods

We utilised the *P. berghei* cerebral malaria model in C57Bl/6J mice to investigate coagulation parameters during infection. We infected mice with 2×10^6 *P. berghei* infected red blood cells. Subsequently, mice were sacrificed at different time points by exsanguination and the activity and levels of coagulation markers were determined by ELISA, coagulation and fluorogenic assays. To investigate whether EPCR plays a role in experimental cerebral malaria, mice were injected with RCR-16 (kind gift from Prof K. Fukudome), an established function blocking antibody against EPCR, or isotype control antibody prior to *P. berghei* infection.

Results

Thrombin anti-thrombin complexes are upregulated in experimental cerebral malaria. Endogenous Thrombin Potential (ETP), APTTs and prothrombin time (PT) are altered following cerebral malaria infection in mice. Soluble thrombomodulin levels are elevated in experimental cerebral malaria. Fibrinogen levels are increased in early *P. berghei* infection. Injection of an EPCR blocking antibody results in significantly shortened survival of *P. berghei* infection in C57Bl/6J mice. Injection of an EPCR blocking antibody does not alter parasitaemias, clinical symptoms or weight change of *P. berghei* infection in C57Bl/6 mice.

CONCLUSIONS

Thrombin anti-thrombin complex, soluble thrombomodulin and fibrinogen levels are elevated in *P. berghei* infection and corresponds to previously published findings in plasma samples from human *P. falciparum* malaria infection. ETPs from *P. berghei*

control vs cerebral malaria mice are not significantly altered, but show a trend toward increased thrombin generation during cerebral malaria. Injection of an EPCR function blocking antibody significantly shortened survival of *P. berghei* infected mice. We conclude that the *P. berghei* cerebral malaria mouse model can be used to study malarial coagulopathy.

3rd Irish Thrombosis and Vascular Biology meeting 2014

Appendix II: Abstracts leading to oral presentations

VON WILLEBRAND FACTOR PLAYS A ROLE IN MEDIATING MALARIA PATHOGENESIS IN THE MURINE CEREBRAL MALARIA MODEL

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²Department of Paediatrics, OLHSC

Introduction

Cerebral malaria (CM) is seen in ~1% of children with *P. falciparum* infection and contributes to almost 1 million malaria related deaths annually. Plasma Von Willebrand Factor (VWF) levels are markedly increased in children with severe *P. falciparum* infection, consistent with acute endothelial cell (EC) activation. The aim of this project is to characterise *in vivo* EC activation in murine cerebral malaria and to elucidate the molecular mechanism(s) through which VWF contributes to the severity of CM.

Materials and Methods

8-10 week old male and female C57BL/6 WT or VWF^{-/-} mice were injected with $\leq 2 \times 10^6$ *P. Berghei* ANKA. CM symptoms were monitored using a well-established clinical scoring system. Blood was taken on day 3 and day 5 of malaria infection and EC activation markers were evaluated using an enzyme-linked immunosorbent assay.

Results

We assessed indices of EC activation including VWF and osteoprotegerin (OPG) levels. VWF levels were markedly increased in day 5 of infection in WT mice compared to uninfected controls ($P < 0.0001$). OPG increased, correlating with disease progression, in both WT and VWF^{-/-} mice ($P < 0.0001$) confirming marked EC activation in murine malaria. Interestingly, VWF^{-/-} mice were significantly protected against a lethal dose of *P. berghei* infection when compared to WT in survival studies. In addition, the onset of experimental CM using a clinical scoring algorithm was significantly delayed in VWF^{-/-} mice.

Conclusions

This data clearly shows EC activation plays a role in experimental CM. VWF might be directly involved in mediating the pathogenesis of infection.

Haematology Association of Ireland Annual Meeting, 2013

Oral Presentation, Presidents Symposium

VON WILLEBRAND FACTOR PLAYS A ROLE IN MEDIATING MALARIA PATHOGENESIS IN THE MURINE CEREBRAL MALARIA MODEL

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Conclusions

This data clearly shows EC activation plays a role in experimental CM. VWF might be directly involved in mediating the pathogenesis of infection.

16th Institute of Molecular Medicine Annual Meeting, 2013

VON WILLEBRAND FACTOR PLAYS A ROLE IN MEDIATING MALARIA PATHOGENESIS IN THE MURINE CEREBRAL MALARIA MODEL

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Results

We assessed VWF levels as indices of EC activation. VWF levels were markedly increased in day 5 of infection in WT mice compared to uninfected controls ($P < 0.0001$). ADAMTS13, the enzyme that cleaves VWF *in vivo* decreased correlating with disease progression. Interestingly, VWF^{-/-} mice were significantly protected against a lethal dose of *P. berghei* infection when compared to WT in survival studies. In addition, the onset of experimental CM using a clinical scoring algorithm was significantly delayed in VWF^{-/-} mice.

Conclusions

This data clearly shows EC activation plays a role in experimental CM. VWF might be directly involved in mediating the pathogenesis of infection.

National Children's Research Centre Annual Symposium, 2013

VON WILLEBRAND FACTOR IN CEREBRAL MALARIA

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Introduction

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Materials and Methods

8-10 week old male and female C57BL/6 WT or VWF^{-/-} mice were injected with $\leq 2 \times 10^6$ *P. Berghei* ANKA. CM symptoms were monitored using a well-established clinical scoring system. Blood was taken on day 3 and day 5 of malaria infection and EC activation markers were evaluated using an enzyme-linked immunosorbent assay.

Results

We assessed indices of EC activation including VWF and osteoprotegerin (OPG) levels. VWF levels were markedly increased in day 5 of infection in WT mice compared to uninfected controls ($P < 0.0001$). OPG increased, correlating with disease progression, in both WT and VWF^{-/-} mice ($P < 0.0001$) confirming marked EC activation in murine malaria. Interestingly, VWF^{-/-} mice were significantly protected against a lethal dose of *P. berghei* infection when compared to WT in survival studies. In addition, the onset of experimental CM using a clinical scoring algorithm was significantly delayed in VWF^{-/-} mice.

Conclusions

This data clearly shows EC activation plays a role in experimental CM. VWF might be directly involved in mediating the pathogenesis of infection.

3rd Irish Thrombosis and Vascular Biology meeting 2014

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

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Cerebral malaria (CM) is a major cause of death in young children in sub-Saharan Africa. Although the pathogenesis of this condition remains poorly understood, *Plasmodium falciparum* infection results in adhesion of infected erythrocytes (IE) to microvascular endothelium, and acute endothelial cell (EC) activation. Recent studies have shown that plasma VWF:Ag and VWF propeptide levels are markedly elevated in children with severe falciparum malaria. Moreover elevated VWF:Ag levels inversely correlate with clinical outcomes. In addition, circulating ultra-large VWF multimers have also been observed in children with severe malaria. Importantly, platelet-decorated UL-VWF strings have been shown to recruit trophozoite-stage falciparum-IE to EC surfaces in shear-based assays. Collectively, these emerging data suggest that VWF may play a novel role in the pathogenesis of CM. To further investigate this hypothesis, we utilized an established murine model of experimental CM which involves wild type (WT) C57BL/6J mice infected with *Plasmodium berghei* ANKA.

In brief, WT C57BL/6J mice were inoculated with 2×10^6 *P. berghei* ANKA via I.P. injection. These mice typically developed CM and died within 6-8 days. In keeping with findings in children with falciparum malaria, acute EC activation was also an early and consistent feature in the murine model of CM. Mean plasma VWF:Ag levels were significantly elevated from Day +3 in infected mice compared to controls (Day +3 1.8 fold increase; Day +5 2.5 fold increase; $p < 0.01$). In addition, plasma angiopoietin-2 and osteoprotegerin levels were also both significantly elevated from Day +3 and Day +5 respectively in mice infected with *P. berghei*. Interestingly, despite the fact that murine plasma ADAMTS13 activity levels were not significantly reduced, pathological ultra-large VWF multimers (UL-VWF) were also observed in murine plasma from Day +3 following *P. berghei* inoculation. Cumulatively these findings suggest that early marked elevation in VWF:Ag levels, and the appearance of pathological UL-VWF multimers in the plasma, represent hallmarks of both human and murine severe malaria infection.

To determine whether VWF plays a direct role in modulating the pathogenesis of CM *in vivo*, we further investigated *P. berghei* infection in VWF^{-/-} C57BL/6J mice. Importantly, although there was no difference in blood parasitemia levels, overall survival was significantly prolonged in VWF^{-/-} mice compared to wild type mice (6 versus 7.25 days; $p = 0.0106$). Moreover, a significant delay in malaria clinical progression in the VWF^{-/-} mice was also observed using a previously validated clinical scoring algorithm for experimental cerebral malaria. (Amante *et al*, Am J Path 2007). Recent studies have demonstrated that platelets may play a direct role in modulating malaria parasite killing. Consequently, to investigate potential mechanisms through which VWF^{-/-} mice are protected against experimental CM, daily platelet counts were determined in WT and VWF^{-/-} mice following infection. In keeping with observations in human patients infected with falciparum malaria, significant thrombocytopenia was also a consistent feature in the murine model. For example, by Day +4 following inoculation of WT mice mean platelet count had fallen by 71.6 ± 26 %. Interestingly however, significant thrombocytopenia was also observed in VWF^{-/-} mice infected with *P. berghei* (84.2 ± 9 %). Despite the significant differences in clinical progression and overall survival no significant differences in platelet counts were observed between VWF^{-/-} and WT mice

at any time point. These findings suggest that VWF^{-/-} mice are protected against CM through a platelet-independent mechanism.

In conclusion, we demonstrate that early and consistent EC activation is a feature of the murine model of experimental CM similar to previous findings of children infected with *P. falciparum*. In addition, our findings show that marked elevation of VWF:Ag, and the appearance of pathological UL-VWF multimers in the plasma, both represent hallmarks of human and murine severe malaria infection. Finally, we report that VWF^{-/-} mice exhibit significantly prolonged survival against CM, and that this effect is mediated through a platelet-independent mechanism. Given the significant morbidity and mortality associated with CM, these novel data have direct translational significance.

56th American Society of Haematology Meeting and Exposition, 2014

MARKED ELEVATION IN PLASMA OSTOPROTEGERIN (OPG) CONSTITUTES AN EARLY AND CONSISTENT FEATURE OF CEREBRAL MALARIA

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Introduction

Although the molecular mechanisms underlying the pathogenesis of severe *P. falciparum* remain poorly understood, previous studies demonstrated a critical role for endothelial cell (EC) activation. In particular, markedly elevated plasma von Willebrand factor (VWF) and VWF:pp levels have been observed in children with cerebral malaria (CM). These proteins are both stored within EC Weibel-Palade bodies (WPB). Osteoprotegerin (OPG) is another protein stored within WPB. Moreover, recent studies have demonstrated roles for OPG in modulating vascular biology. Given the key role of EC activation in the pathogenesis of CM, we investigated OPG levels in children with CM, and in an established murine model of experimental CM.

Methodology

Children with CM were recruited from patients presenting to the Queen Elizabeth Central Hospital in Blantyre, Malawi. Wild type C57BL/6J mice were inoculated I.P. with 2×10^6 *P. berghei* ANKA. *In vivo* markers for EC activation were evaluated by ELISA.

Results

In keeping with previous studies, plasma VWF levels were significantly elevated (3.6-fold) in children with CM compared to healthy controls. In addition, plasma OPG levels were also markedly elevated in children with CM. Interestingly however, in contrast to the rise observed in VWF levels, plasma OPG levels were increased almost 20-fold. Plasma lactate constitutes a useful biochemical prognostic indicator in CM. Unsurprisingly, plasma lactate levels were significantly elevated in children with CM. Importantly, plasma OPG levels were significantly correlated with lactate levels. 60% of children with CM had plasma lactate greater than 5mmol/L, consistent with lactic acidosis. OPG levels in these children with CM and lactic acidosis were markedly higher. Peripheral blood parasitemia levels and platelet count have both also been defined as markers of malaria severity. In children with CM we observed that OPG was significantly correlated with parasite count, and inversely correlated with platelet count.

To further investigate the relationship between CM and OPG, we utilised an established murine model of experimental CM. In keeping with our findings in children with CM, a marked increase in plasma OPG was observed in *P. berghei* infected. By Day +5 following inoculation, murine OPG was increased approximately 18-fold, which was very similar in magnitude to that observed in children with CM. Interestingly, a significant increase in plasma OPG was observed from an early stage following

infection. OPG levels were already increased 4.6-fold within 24 hours following *P. berghei* inoculation.

Conclusions

In conclusion, we demonstrate for the first time that plasma OPG levels are elevated approximately 20-fold in both children with CM, and in a murine model of experimental CM. This marked increase in plasma OPG is evident from an early stage following malaria infection. Furthermore, plasma OPG increases progressively through the course of infection, and correlates with other established markers of outcome in patients with severe malaria. Collectively, these findings demonstrate that OPG constitutes an entirely novel prognostic biomarker in patients with severe malaria infection, and also raise the intriguing possibility that OPG may play a role in modulating malaria pathogenesis.

Haematology Association of Ireland, Annual Meeting 2015.

Appendix III: Manuscripts submitted for publication

A novel role for von Willebrand factor in the pathogenesis of experimental cerebral malaria

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KEY POINTS

Experimental cerebral malaria is associated with an early marked increase in plasma VWF levels and accumulation of UL-VWF multimers.

Following *Plasmodium berghei* infection, VWF^{-/-} 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^{-/-} C57BL/6J mice. Clinical ECM progression was delayed and overall survival was significantly prolonged in VWF^{-/-} mice compared to wild type controls. Despite this protection against ECM, no significant differences in platelet counts, or blood parasitemia levels, were observed between VWF^{-/-} and WT mice. Interestingly however, the degree of ECM-associated enhanced blood brain barrier permeability was significantly attenuated in VWF^{-/-} 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.¹⁻³ Although the biological mechanisms involved in the pathophysiology of severe *P. falciparum* malaria remain poorly understood, previous studies have demonstrated that sequestration of *P. falciparum* infected erythrocytes (IE) within the microvasculature of the brain is important in the development of cerebral malaria (CM).^{4,5} This sequestration involves adhesion of IE to host vascular endothelial cell (EC) surfaces,⁶⁻⁸ and is mediated by a variety of specific EC adhesion molecules including CD36, intercellular adhesion molecule-1 (ICAM-1), and thrombospondin.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.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.^{11,12}

Von Willebrand factor (VWF) is a large sialoglycoprotein synthesized within EC and megakaryocytes.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.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.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.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.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.^{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.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.^{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.^{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.¹⁶ In addition, plasma VWF levels also correlate inversely with platelet count, and with overall clinical outcome.^{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 conformation that promotes platelet GPIb binding.²⁴ This observation is important since platelet adhesion and accumulation have been implicated in facilitating the adhesion of *P. falciparum* IE to activated EC.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.²⁸

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^{-/-} mice were initially obtained from Jackson Laboratories (Bar Harbor, USA). These VWF^{-/-} mice were on a C57BL/6J background and have previously been characterized.^{29,30} Wild type (WT) C57BL/6J and VWF^{-/-} 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°C, aliquoted and stored at -80°C until use.

P. berghei Survival Studies

Mice were infected by intraperitoneal injection of 2×10^6 *Plasmodium berghei* ANKA. Following inoculation, malaria progression was monitored using a previously validated clinical scoring system to assess ECM.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, 0.5 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* parasitemia levels were monitored by examination of Giemsa-stained thin blood smears obtained from tail vein bleeds (VWR International Inc.).

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).^{32,33} 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.^{34,35} 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 50 μ L 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.^{17,36} 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 μ g/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.³⁷ 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.¹⁷

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,^{38,39} 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% tribromoethanol, 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 $\pm$ 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.^{40,41} In keeping with previous findings in children with severe *P. falciparum* malaria,^{16,17} 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.¹⁶ Interestingly, a significant increase in plasma VWF:Ag levels were already evident within 24 hours following *P. berghei* inoculation. This early increase in plasma VWF levels occurred before the appearance of significant blood parasitemia 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*,¹⁷ 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). This observation likely relates to the fact that murine VWF:pp has a much shorter half-life compared to that of human VWF:pp (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.^{16,17,42,43} 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.⁴⁴ 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.^{18,19,45-47} 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.¹⁷ 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). 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 ADAMTS13.⁴⁸ Previous studies have reported modest reductions in plasma ADAMTS13 activity levels in children with *P. falciparum* infection.^{17,21,22} 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 anemia (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^{-/-} mice compared to WT. Using a previously validated clinical scoring algorithm to assess experimental malaria progression,³¹ we found that the clinical features of ECM progressed significantly more slowly in the VWF^{-/-} mice compared to WT mice ($P=0.001$) (Figure 4A). Importantly however, VWF^{-/-} mice still developed typical neurological signs, and died from ECM rather than severe anemia. In addition, overall survival was significantly prolonged in VWF^{-/-} 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^{-/-} mice remained alive.

Thrombocytopenia in ECM is independent of plasma VWF

Significant thrombocytopenia is common feature in patients with *P. falciparum* malaria.^{49,50} This thrombocytopenia is predominantly due to increased platelet clearance.^{24,51} A putative role for UL-VWF in contributing to this enhanced platelet clearance has been proposed.^{23,24} Furthermore, recent studies have identified novel roles for platelets in killing intraerythrocytic malaria parasites,⁵² and in modulating host immune responses during the early stages of *P. falciparum* infection.⁵³ Consequently, to investigate potential mechanisms through which VWF^{-/-} mice were protected against ECM, daily platelet counts were performed in WT and VWF^{-/-} 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^{-/-} 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^{-/-} and WT mice. Finally, we observed no significant differences in blood *P. berghei* parasitemia levels between VWF^{-/-} 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.⁵⁴ In addition, endothelial VWF has recently been shown to play an important role in modulating permeability of the BBB under different pathological conditions.^{38,39} Although the underlying biological mechanisms remain poorly understood, enhanced permeability of the BBB has also been implicated in the pathogenesis of CM.⁵⁵⁻⁵⁸ We therefore investigated BBB permeability in VWF^{-/-} 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^{-/-} and WT mice prior to *P. berghei* inoculation (Figure 6). 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^{-/-} 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 *P. berghei* ANKA typically develop a complex neurological syndrome involving clinical features similar to those observed in human CM^{40,41,59}. These include ataxia, paralysis, seizures and coma. Depending upon the dose of *P. berghei*, infected mice typically die within 6 to 10 days. Although there are important differences between human CM and the ECM model,^{60,61} both *P. falciparum* and *P. berghei* infections are associated with pronounced effects on EC biology.^{40,41,62,63} In this study, we demonstrate that *P. berghei* infection mirrors the activated EC phenotype observed in African children with severe *P. falciparum* malaria, with significant increases in plasma VWF:Ag and VWF:CB levels.¹⁶⁻²⁰ In addition, and in keeping with previous data from human volunteers infected with *P. falciparum*,²³ these increased plasma VWF levels were observed from a very early stage following *P. berghei* infection. Thus, the early acute EC activation and WP body secretion which constitute hallmarks of *P. falciparum* infection in humans are replicated in this ECM model. Interestingly, *P. falciparum*-infected humans and *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,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 *P. falciparum* malaria is associated with accumulation of abnormal UL-VWF multimers in the plasma.^{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. We observed no significant reduction in murine ADAMTS13 activity following *P. berghei* infection. In contrast, previous studies did report a significant reduction in plasma ADAMTS13 activity in children and adult patients with severe malaria compared to healthy controls.^{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.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,⁶⁵⁻⁶⁹ we previously showed that those described to date could not explain the accumulation of UL-VWF multimers observed in children with CM.¹⁷ Interestingly, 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.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*.

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,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.²⁸ In this study, we demonstrate that VWF^{-/-} mice are significantly protected against *P. berghei* infection compared to WT controls. This survival difference was not explained by a difference in *P. berghei* parasitemia levels, which were similar in both VWF^{-/-} and WT mice. Significant thrombocytopenia is a common feature of both human and murine malaria.^{49,50} Moreover, recent studies have described a role for platelets in modulating the innate host defense during the early stages of malaria infection.^{52,53} Interestingly, although previous studies suggested that the UL-VWF might be important in mediating this malaria-associated thrombocytopenia,^{23,24} we observed no significant difference in *P. berghei*-induced thrombocytopenia in VWF^{-/-} 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^{-/-} mice are clearly protected against ECM through a platelet-independent mechanism.

Severe malaria infection has been associated with enhanced permeability of the BBB.⁵⁵⁻⁵⁷ In addition, recent studies have demonstrated that VWF plays an important role in regulating the BBB.^{38,39} For example, significantly attenuated BBB permeability was observed in VWF^{-/-} mice compared to WT controls in a hypoxia/reoxygenation model, and also in an experimental model of status epilepticus.³⁸ 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^{-/-} mice compared to WT controls.³⁸ 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.⁷¹ Consequently, VWF^{-/-} mice are not only deficient in plasma and platelet VWF, but also do not possess WP bodies.²⁹ Importantly, in addition to VWF and VWF:pp, WP bodies also normally store other proteins including P-selectin, Ang-2 and osteoprotegerin. 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.³⁸ 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.^{18,19,45-47} Interestingly, Ang-2 has also been shown to function as an autocrine regulator by sensitizing EC to activation by tumor necrosis factor (TNF),⁷² and thus may play a role in modulating the early acute EC activation associated with both *P. falciparum* and *P. berghei* infections.

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. We postulate that during the early stages following malaria infection, formation of UL-VWF strings on activated endothelial cell surfaces may be important in recruiting platelets (Figure 7). These tethered platelets result in further EC activation, and more WP body secretion. In addition, platelet-decorated VWF strings play a role in modulating subsequent microvasculature sequestration by recruiting malaria-infected erythrocytes, and also by binding to granulocytes and activated monocytes respectively.^{73,74} The VWF-mediated sequestration of platelets, infected erythrocytes and leucocytes leads to further enhanced EC activation, increased EC permeability, and ultimately results in vessel occlusion.

AUTHORSHIP

Contribution: N. O'R., K. G., J. M. O'S., N. D., T. M. B., U. B., G. E. G., S. M. and A. C. performed experiments; N. O'R., K. G., J. M. O'S., T. M. B., P. G. F., U. B., G. E. G., R. J. S. P., O. P. S., A. G. C., and J. S. O'D. designed the research and analyzed the data. All authors were involved in writing and reviewing the paper.

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 2×10^6 *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 $\pm$ SEM unless otherwise stated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ respectively). (B) Peripheral blood *P. berghei* parasitemia 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^{-/-} mice was investigated. (A) Following inoculation with *P. berghei*, clinical phenotype and progression in VWF^{-/-} mice ($n = 10$) and WT C57BL/6J mice ($n = 16$) were compared using a validated clinical scoring algorithm. Results presented represent the mean values $\pm$ SEM. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$

respectively). (B) In addition, overall survival in WT (n=16) and VWF^{-/-} (n=10) mice infected with 2×10^6 *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^{-/-} mice and WT C57BL/6J mice following *P. berghei* infection. (n = 4-5 mice per time point). (D) Peripheral blood *P. berghei* parasitemia levels were determined following inoculation in both VWF^{-/-} (n=10) and WT C57BL/6J mice (n=10) at specified time points from Giemsa-stained smears. Results presented illustrated represent the mean values $\pm$ 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. UL-VWF strings on the surface of activated EC then 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 strings modulate 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, increased EC permeability (4), and ultimately results in vessel occlusion (5).

Supplementary data – Figure 1

(1A) Murine plasma VWF:pp were determined by ELISA. In contrast to the marked increase in plasma VWF:Ag associated with *P. berghei* infection, and in contrast to the increase in plasma VWF:pp levels we have previously reported in children with CM, no significant increase in murine plasma VWF:pp levels were observed in ECM. To study the mechanism underlying this observation, we investigated the half-life of murine VWF:pp. (1B) Treatment with histamine resulted in a marked increase in both plasma VWF:Ag and VWF:pp levels. However by 10 minutes following histamine administration, murine plasma VWF:pp levels had already fallen by almost 50%. In contrast, (but in keeping with previous reports suggesting that murine VWF:Ag has a plasma half-life of approximately 11 hours,⁷⁵) murine VWF:Ag levels had only fallen by a small amount. Cumulatively, these data suggest that the plasma half-life of murine VWF:pp is much reduced compared to that of human VWF:pp.

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Figure 1

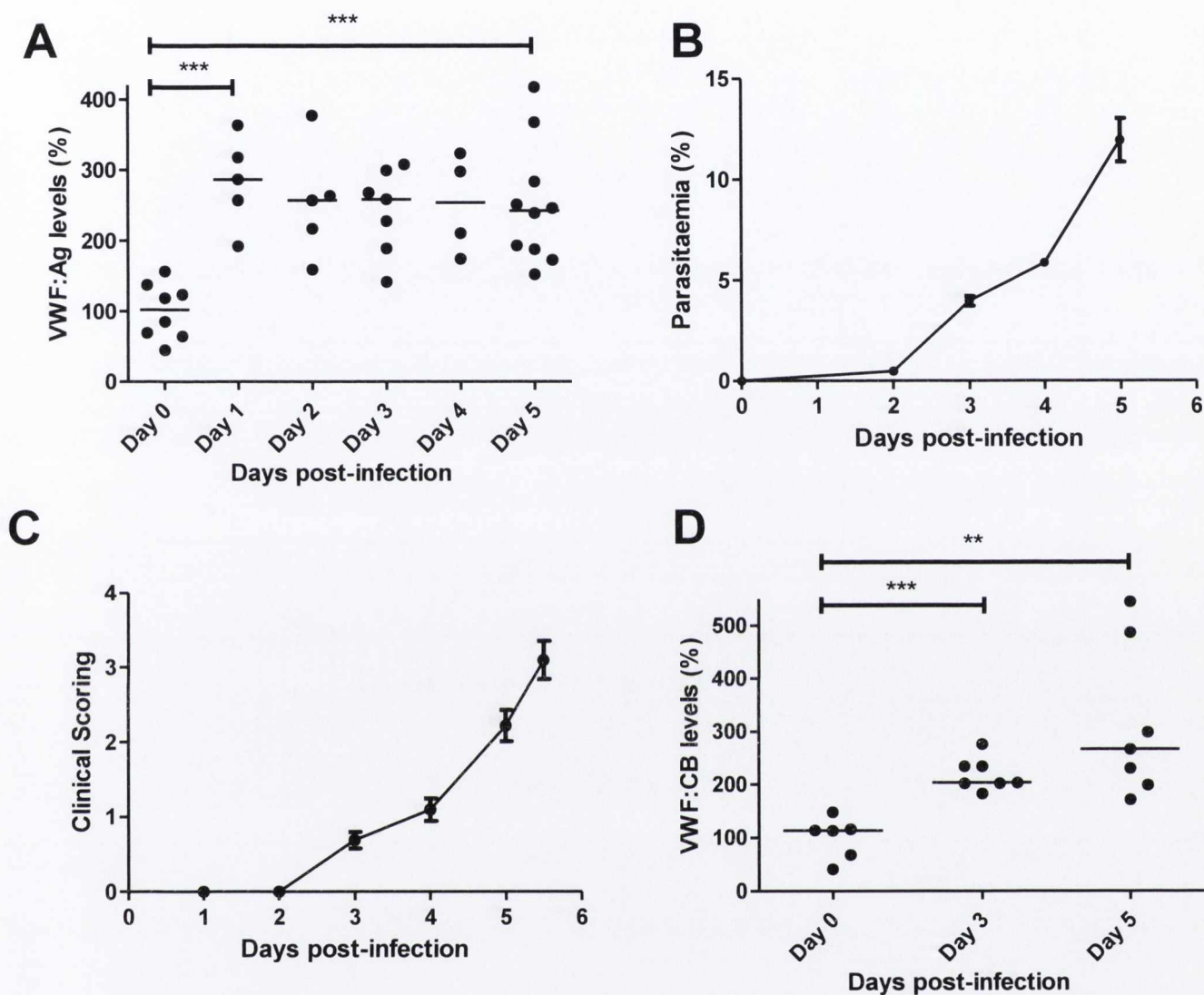


Figure 2

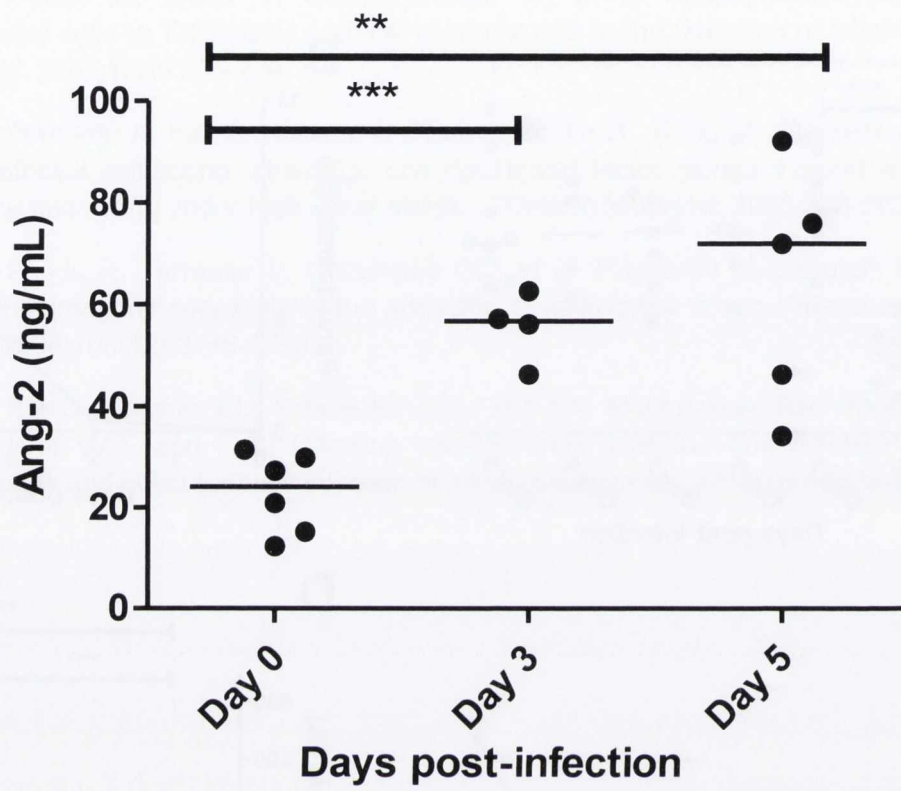


Figure 3

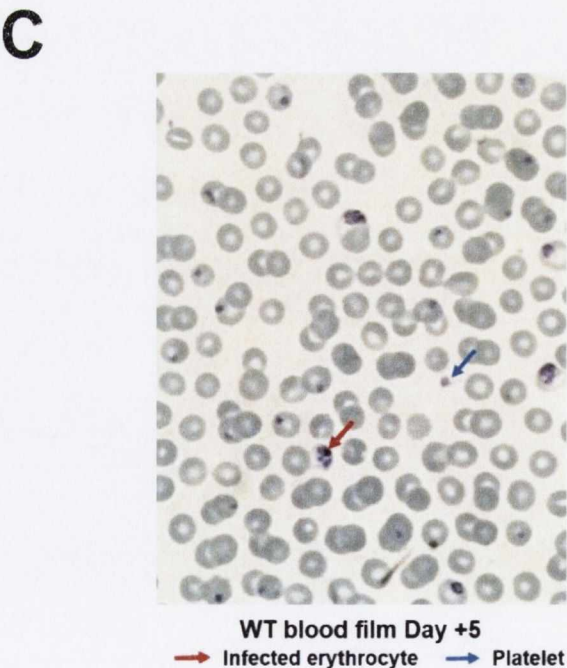
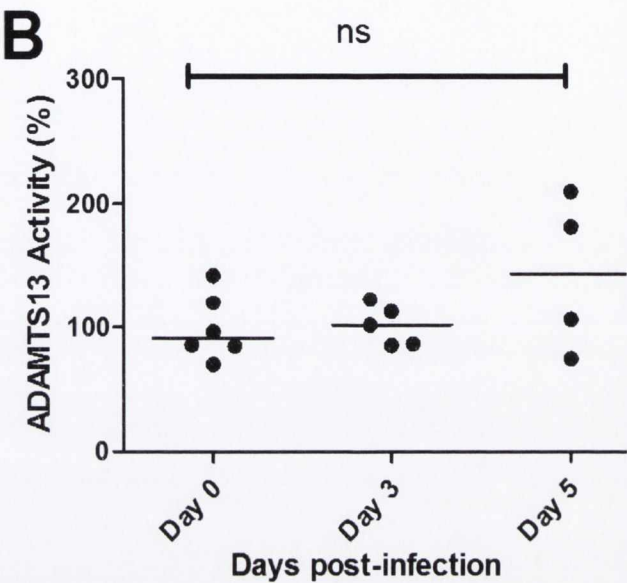
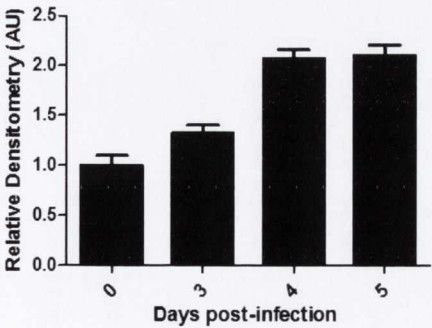


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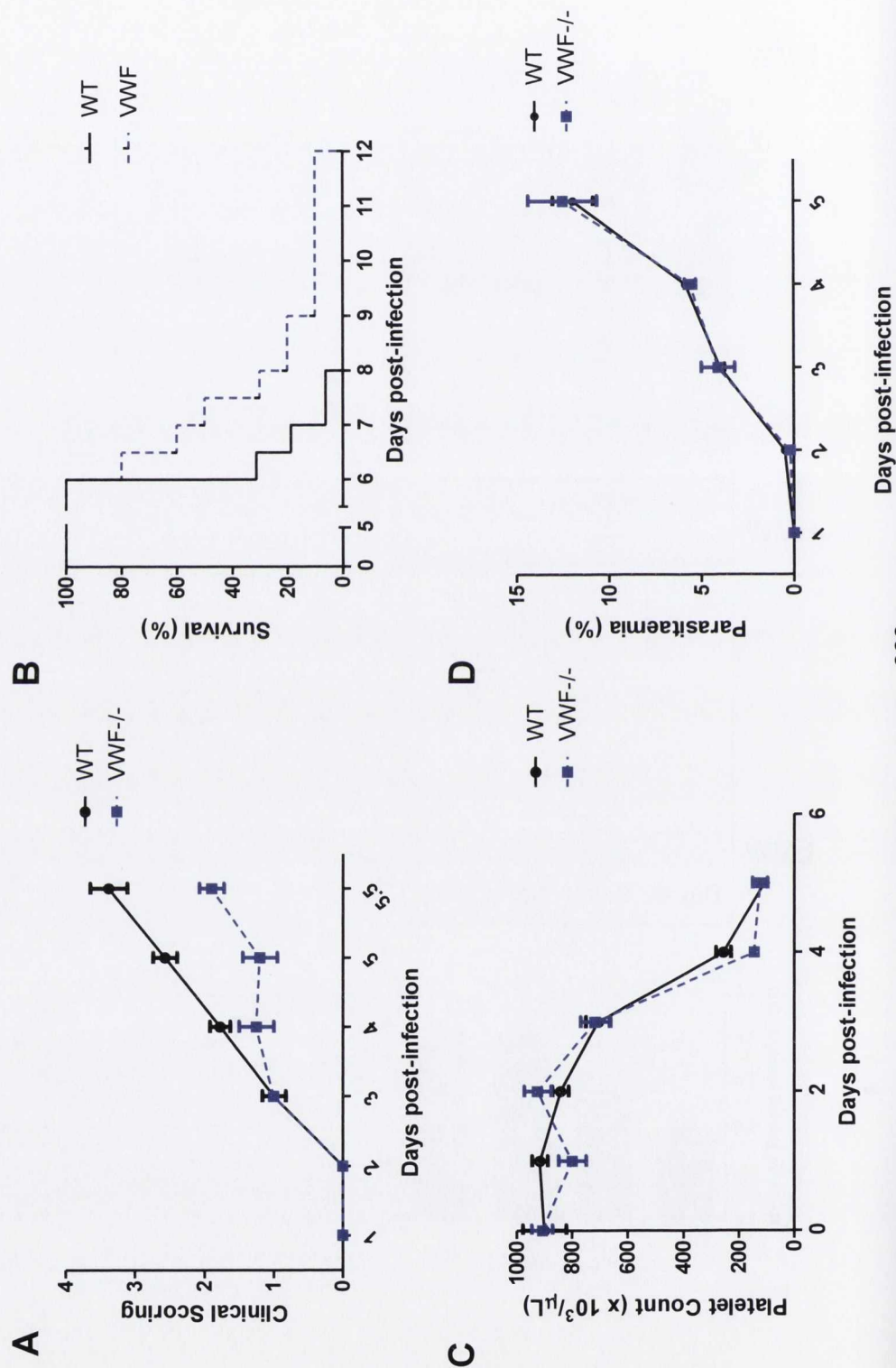


Figure 5

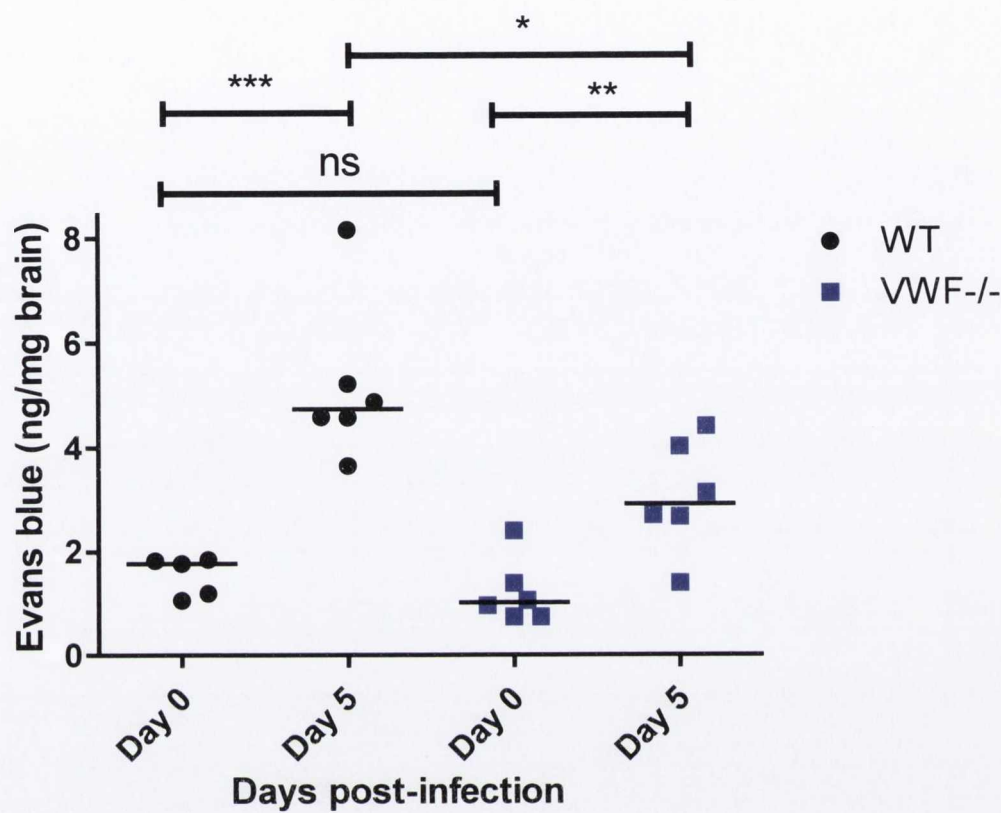
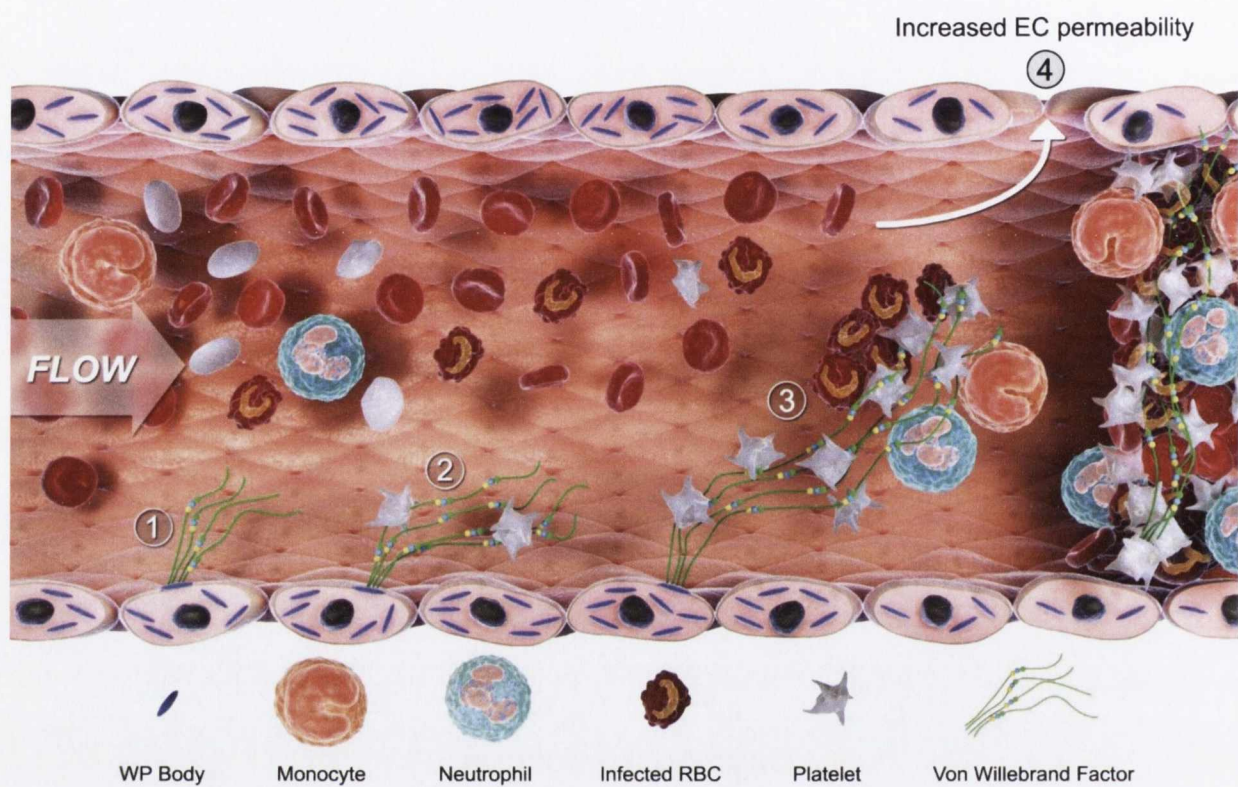
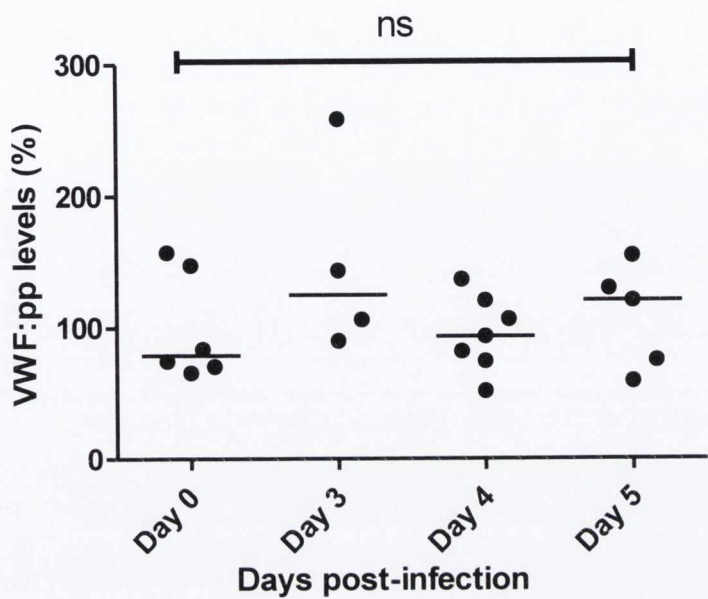


Figure 6

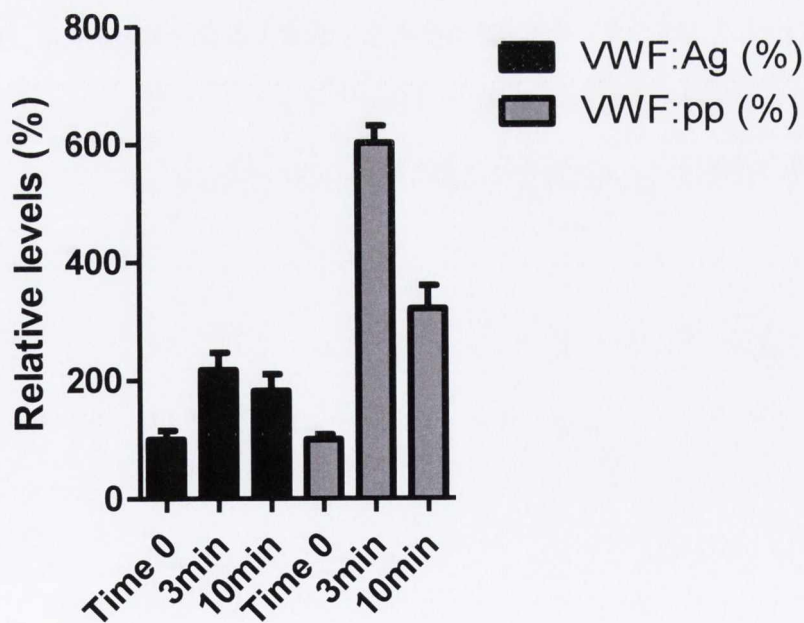


Supplementary Figure 1

A



B



**MARKED ELEVATION IN PLASMA OSTOPROTEGERIN (OPG) CONSTITUTES AN
EARLY AND CONSISTENT FEATURE OF CEREBRAL MALARIA**

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ABSTRACT

Adherence of infected erythrocytes to vascular endothelium causes acute endothelial cell (EC) activation during *Plasmodium falciparum* infection. As a result, proteins stored in Weibel Palade (WP) bodies within EC are secreted into the plasma. Osteoprotegerin (OPG) binds to VWF and consequently is stored within WP bodies. Given the critical role of EC activation in the pathogenesis of severe malaria, we investigated plasma OPG levels in children with *Plasmodium falciparum* malaria. At presentation, plasma OPG levels were significantly elevated in children with cerebral malaria (CM) compared to healthy controls (means 16.0 versus 0.8ng/ml; $p < 0.01$). Importantly, OPG levels were also significantly higher in children with CM who had a fatal outcome, compared to children with CM who survived. Finally, in children with CM, plasma OPG levels correlated with other established prognostic indices (including plasma lactate levels and peripheral parasite density). To further investigate the relationship between severe malaria and OPG, we utilized a murine model of experimental CM in which C57BL/6J mice were infected with *P. berghei* ANKA. Interestingly, plasma OPG levels were increased 4.6 fold within 24 hours following *P. berghei* inoculation. This early marked elevation in OPG levels was observed before any objective clinical signs were apparent, and preceded the development of peripheral blood parasitemia. As the mice became increasingly unwell, plasma OPG levels progressively increased. Collectively, these data suggest that OPG constitutes a novel biomarker with prognostic significance in patients with severe malaria. In addition, further studies are required to determine whether OPG plays a role in modulating malaria pathogenesis.

Keywords

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

INTRODUCTION

Plasmodium falciparum malaria continues to be associated with significant morbidity and mortality (1, 2). In particular cerebral malaria (CM), a severe encephalopathy caused by *P. falciparum* infection, has a mortality of 10 – 20% and a high rate of neurological sequelae in survivors (3). Although the molecular mechanisms underlying the pathogenesis of CM remain poorly understood, previous studies have demonstrated a critical role for endothelial cell (EC) activation (4, 5). In particular, a number of specific EC surface receptors, including CD36, endothelial protein C receptor (EPCR), E-selectin and intercellular adhesion molecule-1 (ICAM-1), have been shown to regulate the adhesion and sequestration of *P. falciparum*-infected erythrocytes (IE) within the microvasculature (6). Quantitative expression of these different EC surface receptors on EC surfaces is modified by inflammatory cytokines, such as interleukin-1 and TNF respectively (7). In addition to these IE surface receptors, EC also contain intracellular storage organelles known as Weibel-Palade (WP) bodies (8). These WP bodies are rod shaped organelles that can be up to 5µm in length and are specific to EC. von Willebrand factor (VWF) synthesis within EC is a prerequisite for WP body formation (9, 10). In normal plasma, VWF circulates as a series of heterogeneous multimers. In contrast, VWF stored within WP bodies is enriched in high molecular weight multimers (HMWM) that demonstrate enhanced binding affinities for both collagen and platelets (8, 10). In addition, a number of other EC proteins have also been identified as WP body constituents. These include VWF propeptide (VWF:pp), P-selectin, factor VIII, angiopoietin-2, tissue-type plasminogen activator (tPA), interleukin-8, galectin-3 and osteoprotegerin (OPG) (8, 10). Following acute EC activation, WP bodies fuse with the EC surface membrane, and their contents are secreted directly into the plasma.

Previous studies from our laboratory and others have demonstrated that plasma levels of HMWM VWF and VWF:pp are both markedly elevated in patients with severe *P. falciparum* malaria (11-17). Furthermore, plasma VWF:Ag and VWF:pp levels have also been shown to correlate with other biochemical prognostic markers in patients with severe malaria (14, 18). Interestingly, in a study of healthy volunteers infected with *P. falciparum*, de Mast *et al* observed that the increase in plasma VWF:Ag and VWF:pp levels was evident from a very early stage following *P. falciparum* infection (19). The molecular mechanism(s) underlying this early marked EC activation and WP body secretion have not been defined. Nevertheless, emerging data suggest that the early release of WP body contents, and in particular VWF, may play a role in malaria pathogenesis. We recently reported that platelet-decorated HMWM VWF strings on the surface of activated EC can directly adhere to trophozoite-stage *P. falciparum* IE in a shear-based assay (20). Thus, the rapid release of HMWM VWF multimers from WP bodies, which occurs at an early stage in the clinical course of *P. falciparum* infection, may provide a novel mechanism through which IE can adhere to activated EC surfaces.

In keeping with the marked elevated VWF and VWF:pp levels observed in children with cerebral malaria (CM), plasma levels of Angiopoietin-2 (Ang-2), another protein stored within WP bodies, are also significantly elevated (11, 13, 21, 22). Moreover,

higher plasma Ang-2 levels have also been associated with a worse clinical outcome (21-23). Osteoprotegerin (OPG) is another EC protein stored within WP bodies (24). OPG is recruited into WP bodies by binding directly to the A1 domain of VWF (24, 25). OPG plays a key role in regulating bone remodelling by acting as a soluble decoy receptor for Receptor Activator of NF κ B Ligand (RANKL) expressed on osteoblasts (26). Recent studies have also identified functional roles for OPG in modulating vascular biology (27, 28). In particular, OPG has been shown to upregulate expression of adhesion molecules on EC surfaces, and thereby facilitate leucocyte adhesion (29, 30). Furthermore, OPG influences EC apoptosis, and elevated OPG levels have been associated with vascular disease (27, 28). Given the key role of EC activation in the pathogenesis of severe *P. falciparum* malaria, we have investigated plasma OPG levels in children with cerebral malaria. To further understand the relationship between WP body secretion and malaria, we have investigated OPG levels in an established murine model of experimental CM.

MATERIALS AND METHODS

Patient samples

Children aged 6 months to 12 years were recruited at Queen Elizabeth Central Hospital, Blantyre, Malawi between 2008 and 2011. Children were recruited into one of 4 prospectively defined groups: [1] CM, defined as Blantyre coma score ≤ 2 , peripheral *P. falciparum* parasitemia and an absence of clinical evidence for another cause of coma; [2] uncomplicated malaria, defined as an acute febrile illness in a child with peripheral *P. falciparum* parasitemia and no evidence of organ compromise; [3] non-malarial febrile illness, defined acute febrile illness in a aparasitaemic child with no evidence of organ compromise and; [4] healthy controls, recruited from otherwise well children having elective surgery. We screened for parasitemia using a rapid diagnostic test that employs a *P. falciparum* specific antigen (First Response, Premier Medical, India) and if positive, peripheral parasite density was confirmed and quantified by thick and thin blood smears, as described previously (31). We have published data examining coagulation factors and soluble receptors in this cohort previously (31, 32) but the data presented here on VWF and OPG levels is presented for the first time. The study was approved by the ethical boards at the College of Medicine in Malawi (no. P.02/ 10/860) and Liverpool School of Tropical Medicine in the United Kingdom (no. 09.74). Informed consent was obtained from the parents or legal guardians of all the children enrolled in this study in accordance with the Declaration of Helsinki.

Murine studies

Previous studies have demonstrated that wild type (WT) C57BL/6 mice infected with *P. berghei* ANKA develop a complex neurological syndrome that includes many similar clinical features (including paralysis, seizures and coma) to those observed in patients with CM (33-35). Depending upon the *P. berghei* inoculation dose, mice typically die within 6 to 10 days from this experimental CM. Consequently we used this validated murine model of experimental CM to further investigate the relationship between severe malaria and OPG. All mouse experiments were performed in compliance with Irish Medicines Board regulations, and were reviewed and approved by the Trinity College Dublin BioResources Ethical Committee. WT C57BL/6J mice were bred in-house under standard pathogen-free conditions, and all experiments were performed on mice aged 8 to 10 weeks. Mice were infected by intraperitoneal injection of 2×10^6 *P. berghei* ANKA. Following inoculation, the mice were monitored using a previously validated clinical scoring system to assess progression of experimental CM (36)(Amante *et al*, 2007). In addition, peripheral blood *P. berghei* parasitemia levels were monitored daily by examination of Giemsa-stained thin blood smears obtained from tail vein bleeds. Blood samples at predefined time points following *P. berghei* infection were also collected by cardiac puncture into acid citrate dextrose (ACD) anticoagulant (85mM trisodium citrate, 65mM citric acid, 100mM glucose) (Sigma, Ireland). Platelet-poor plasma was prepared by centrifugation of blood samples at 1500 g for 15 min at 20°C. The samples were then aliquoted and stored at -80°C until use.

Determination of plasma VWF:Ag and OPG levels

Human plasma VWF:Ag levels were measured using a previously described enzyme-linked immunosorbent assay (ELISA) (37). In brief, Maxisorp plates (Nunc, Denmark) were coated with rabbit anti-human VWF antibody (Dako, Denmark) in 50mM carbonate buffer. After blocking with 3% bovine serum albumin (Sigma, Ireland) test samples or reference plasma were then added at appropriate dilutions. Bound VWF was subsequently detected using horseradish peroxidase–conjugated (HRP) rabbit anti-human VWF antibody (Dako, Denmark). Following further washing, HRP substrate 3,3',5,5'-Tetramethylbenzidine (TMB; Substrate Reagent Pack, R&D Systems, UK) was then added, and the reaction finally terminated by addition of 1M H₂SO₄. Absorbance was read at 450nm using a VERSAmax microplate reader (Molecular Devices, UK). Human and murine plasma OPG levels were determined using commercial OPG ELISAs (Osteoprotegerin/TNFRSF11B DuoSet®, R&D Systems, UK) performed in accordance with the manufacturer's guidelines.

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) and statistical significance was assigned at a value of $P < 0.05$. Normally distributed data were expressed as mean values $\pm$ standard error of the mean (SEM). To assess statistical differences, data were analysed using Student's unpaired 2-tailed t test. The Spearman correlation coefficient was used to determine linear association between different variables.

RESULTS

Clinical characteristics

A total of 135 children with cerebral malaria, 45 children with non-malarial febrile illness, 52 children with uncomplicated malaria and 26 healthy controls were enrolled in this study. Baseline clinical and laboratory characteristics are summarized in Table 1.

Plasma VWF:Ag and OPG levels are markedly elevated in children with CM

At presentation, plasma VWF:Ag levels were significantly elevated in Malawian children with CM compared to healthy control children (means 29.7 versus 8.3µg/mL; $p<0.001$) (Figure 1A). This ~3.6 fold increase is similar in magnitude to that previously reported in other cohorts of paediatric and adult patients with severe *P. falciparum* infection (12, 14-16). Plasma VWF:Ag levels were also mildly increased in children with uncomplicated malaria (UM). However the elevation in plasma VWF:Ag observed in children with UM was similar to that observed in children with other non-malarial febrile illnesses (NMFI). Plasma OPG levels were also markedly elevated in children with CM compared to healthy controls (means 16.0 versus 0.79ng/mL; $p<0.01$) (Figure 1B). However in contrast to the 3.6 fold increase observed in VWF:Ag levels, plasma OPG levels were increased more than 20 fold. Plasma OPG levels were also significantly elevated in children with UM compared to healthy controls ($p<0.05$). Although plasma VWF:Ag levels were significantly elevated in children with NMFI, plasma OPG levels were not significantly increased in this cohort. Thus, the marked increase in OPG seems to constitute a relatively malaria-sensitive phenomenon. Collectively, these findings support the hypothesis that acute endothelial cell activation and WP body secretion constitute characteristic features of severe *P. falciparum* malaria. In addition, these data further demonstrate for the first time that plasma OPG levels are markedly elevated in children with *P. falciparum*.

Plasma OPG levels relate to malarial severity in children with P. falciparum malaria

In patients with severe *P. falciparum* malaria, it is well established that plasma lactate levels constitute a useful biochemical prognostic indicator (38). Unsurprisingly, plasma lactate levels were significantly elevated in children with CM compared to healthy controls (means 6.9 versus 2.0mmol/L; $p<0.001$) (Figure 2A). In addition, plasma lactate levels were significantly higher in children with CM compared to those with UM respectively ($p<0.001$). Among the 135 children with confirmed CM, plasma OPG levels and lactate levels were significantly correlated ($r=0.42$, $p<0.001$) (Figure 2B). Eighty four children with CM demonstrated plasma lactate levels greater than 5mmol/L consistent with lactic acidosis. Plasma OPG levels in children with CM and lactic acidosis were higher compared to levels in children with CM uncomplicated by lactic acidosis (means 19.6 versus 10.2; $p=0.06$). Importantly, plasma OPG levels at admission were also significantly higher in the 21 children with CM who went on to die, than in the 114 children with CM who survived (means 29.6 versus 14ng/mL; $p=0.026$).

(Figure 3A). Peripheral parasite density and platelet count have previously been defined as markers of disease severity in patients with severe *P. falciparum*. In previous studies, we found no significant correlation between plasma VWF:pp levels and peripheral parasite density (14, 15). In contrast, in children with CM we observed an association between OPG levels and parasite density ($r=0.65$; $p<0.001$) (Figure 3B). OPG levels were also negatively associated with platelet count ($r= -0.5$; $p<0.001$) (Figure 3C).

Plasma OPG levels are markedly elevated in experimental CM

To further investigate the relationship between severe malaria and OPG, we utilised an established murine model of experimental CM. In this model, C57BL/6J mice were infected with *P. berghei* ANKA. Malaria progression was then assessed using a previously validated clinical scoring algorithm (36). Using this algorithm, overt clinical signs of malaria were first observed on Day +3 following *P. berghei* infection. Subsequently, the infected mice became progressively unwell, with death typically occurring 6-7 days after infection (Figure 4A). Peripheral blood smear analysis demonstrated that *P. berghei* parasitemia levels were first detectable 48 hours following inoculation (Figure 4B). Thereafter, blood parasitemia levels progressively increased as the malaria infection progressed. In keeping with our findings in children with CM, a significant increase in plasma OPG levels was also observed in wild type C57BL/6J mice following *P. berghei* infection (Figure 4C). For example, by Day +5 following inoculation, mean murine plasma OPG levels were increased approximately 18 fold (1.38ng/ml at Day 0 versus 25.5ng/ml at Day+5; $p<0.001$). This increase in murine plasma OPG levels seen was thus strikingly similar in magnitude to that observed in children with *P. falciparum* CM.

Interestingly, a significant increase in plasma OPG levels was observed from a very early stage following *P. berghei* infection. Plasma OPG levels were already increased 4.6 fold (1.38 versus 6.4ng/ml; $p<0.05$) within 24 hours following *P. berghei* inoculation. This early marked elevation in murine plasma OPG levels was established before any objective clinical signs were apparent (Figure 4A). Furthermore, the increase in plasma OPG levels also preceded the development of significant peripheral blood parasitemia levels (Figure 4B). As the mice became increasingly unwell, plasma OPG levels progressively increased. Finally, and again in keeping with our observations in children with CM, a correlation between plasma OPG levels and peripheral blood parasitemia levels was also observed in the murine ECM model ($r=-0.9$; $p=0.08$). Collectively, these findings confirm that marked elevation in plasma OPG constitutes a consistent finding in both human and experimental murine CM. Moreover, the increase in plasma OPG occurs at an early stage during the pathogenesis of severe malaria infection.

DISCUSSION

EC activation plays a critical role in modulating the pathogenesis underlying severe *P. falciparum* malaria (4, 5). This EC activation triggers the secretion of WP body contents into the plasma. Previous studies have consistently shown that plasma levels of WP body-stored HMW VWF are significantly increased in patients with both *P. falciparum* and *P. vivax* malaria (12, 14-16). Furthermore, significant elevations in the plasma levels of other WP components (including VWFpp and Ang-2) have also been previously reported in patients with malaria infection (13, 14, 21). In this study, we demonstrate for the first that severe *P. falciparum* malaria is also associated with a marked increase in plasma OPG levels. Although the biological mechanism(s) responsible for this increase in OPG have not been defined, it seems likely that fulminant acute endothelial cell activation is at least in part responsible. Interestingly, we observed a similar marked increase in plasma OPG levels in a murine model of experimental CM. In addition, using this murine model we demonstrated that the increase in plasma OPG develops at an early stage following malaria infection. Indeed, plasma OPG levels were significantly increased before parasitemia was detectable, and well before the onset of any clinical signs. This finding is consistent with previous studies in which elevations in plasma VWF levels were observed at a surprisingly early stage in both human volunteers infected with *P. falciparum*, and in mice infected with *P. berghei* (19).

The early and marked increase in plasma OPG levels associated with malaria may represent an epiphenomenon, consistent with acute EC activation and WP body secretion. Alternatively, it is possible that OPG may have a role in the pathogenesis of severe malaria. Although OPG has an established role in regulating osteoclast differentiation, more recent studies have identified additional novel biological functions. Of particular relevance, OPG has been shown to influence the expression of adhesion molecules on EC surface, and to modulate EC apoptosis (28-30). For example, *in vitro* studies have shown that OPG increases expression of Ang-2, VCAM-1, and ICAM1 on HUVEC (29). Interestingly, in this study we found significant correlations between plasma OPG levels and several other prognostic markers (including plasma lactate levels, parasitemia and thrombocytopenia) in children with CM. Further studies will be required to determine whether OPG plays any direct role in contributing to malaria pathogenesis.

Notwithstanding any putative role of OPG in malaria pathogenesis, our combined human and animal data support the hypothesis that plasma OPG levels may constitute a novel biomarker with prognostic significance in malaria. In this context, it is interesting that although both VWF and OPG are stored together within WP bodies, the increase in plasma OPG levels in children with CM was much more marked than that in VWF levels. This discrepancy may relate to different relative concentrations of VWF and OPG being stored within WP bodies. Alternatively, it is not known whether significant differences in plasma half-life may exist between OPG and VWF. Finally,

significant OPG synthesis has also been described in other non-EC tissues (e.g. smooth muscle cells) (39).

The combined increases in plasma levels of both VWF and OPG observed in severe malaria are of further interest in that previous studies have demonstrated that OPG can bind directly to the A1 domain of VWF (24, 25). This OPG-VWF binding occurs within EC prior to secretion from WP bodies (25). Due to the ionic conditions existing in normal plasma, it seems unlikely that any further OPG binding to VWF takes place happens in the peripheral circulation. Nevertheless, VWF-OPG complexes formed prior to secretion from WP bodies have been shown to remain relatively stable in normal plasma (25). Further studies will be required to determine whether increased levels of this VWF-OPG complex are circulating in patients with severe malaria. Moreover, it remains unclear whether the formation of this complex has any effect upon the normal functional properties of either VWF and/or OPG respectively.

In conclusion, we have shown that plasma OPG levels are elevated approximately 20 fold in both children with CM, and in a murine model of experimental CM. This marked increase in plasma OPG is evident from an early stage following malaria infection. Furthermore, plasma OPG levels increase progressively through the course of infection, and correlate with other established markers of outcome in patients with severe malaria. Collectively, these findings suggest that OPG may have a role as a novel biomarker in patients with severe malaria infection, and also raise the intriguing possibility that OPG may play a role in modulating malaria pathogenesis.

AUTHORSHIP

Contribution: *N. O'R., C. A. M., K. G., J. M. O'S., A. C., and T. M. B.* performed experiments; *N. O'R., C. A. M., K. G., T. E. T., K. B. S., J. M. O'S., T. M. B., R. J. S. P., O. P. S., A. G. C., and J. S. O'D.* designed the research and analyzed the data. All authors were involved in writing and reviewing the paper.

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

Table 1: Patient characteristics and baseline data for children enrolled.

(n.a. = not applicable; n.d. = not done).

Figure 1: Plasma VWF:Ag and OPG are markedly elevated in children with CM

(A) Plasma VWF:Ag and (B) OPG levels were determined by ELISA in children presenting with cerebral malaria (CM), uncomplicated malaria (UM), or non-malarial febrile illness (NMFI) as well as in a cohort of healthy control children. Each plasma was tested in duplicate at three dilutions, and mean values for each group are shown. VWF:Ag and OPG levels were significantly elevated in children with CM compared to controls. An unpaired student's t-test was performed for statistical analysis and significance is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ respectively.

Figure 2: Relationship between plasma OPG levels and lactate levels in children with CM

(A) Plasma lactate levels were performed by ELISA in children with CM, UM, NMFI and healthy controls. (** $P < 0.01$, *** $P < 0.0001$, n.s. = not significant).

(B) Relationship between plasma OPG levels and lactate levels in children with CM. The hashed red line at 5mmol/L defines plasma lactate level considered diagnostic of lactic acidosis.

Figure 3: Plasma OPG levels relate to malaria severity in children with *P. falciparum* malaria

(A) Plasma OPG levels at presentation in children with CM and a fatal outcome were compared to those of children with CM and a non-fatal outcome.

(B) Plasma OPG levels in children with CM correlated with peripheral blood *P. falciparum* levels (C) Plasma OPG levels inversely correlated with platelet count in children with CM.

Figure 4: Marked elevation in plasma OPG levels constitutes an early and characteristic feature of experimental CM

WT C57BL/6J mice (n=12) mice were inoculated with 2×10^6 *P. berghei* ANKA parasites. (A) Clinical progression of the malaria infection was assessed were compared using a validated clinical scoring algorithm. Results presented illustrated represent the mean values $\pm$ SEM unless otherwise stated.

(B) Following infection with *P. berghei*, peripheral blood parasitemia levels were determined on a daily basis from Giemsa-stained smears.

(C) Whole blood samples were collected from infected mice by cardiac puncture, and plasma OPG levels were then measured at pre-defined time points using ELISA. All ELISAs were performed in triplicate, and results presented represent the mean values $\pm$ SEM (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ respectively).

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Table 1

	Healthy	NMFI	UM	CM
N	26	45	52	135
Age (Years)	5.7 ± 0.4	4.1 ± 0.2	5.5 ± 0.3	4.2 ± 0.1
Sex, female (%)	10 (38%)	27 (52%)	22 (49%)	64 (47%)
Mortality (%)	n.a.	n.a.	n.a.	20 (15%)
Temperature (°C)	36.6 ± 0.1	38.4 ± 0.1	38.5 ± 0.1	38.6 ± 0.1
Parasitemia (x10 ³ /µl)	n.d	n.d	117 ± 30	200 ± 25
Haemoglobin (g/dl)	9.9 ± 1.27	10.6 ± 0.2	8.8 ± 0.3	7.1 ± 0.2
Platelets (x10 ³ /µl)	413.7 ± 102.9	332.5 ± 20.0	140.8 ± 12.8	102.8 ± 6.3
Lactate (mmol/l)	2.0 ± 0.1	1.8 ± 0.1	2.5 ± 0.1	6.9 ± 0.3
VWF:Ag (µg/ml)	8.2 ± 1.0	24.7 ± 1.6	15.4 ± 1.4	29.7 ± 1.2

Figure 1

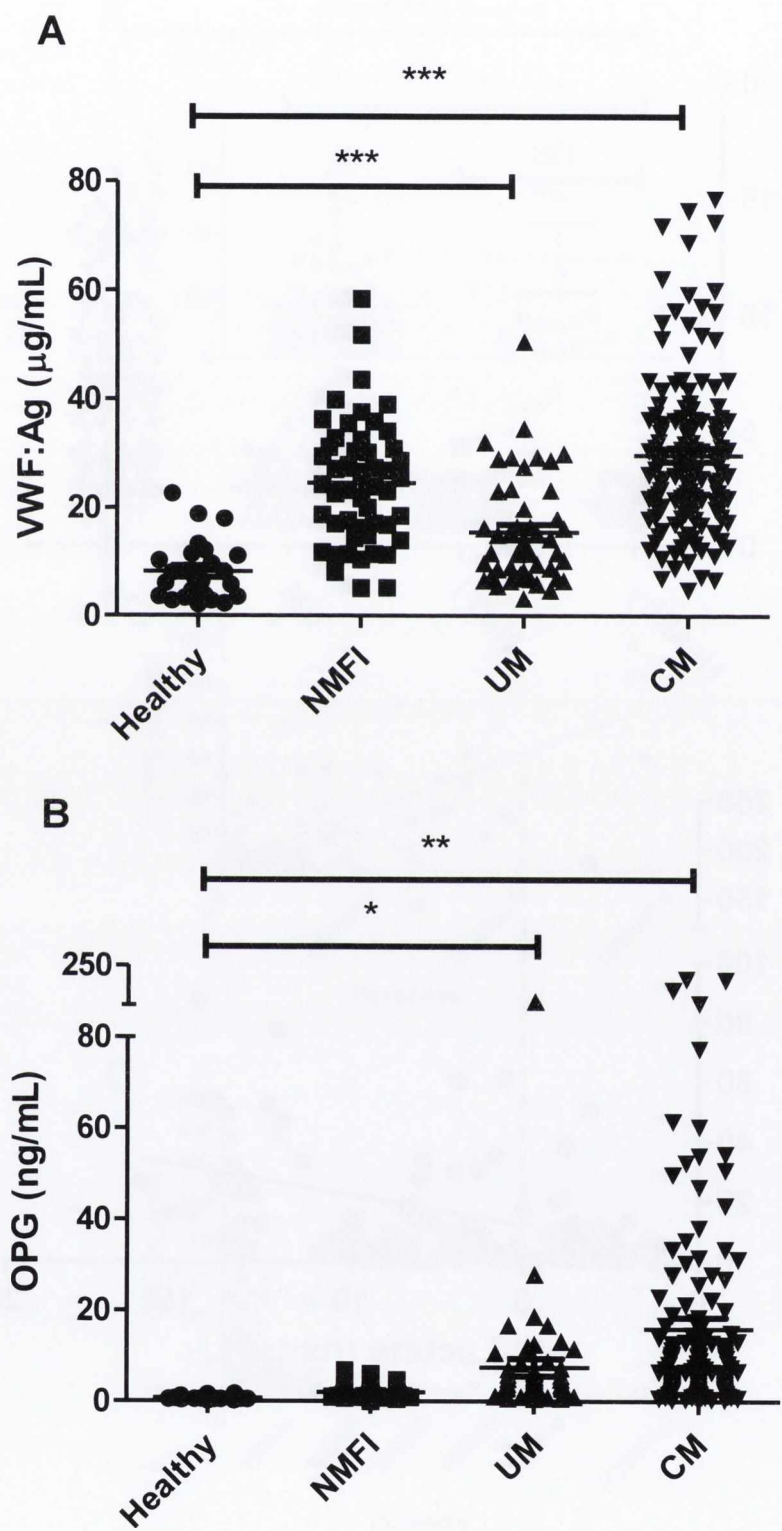


Figure 2

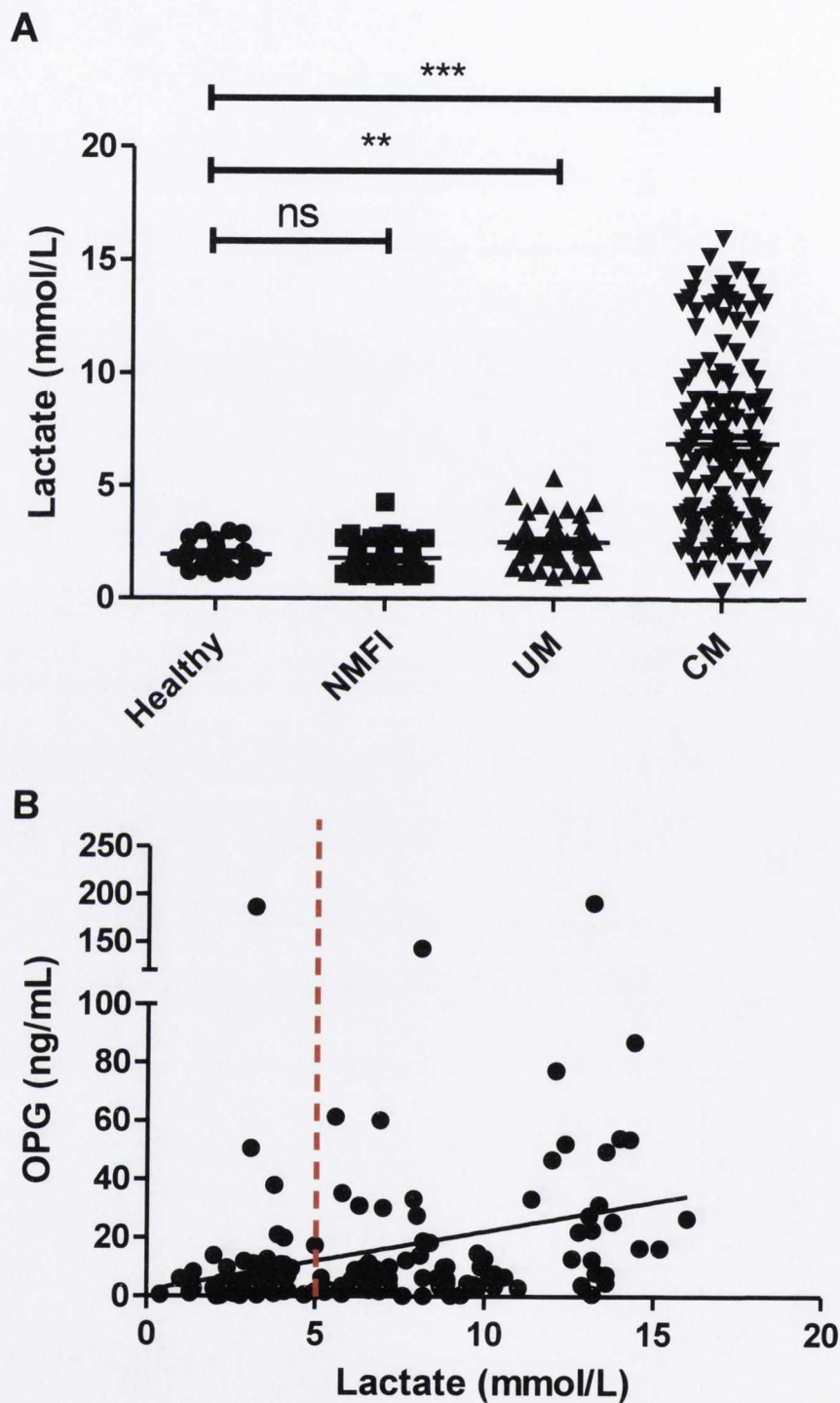


Figure 3

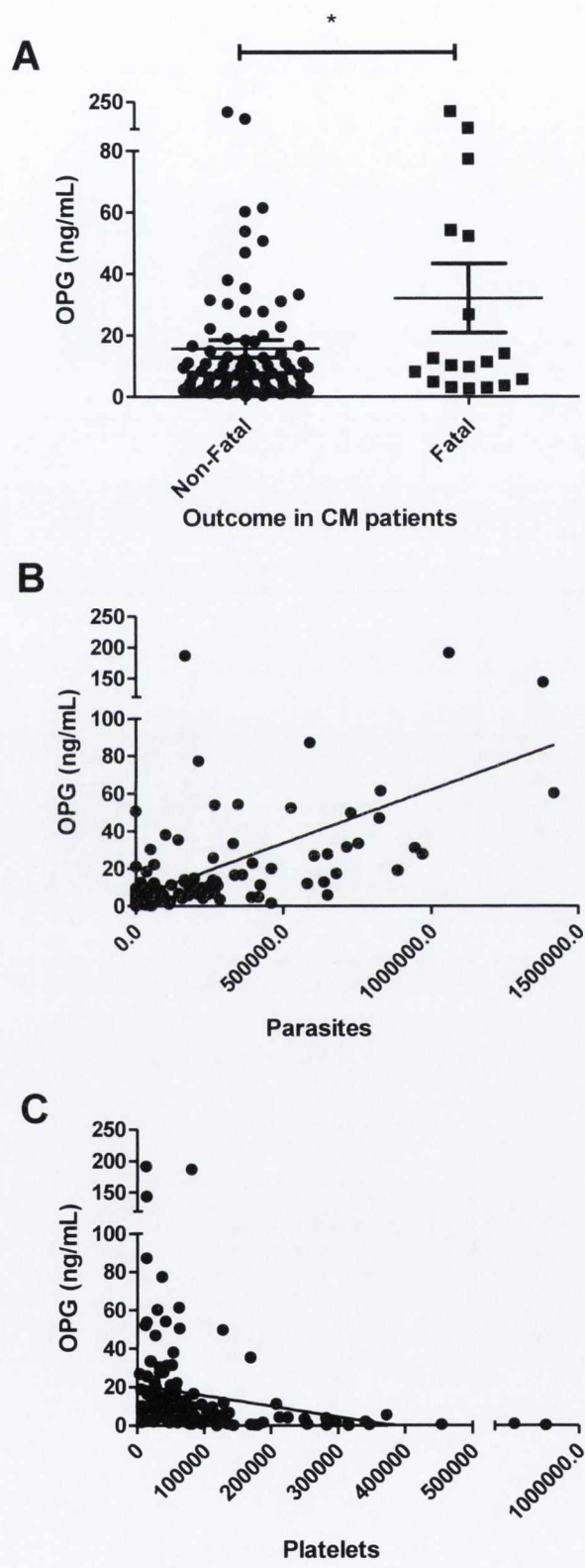
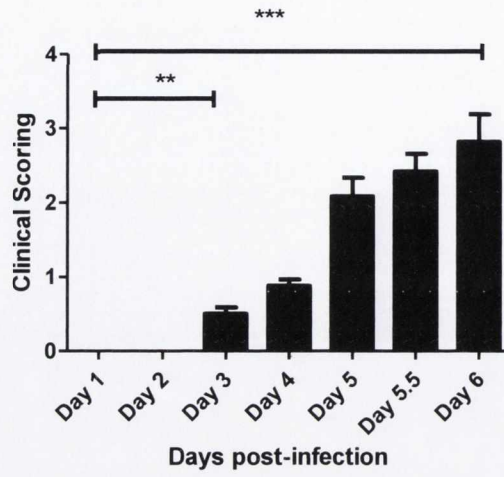
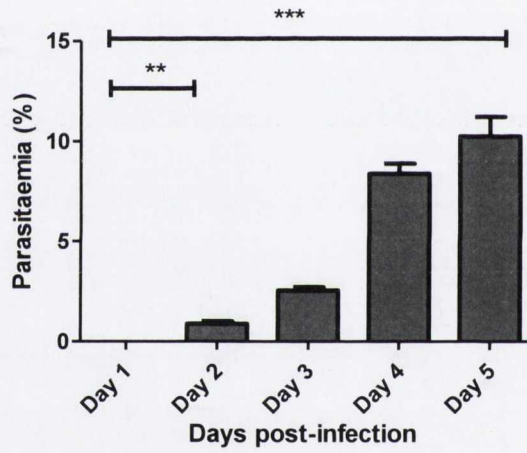


Figure 4

A



B



C

