Host-pathogen interactions in malaria and tuberculosis: experimental models and translation to novel adjunctive therapies

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Sciences
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Abstract

Malaria and tuberculosis together account for more than 2 million deaths worldwide each year. The present body of work examines interactions between these leading pathogens and cross-cutting themes in innate immunity to both diseases. Not only are malaria and tuberculosis important threats to public health in their own right, but malaria-tuberculosis co-infection appears to generate more severe pathology than either disease on its own, and malaria may exacerbate primary or re-activation tuberculosis (Chapter 2). Moreover, both diseases appear to share common host defense pathways, including CD36, a macrophage cell surface receptor important for innate immunity (Chapter 3). Biomarkers of host defense pathways common to both malaria and tuberculosis distinguish between clinical disease phenotypes and predict mortality in severe malaria (Chapters 4-7). Biomarker discovery led to the identification of angiopoietin-2 (Ang-2) as a surrogate marker of disease severity in malaria and a potential therapeutic target. Nitric oxide, in addition to its antimycobacterial properties, is known to inhibit Ang-2 release from the endothelium, and is therefore hypothesized to improve outcomes in African children with severe malaria (Chapters 8 and 9). A broad range of methods are applied to address these diseases and their interactions, ranging from mammalian cell culture experiments
*in vitro*, animal models of disease, analysis of human samples, and clinical epidemiology (randomized controlled trial). Translational aspects of this research are emphasized, outlining how advances in understanding of infectious disease pathogenesis can be applied to improved diagnosis, prognosis, and novel adjunctive therapies for two of the leading global infectious threats.
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Dissemination of Thesis Contents and Author Contributions

Chapter 2:


MH designed and executed the experiments, analyzed the data, and wrote the manuscript.

Chapter 3:


MH designed and executed the experiments, analyzed the data, and wrote the manuscript.

Chapter 4:


MH assisted with the experimental design and statistical analysis, and reviewed the manuscript.

Chapter 5:


MH assisted with the experimental design and statistical analysis, and reviewed the manuscript.
Chapter 6:


MH assisted with the experimental design and statistical analysis, and reviewed the manuscript.

Chapter 7:


MH assisted with the experimental design and statistical analysis, and reviewed the manuscript.

Chapter 8:


MH designed the study and wrote the manuscript.

Chapter 9:


MH designed the study and wrote the manuscript.

Additional publications arising from thesis work:


Chapter 1
Malaria and Tuberculosis

The three leading infectious diseases of humans, malaria, tuberculosis and HIV/AIDS, together account for over five million deaths each year. *Plasmodium falciparum*, the causative agent of malaria, and *Mycobacterium tuberculosis*, the causative agent of tuberculosis, are the focus of the present thesis. *P. falciparum* is the leading parasitic cause of mortality worldwide, causing approximately 300 million new infections and one million deaths per year (WHO 2009). *M. tuberculosis* infects an estimated 2 billion people worldwide, one third of the human population, and is responsible for the most deaths annually (1.6 million/year) of any single bacterial pathogen (Noss, Pai et al. 2001).

Considerable geographic overlap exists in the distribution of malaria and tuberculosis. Based on data reported to the World Health Organization (WHO), Figure 1.1 and Figure 1.2 show the areas of malaria and tuberculosis endemicity, respectively. These maps demonstrate the concentration of both diseases in Africa, Asia, and South America. Individuals residing in zones of high transmission may become co-infected with both pathogens, and biological interactions may exist between *P. falciparum*, *M. tuberculosis*, and a shared human host. While multiple socio-economic factors may also in part explain the observed pattern of co-endemicity, we show in Chapter 2 that malaria exacerbates acute and recrudescent mycobacterial infection in an experimental model system, suggesting that malaria may fuel the spread of tuberculosis, with direct relevance to the observed spatial epidemiology.
Figure 1.1. Global distribution of malaria.
Incident cases by country, 2003.

Figure 1.2. Global distribution of tuberculosis.
Incident cases per 100,000, 2006.
Although potent anti-infective agents are available for malaria and tuberculosis, severe manifestations of both diseases continue to cause significant morbidity and mortality. Severe malaria carries a mortality of approximately 20% in sub-Saharan Africa despite treatment with effective antimalarials (Kyu and Fernandez 2009). Serious complications of infection with *M. tuberculosis*, including tuberculous meningitis and miliary tuberculosis are frequently lethal or disabling to survivors. Furthermore, the emergence of resistance to conventional antimicrobials threatens the current therapeutic armamentarium. Malaria treatment and control programs are hampered by parasite resistance to antimalarial drugs; as a striking example, the rapid worldwide spread of chloroquine resistance (Marsh 1998) has limited the utility of this agent to highly restricted geographic zones. More recently, resistance to artemisinin combination therapy, currently the best available treatment, has emerged in Southeast Asia (Rogers, Sem et al. 2009). Similarly, multi-drug resistant (MDR) and extremely drug resistant (XDR) *M. tuberculosis* is associated with increased mortality and alternative treatment regimens to circumvent resistance are toxic, expensive, and unavailable in many areas of the world (Raviglione and Smith 2007).

In this context, advances in the management of the world’s major infectious diseases will require an improved understanding of pathogen and host interactions, particularly host response which determines, to a large extent, the manifestations and outcome of an infectious challenge. Host response to malaria and tuberculosis may result in clearance of the microbe without clinical manifestations, mild or uncomplicated disease without lasting sequellae, or severe complications with the development of pathology or death. The present thesis examines selected mechanisms of host response to malaria and tuberculosis, and novel interventions that target key pathways in disease pathogenesis.

We begin by introducing malaria and tuberculosis from a clinical perspective, with emphasis on aspects of etiology, clinical features, pathogenesis, diagnosis, treatment and prognosis most relevant to subsequent thesis chapters. Next, we review selected aspects of the pathobiology of malaria and tuberculosis, highlighting host-pathogen interactions that feature prominently in subsequent chapters. Chapter 2 deals with malaria and tuberculous co-infection using *in vitro* methods and an animal model of disease. Chapter 3 discusses *in vitro* and *in vivo* results that demonstrate a role for the class B scavenger receptor CD36 as a determinant of susceptibility to experimental mycobacterial infection. Chapters 4, 5, 6 and 7 report findings from observational
studies in five distinct cohorts of African children and Asian adults, identifying biologically and clinically informative biomarkers involved in the critical pathogenic pathways of endothelial activation, inflammation, and coagulation in severe and fatal malaria. Chapter 8 outlines the hypothesis and rationale for a randomized controlled trial of nitric oxide for the adjunctive treatment of severe malaria in African children, followed by a detailed protocol to test this hypothesis in Chapter 9. Chapter 10 is a discussion and synthesis of this work, exploring some of the unifying themes between the host response to malaria and tuberculosis, and emphasizing the translation of laboratory findings to clinical practice using a broad range of methodologies, from *in vitro* and animal models to observational studies of human disease and an interventional trial.

### 1.1 Malaria

#### 1.1.1 Etiology

Human malaria is caused by infection with one of five species of *Plasmodium*. *P. falciparum*, endemic in most of sub-Saharan Africa and throughout the tropics, may cause severe pathology and mortality, and is the primary focus of the present thesis. *Plasmodium vivax* is a common cause of severe, acute febrile illness, especially in Asia and South America. Infection with *P. vivax* is rarely lethal, although severe and fatal cases have been recognized (Barcus, Basri et al. 2007; Valecha, Pinto et al. 2009). Its distribution in Africa may be restricted by the absence in several populations of the Duffy antigen, the receptor for entry into erythrocytes. Unlike *P. falciparum*, *P. vivax* forms dormant stages that persist in hepatocytes (hypnozoites) and therefore may relapse months to years after apparent resolution of infection. *Plasmodium malariae* is an infrequent cause of clinical malaria, with most cases found in Africa. It may produce a chronic parasitemia persisting for decades if untreated, but is rarely if ever fatal. *Plasmodium ovale* is an uncommon cause of mild to moderate relapsing malaria and is often found in the context of mixed *Plasmodium* infections. Finally, *Plasmodium knowlesi* has recently been recognized as a significant agent of severe malaria in Southeast Asia (Cox-Singh, Davis et al. 2008). Prior to the use of molecular diagnostic techniques, it was frequently misdiagnosed as *P. malariae*. Because *P. knowlesi* replicates every 24 h, hyperparasitemia may develop rapidly, and fatal cases have been reported (Cox-Singh, Davis et al. 2008).
Plasmodia are eukaryotic apicomplexan parasites with a life cycle involving both human and *Anopheles* mosquito hosts. Following the bite of a female anopheline mosquito, sporozoites harbored in the mosquito salivary glands are injected into the human host. Fewer than 25 sporozoites are inoculated in an infective bite, and travel to the liver through the bloodstream in less than one hour. After invasion of a hepatocyte, the parasite multiplies asexually to produce more than 10,000 infective merozoites, which represent the smallest extracellular parasite form. Merozoites released into the bloodstream invade erythrocytes by a complex, dynamic process that is completed within seconds to minutes of egress (Dvorak, Miller et al. 1975). Coordinated events in merozoite invasion include: (1) non-oriented surface attachment; (2) apical re-orientation such that specialized secretory organelles (micronemes, rhoptries and dense granules) are directed toward the erythrocyte; (3) early tight-junction formation; (4) active invasion through the junction into the nascent parasitophorous vacuole (PV) using the parasite’s actomyosin motor (Baum, Richard et al. 2006); and (5) sealing of the parasite within the PV (Riglar, Richard et al. 2011). Within the erythrocyte, merozoites develop into trophozoites, reproduce asexually (schizogony), and release progeny merozoites that can go on to invade fresh erythrocytes. The cycle of erythrocyte invasion and asexual multiplication is responsible for the pathogenesis of malaria. In some cases, a merozoite entering an erythrocyte does not undergo asexual reproduction, but differentiates into male or female gametocytes. Upon ingestion by the female mosquito, male gametocytes divide into eight flagellated microgametes which escape from the erythrocyte (exflagellation). The motile microgametes fertilize the female macrogamete, resulting in a motile zygote (ookinete) which moves between or through the cells of the mosquito stomach wall and encysts to form an oocyst. The oocyst undergoes asexual reproduction to form threadlike sporozoites that are released into the mosquito body cavity and migrate to the salivary glands. The life cycle is completed when an infected anopheline mosquito takes a blood meal and sporozoites are again injected into the human host.

### 1.1.2 Clinical features and pathophysiology

Typical symptoms of malaria are high fever with chills, rigor, sweats, and headache. Classically, symptoms are paroxysmal and periodic, recurring approximately every 48 hours in *P. falciparum*, *P. vivax* and *P. ovale* and every 72 hours for *P. malariae*. This distinctive fever pattern is characteristic of untreated infection in semi-immune individuals living in highly
 endemic regions; in non-immune individuals, such as children less than 5 years of age or travelers, the fever may not be paroxysmal or periodic. Periodicity of the fever may be explained by the synchronized rupture of schizonts with release of parasite products such as glycophosphatidylinositol (GPI), which stimulate a pyrogenic host response. Other non-specific symptoms include nausea, vomiting, diarrhea, cough, tachypnea, arthralgia, myalgia, abdominal pain and back pain. Anemia, thrombocytopenia, jaundice caused by intravascular hemolysis are common findings. Hepatosplenomegalaly may be present on physical examination.

Infection with *P. falciparum* is potentially fatal. Individuals at increased risk of severe disease include young children and primigravid pregnant women living in endemic areas, as well as non-immune travelers to transmission areas. In children, the most common severe manifestations of falciparum malaria are cerebral malaria, severe anemia, and respiratory distress with metabolic acidosis (Marsh, Forster et al. 1995). Here, the clinical features and pathophysiology of cerebral malaria and respiratory distress with acidosis are discussed in some detail, since these syndromes relate to the clinical trial discussed in Chapters 8 and 9.

1.1.2.1 Cerebral malaria

The clinical features of cerebral malaria are those of a diffuse symmetric acute encephalopathy. As such, the differential diagnosis is broad and clinical features overlap with other infectious and metabolic causes of encephalopathy such as hypoglycemia, bacterial meningitis, and viral encephalitis (White, Krishna et al. 1992; Wright, Avery et al. 1993). Unrousable coma is the hallmark of cerebral malaria (WHO 1990). Seizures are common at presentation, occurring in up to 82% of children prior to hospitalization in one large series (Newton and Krishna 1998). Neurologic signs include altered level of consciousness, seizures, symmetric upper motor neuron signs and brainstem disturbances, including dysconjugate gaze, palsies, hypotonicity or hypertonicity, extensor plantar responses, and absent abdominal reflexes. Retinal hemorrhages are frequently present, and decorticate and decerebrate posturing are seen in severe cases (Molyneux, Taylor et al. 1989; Lewallen, Taylor et al. 1993).

The current understanding of the pathophysiology of cerebral malaria is based on case-control studies, clinical case series, post-mortem surveys, animal models, and *in vitro* studies. Impairment of consciousness may be due to one or more of the following mechanisms:

Post-mortem studies have demonstrated that there are large numbers of parasitized red blood cells within capillaries in fatal cerebral malaria. However, simple mechanical occlusion of the vasculature by parasitized erythrocytes does not fully explain the clinical manifestations (Aikawa, Iseki et al. 1990; Phillips and Solomon 1990). Diffuse encephalopathy, rather than focal neurologic abnormalities, and rapidly reversible obtundation, including those who have been in deep coma, are the usual manifestation of cerebral malaria. This pattern of neurological deficits and recovery would be unlikely if thrombotic occlusion of the vasculature had occurred. Adherence of parasitized red cells to the endothelium occurs through specific, receptor-mediated interactions. Many different endothelial receptors for infected red cells have been identified (Nakamura, Hasler et al. 1992), including CD36, thrombospondin, and intercellular adhesion molecule-1 (ICAM-1). Binding to ICAM-1 is highest in patients with cerebral malaria, with the degree of binding correlating with the severity of the illness (Newbold, Warn et al. 1997).

Metabolic factors have also been implicated in the pathophysiology of cerebral malaria. Hypoglycemia is found in almost one-third of children with cerebral malaria, and children who are hypoglycemic on admission are at significantly greater risk of death (Molyneux, Taylor et al. 1989; Jaffar, Van Hensbroek et al. 1997). Mechanisms underlying hypoglycemia may include inadequate intake, depletion of glycogen stores, impaired gluconeogenesis, and quinine-induced hyperinsulinemia (Idro, Jenkins et al. 2005). Elevated plasma and CSF lactate levels, as well as acidosis are associated with poor outcome (Molyneux, Taylor et al. 1989; Maitland and Marsh 2004). Competition between parasites and host cells for essential nutrients may contribute to the metabolic derangement, as may the release of toxic metabolites that impair brain cell metabolism (Phillips and Solomon 1990).
Cerebral edema has been advanced as another possible mechanism in the pathophysiology of cerebral malaria, although current evidence is conflicting. Experimental cerebral malaria is associated with enlarged perivascular spaces and upregulation of aquaporin-4, a glial membrane water channel which modulates brain water transport and a critical mediator of cerebral edema (Manley, Fujimura et al. 2000; Ampawong, Combes et al. 2011). Human studies have also demonstrated alterations of blood brain barrier structure and function, with a generalized reduction in the cell junction proteins ZO-1, occludin, and vinculin in the endothelial lining of the cerebral vasculature of patients who died from CM (Brown, Hien et al. 1999). In African children with CM, disruption of endothelial intercellular junctions occurs in areas of parasite sequestration, but without gross leakage of plasma proteins (Brown, Rogerson et al. 2001). However, in another autopsy study of fatal falciparum malaria in Asian adults, histological evidence of cerebral edema or immunohistochemical staining for aquaporin-4 did not correlate with pre-mortem alteration of CNS function (Medana, Day et al. 2011). Neuroimaging demonstrates increased brain volume during the acute phase of cerebral malaria which may be attributable to cerebral edema, or an increase in the volume of intracerebral blood as a consequence of the sequestration of parasitized erythrocytes (Looareesuwan, Wilairatana et al. 1995). Cerebral swelling on computed tomography brain scans is a common finding in adult patients with cerebral malaria (approximately 60% of patients) but is not related to coma depth or survival (Mohanty, Mishra et al. 2011). Finally, therapies aimed at correction of cerebral edema have not improved outcomes in clinical trials. Osmotic diuretics as adjunctive therapy in cerebral malaria did not reduce mortality, and prolonged coma resolution time, based on two randomized clinical trials of mannitol in African children and Indian adults (Okoromah and Afolabi 2004; Namutangula, Ndeezi et al. 2007; Mohanty, Mishra et al. 2011).

1.1.2.2 Pulmonary manifestations of malaria

Children with malaria and respiratory distress with deep acidotic breathing have a mortality rate higher than that associated with isolated cerebral malaria (Marsh, Forster et al. 1995). In sub-Saharan Africa, cohort studies indicate that 17-29% of patients with severe malaria presenting to hospital emergency facilities have respiratory distress (Idro and Aloyo 2004; Idro, Aloyo et al. 2006). The following section highlights important differences between the respiratory manifestations of severe malaria in children and adults.
Respiratory distress is a clinically useful summary description with good inter-observer consistency among experienced medical practitioners (English, Waruiru et al. 1996). The following clinical signs may indicate increased work of breathing: sustained nasal flaring; indrawing (recession) of the bony structures of the chest wall (subcostal, intercostal, supraclavicular) on inspiration; tracheal tug; and deep breathing (acidotic or Kussmaul breathing). Respiratory distress is a sign that one or more serious pathological processes are at play: metabolic acidosis, fluid overload, acute lung injury, and/or co-morbid pneumonitis. In children with malaria, the presence of deep breathing, characterized by increased inspiratory and expiratory excursion of the chest is generally associated with metabolic acidosis and portends a high mortality (English, Waruiru et al. 1996).

Pulmonary edema is a well recognized complication of malaria in adults, but appears to be less common in children. Pulmonary edema has been described in non-immune individuals with *P. falciparum* infections as a component of a severe systemic illness with multi-system involvement, or as the primary feature (Taylor, Canon et al. 2006). A clinical diagnosis of pulmonary edema may be suggested by diffuse crepitations on chest auscultation, in contrast to Kussmaul breathing in which chest auscultation does not reveal adventitious sounds. Nonetheless, accurate diagnosis of pulmonary edema requires radiographic confirmation (WHO 1990). The underlying mechanism is usually related to alveolar-capillary fluid leak, rather than cardiogenic pulmonary edema, with raised central venous or pulmonary artery wedge pressures and grossly positive fluid balance (WHO 1990). Thus, the classical pulmonary manifestation of severe malaria in adults is non-cardiogenic pulmonary edema, resembling the adult respiratory distress syndrome (ARDS) or acute lung injury (ALI) also observed in bacterial sepsis (Deaton 1970). In this setting, fluid balance is normal or negative with normal or reduced pulmonary wedge pressure. Non-cardiogenic pulmonary edema may occur one to two days following the initiation of antiparasitic therapy, after parasite clearance, suggesting an immunologically-mediated injury rather than a direct effect of the parasite.

In children hospitalized with malaria in sub-Saharan Africa, metabolic acidosis and hypovolemia are common presenting signs (Maitland, Pamba et al. 2003; Pamba and Maitland 2004), and echocardiography reveals severe tachycardia, low stroke volume index, and high inferior vena cava collapsibility index (Yacoub, Lang et al. 2010). Thus, impaired tissue perfusion, metabolic
acidosis, and Kussmaul breathing, representing an acute pulmonary compensation, appear to account for most cases of respiratory distress in children. In contrast, acute lung injury appears to play a more important role in adult patients with severe malaria. Of note, lactic acidosis is a prognostic marker for mortality in children with severe malaria (Newton, Valim et al. 2005), and its association with respiratory distress represents a final common pathway of decompensated shock, cardiopulmonary insufficiency and impending death.

On the other hand, evidence from a recent clinical trial of fluid bolus therapy in critically ill African children, many of whom had severe malaria, demonstrated increased mortality with rapid fluid replacement, challenging the view that hypovolemia is a key mechanism along the causal pathway to mortality in severe malaria (Maitland, Kiguli et al.). This finding from a large, multi-centre, well designed randomized clinical trial is surprising in light of previous evidence that fluid replacement therapy restores circulatory homeostasis in children with severe malaria (Maitland, Pamba et al. 2003). In this study, the cause of death did not appear to be related to pulmonary or cerebral edema. The authors proposed alternative explanations: (1) the vasoconstrictor response in shock, which confers protection by reducing perfusion to nonvital tissues, and the rapid reversal of which with fluid resuscitation may be damaging; (2) reperfusion injury; or (3) subclinical effects on pulmonary compliance, myocardial function, or intracranial pressure (Maitland, Kiguli et al.). New evidence from this clinical trial does not contradict the importance of hypovolemia, acidosis and respiratory distress in the pathogenesis of pediatric malaria, but suggests that rapid volume correction may be deleterious.

Finally, irrespective of the etiology of respiratory compromise in a patient with malaria, hypoxia may itself provoke convulsions and deterioration in the level of consciousness and may be the immediate precipitant of death.

1.1.2.3 Severe malarial anemia

Hemoglobin levels less than 50g/L define severe anemia. Patients with severe anemia tend to be younger (age 16 to 31 months in one series (Thuma, van Dijk et al. 2011)) than those with cerebral malaria or respiratory distress. Pallor of the conjunctivae and/or palms may be clinical clues to the presence of profound anemia. Non-specific findings such as lethargy, poor feeding are common in anemic infants. Signs of high output congestive heart failure, including
tachypnea, tachycardia, hepatomegaly, gallop rhythm on cardiac auscultation, and cardiomegaly on chest x-ray may be manifestations of severe anemia.

Increased clearance of both infected and uninfected erythrocytes, as well as inhibition of erythrocyte production contribute to anemia associated with malaria (McDevitt, Xie et al. 2004). Bone marrow abnormalities observed in the setting of severe malarial anemia include ineffective erythropoiesis, dyserythropoiesis, and lower erythroblast proliferative rates (Thuma, van Dijk et al. 2011). Immune responses to *Plasmodium* may affect bone marrow function through alterations of the cytokine milieu. Elevated levels of tumor necrosis factor (TNF) and macrophage migratory inhibitory factor (Nussenblatt, Mukasa et al. 2001; McDevitt, Xie et al. 2006), and low circulating levels of interleukin (IL)-10 (Kurtzhals, Adabayeri et al. 1998) may contribute to suppression of erythropoiesis.

### 1.1.3 Diagnosis

The relevance of accurate yet practical malaria diagnostics to the present thesis relates to recruitment of patients for the clinical trial discussed in Chapters 8 and 9. The trial is being conducted in a peripheral centre in sub-Saharan Africa with limited laboratory support for the timely parasitological diagnosis of malaria. In order to enrol and randomize critically patients with severe malaria, clinical acumen needs to be complemented with parasite detection techniques that are amenable to use in a low resource setting. Microscopy, the century-old standard, as well as rapid diagnostic immunochromatographic tests will be used, and are reviewed in some detail here, in the context of the evolving field of malaria diagnostics.

Accurate diagnosis of malaria is a cornerstone of individual patient management and of control efforts globally. However, in resource-poor endemic areas, misdiagnosis and empirical overtreatment of fever with antimalarials in resource-poor endemic areas is rampant, such that 32 to 96% of clinical cases are negative by laboratory testing (Amexo, Tolhurst et al. 2004). Worldwide, an estimated 100,000 malaria-related deaths and 400 million unnecessary treatments could potentially be averted with sensitive and specific diagnostic tools (Rafael, Taylor et al. 2006). In non-endemic areas, diagnosis of imported malaria poses different challenges, due to lack of experience with clinical malaria among practitioners. Finally, clinical trial results can be
affected by inaccurate diagnosis (Ohrt, Purnomo et al. 2002), emphasizing the importance of accurate malaria diagnosis for valid knowledge generation.

Clinical diagnosis of malaria is notoriously unreliable given the non-specific nature of signs and symptoms of disease, which overlap with other common, as well as potentially life-threatening conditions such as pneumonia, bacterial sepsis, or meningitis. As such, standardized clinical algorithms (e.g., the WHO Integrated Management of Childhood Illness) were sensitive (100%), but strikingly non-specific (0-9%) when evaluated systematically in two African settings (Perkins, Zucker et al. 1997; Weber, Mulholland et al. 1997).

Microscopic detection of parasites on Giemsa-stained blood smears remains the reference standard for malaria diagnosis in laboratories around the world, as it has been for over a century. Microscopy is widely available, relatively inexpensive, and allows not only the identification of malaria species, but also the quantification of peripheral parasite density. However, the staining and interpretation process is labour-intensive and requires considerable expertise, particularly for accurate species identification at low parasitemia and when mixed infections occur. Quality control of this highly technician-dependent modality is problematic in resource-poor areas, and the ability to maintain the required level of expertise is challenging in areas where the disease is encountered infrequently (Hawkes and Kain 2007).

Immunochromatographic tests (ICTs) for malaria rely on the detection of malaria antigen, such as parasite histidine-rich protein-2 (HRP-2), parasite lactate dehydrogenase (pLDH), or Plasmodium aldolase, in whole blood. Monoclonal antibodies, conjugated to either gold particles or liposomes containing selenium dye, capture the malaria antigen of choice. Migration of liquid across a nitrocellulose membrane allows captured malaria antigen to bind a second immobilized monoclonal antibody, producing a visible coloured line. Several formats are commercially available, including dipsticks, cards and cassettes.

HRP-2 is a P. falciparum-specific antigen that may persist for up to 28 days in peripheral blood after the resolution of clinical symptoms and parasitemia. Therefore, assays limited to the detection of HRP-2 are unable to detect other species of Plasmodium, cannot be used as a test of cure, and may produce false positive results in the setting of recently treated infection. Additional “three-band” assays have been developed to also include a pan-species malaria
antigen, such as aldolase or pLDH. The ability to detect both HRP-2 and a pan-plasmodium antigen on a single test strip permits the identification of non-falciparum infections. Furthermore, confirmation of *P. falciparum* antigenemia using a second molecular target reduces the probability of a false positive diagnostic test. Other ICTs detect species-specific isomers of parasite LDH from the parasite glycolytic pathway. Because pLDH has been found to decrease in proportion to parasitemia (Grobusch, Hanscheid et al. 2003), this test may be useful for monitoring response to therapy. However, persistence of gametocytes post-infection, which is common in endemic regions, may also produce pLDH, potentially complicating interpretation of test results (Miller, McDaniel et al. 2001; Mueller, Betuela et al. 2007).

HRP-2-based assays have excellent sensitivity and specificity across a variety of clinical and research settings. According to two systematic reviews of the literature involving over 15,000 patients and 38 studies, these assays had a sensitivity of 90 to 93% and a specificity of 94 to 99% (Cruciani, Nardi et al. 2004; Ochola, Vounatsou et al. 2006). In endemic areas, HRP-2-based assays were superior to microscopy and pLDH based assays (Ochola, Vounatsou et al. 2006). Three-band ICTs (HRP-2 and aldolase detection) have similar sensitivity to two-band ICTs (HRP-2 detection alone) but are more specific to confirm the presence of *P. falciparum* (Marx, Pewsner et al. 2005). Assays for the detection of pLDH have lower sensitivity (67%) but a similarly high specificity (98%) for *P. falciparum* in endemic areas (Ochola, Vounatsou et al. 2006).

Additional diagnostic modalities, including polymerase-chain reaction, mass spectrometry and flow cytometry have been applied to the diagnosis of malaria and are reviewed elsewhere (Hawkes and Kain 2007). While these more sophisticated molecular techniques demonstrate improved sensitivity and unambiguous interpretation of clinical specimens, cost and the need for laboratory equipment limit their use in resource constrained settings.

### 1.1.4 Treatment

Treatment of malaria is briefly reviewed here, in relation to the choice of primary anti-parasitic therapy for the clinical trial described in Chapters 8 and 9. In addition, previous studies of adjunctive therapies for severe malaria are reviewed, in order to situate our trial of a novel
adjuvant (inhaled nitric oxide) in relation to other strategies that have attempted to modulate the critical host pathways that govern malaria pathogenesis.

Drugs targeting the parasite are currently the only specific therapy known to arrest the infection, and remain the primary treatment modality. As such, children suspected of having severe malaria must receive the best available antimalarials by the parenteral route as quickly as possible and specific therapy should not be delayed. The design of our clinical trial therefore randomizes children to receive a novel adjunctive therapy, inhaled nitric oxide, in addition to a potent anti-parasitic drug, artesunate. Moreover, the trial protocol prioritizes the emergency management of patients, including the early administration of an anti-parasitic agent, during the recruitment process.

Whereas uncomplicated malaria may be treated with oral anti-parasitic agents, severe malaria requires parenteral therapy. The WHO recommends artemisinin combination therapy (ACT) as first line therapy for uncomplicated malaria (WHO 2010) and parenteral artesunate is the most potent agent for the treatment of severe malaria (Dondorp, Nosten et al. 2005; Dondorp, Fanello et al. 2010). Thus, both uncomplicated and severe malaria are best treated with the artemisinin derivatives. Artemisinin is a sesquiterpene lactone extracted from the leaves of Artemisia annua (sweet wormwood), which has been used for centuries in China for the treatment of fever. It is the most potent and rapidly acting agent for elimination of blood stage parasites, with a broad spectrum of activity against asexual forms from young rings to mature schizonts, as well as gametocytes of *P. falciparum*. The mechanism of action appears to be due to the inhibition of an essential calcium adenosine triphosphatase, PfATP6, outside the food vacuole (Eckstein-Ludwig, Webb et al. 2003).

Several fixed-dose combinations of artemisinin derivatives are available for the treatment of uncomplicated malaria, including artemether-lumefantrine, which will be the agent used for follow-on therapy in the clinical trial described in Chapters 8 and 9. Because of their short half-life and in order to avoid the emergence of resistance, it is recommended that artemisinins be used in combination with a second antimalarial agent. Alternative oral antimalarials include mefloquine, atovaquone-proguanil, quinine, doxycycline and clindamycin.
Artesunate, the sodium salt of the hemisuccinate ester of artemisinin, is superior to the classical antimalarial quinin for the treatment of severe malaria in adults (Dondorp, Nosten et al. 2005) and children (Dondorp, Fanello et al. 2010). The compound is converted in vivo to dihydroartemisinin, which is the active form of the drug. Parenteral therapy is usually given until patients regain consciousness (in the case of cerebral malaria with coma), or until clinical improvement (in other severe malaria syndromes), and is generally followed by a treatment course of effective oral antimalarials.

In addition to specific therapy with anti-malarial medications, supportive therapy plays an important role in severe malaria, including control of convulsions and fever, attention to fluid and electrolyte balance, transfusions for severe and symptomatic anemia, maintenance of the airway, and good nursing care. Our clinical trial, described in Chapters 8 and 9 seeks to optimize the supportive care of patients in the resource-limited local context in order to improve outcomes for trial participants and build capacity for ongoing clinical management beyond the duration of the study.

A large number of adjunctive agents have been investigated for the treatment of severe malaria and are reviewed elsewhere (John, Kutanda et al. 2010). These include corticosteroids, heparin, mannitol, sodium bicarbonate, low-molecular-weight dextran, prostacyclin, anti-TNF antibodies, pentoxifylline, phenobarbital, dichloxacetate and desferrioxamine. Many of these agents have not been subjected to controlled clinical trials, or else the trials were powered to detect only unrealistically large treatment effects (Enwere 2005). A single study that enrolled a limited number of patients demonstrated a mortality benefit of albumin versus crystalloid as fluid resuscitation with in severe malaria (Maitland, Pamba et al. 2005). However, a subsequent larger trial demonstrated that both albumin and normal saline fluid bolus therapy increased mortality in African children with severe infection including malaria (Maitland, Kiguli et al. 2011). Additional therapies that target the underlying disease pathogenesis warrant further investigation.

1.1.5 Prognosis

The mortality for pediatric patients hospitalized for severe malaria in sub-Saharan Africa ranges from 6% to 29% in published randomized trials (Kyu and Fernandez 2009). Predicting which patients will succumb to their illness and which will survive remains challenging. Nonetheless,
accurate prognosis has practical implications for triage and resource allocation. New methods are needed to determine at the time of presentation which patients will progress to serious complications and death.

Clinical observations made at the time of presentation are useful but imperfect prognostic tools. Basic demographic information including young age and low weight-for-age identifies patients at increased risk of death. Unsurprisingly, observational studies confirm that the severity of CNS manifestations is related to the risk of death in CM (Molyneux, Taylor et al. 1989). Likewise, as expected, vital sign abnormalities (Waller, Krishna et al. 1995) and signs of shock, including prolonged capillary refill time, acidosis and respiratory distress are associated with an increased risk of death in severe malaria (Evans, May et al. 2006). Respiratory distress is consistently reported as a harbinger of death in multiple reports (Marsh, Forster et al. 1995; Waller, Krishna et al. 1995). Characteristic findings on fundoscopic examination, including retinal whitening, vessel discoloration, areas of hypoperfusion, and retinal hemorrhages, are associated with sequestration of parasites in the brain and have prognostic significance. In patients with CM, retinopathy is associated with an increased risk of mortality (Beare, Southern et al. 2004). Integrating independent clinical findings into a bedside index (Molyneux, Taylor et al. 1989) or summary score improves the predictive value of clinical signs. A simple clinical score, the Lambaréné Organ Dysfunction Score (LODS), has been developed in a multi-centre observational study involving over 20,000 African children with severe malaria, and predicts death in severe malaria with reasonable accuracy (Helbok, Kendjo et al. 2009). Nonetheless, clinical observations remain limited in their ability to correctly classify fatal and non-fatal cases, emphasizing the need for improved prognostic tools. Chapters 4-7 of the present thesis report on novel biomarkers associated severe and fatal disease which could serve as clinically informative predictors of poor outcomes in malaria.

Laboratory findings predictive of a poor outcome include indices of impaired tissue perfusion such as acidosis (Taylor, Borgstein et al. 1993; Newton, Valim et al. 2005) and elevated plasma or CSF lactate (White, Warrell et al. 1985). Other severe metabolic derangements including hypoglycemia are associated with increased risk of death (Waller, Krishna et al. 1995; Ogetii, Akech et al. 2010). Leukocytosis (independent of secondary bacterial infection) (Ladhani, Lowe et al. 2002), hyperparasitemia, and pigmented monocytes and neutrophils (Molyneux, Taylor et
al. 1989; Lyke, Diallo et al. 2003; Lovegrove, Tangpukdee et al. 2009) also predict mortality. Of note, some studies have demonstrated that lactate and acid-base add little to straightforward clinical predictors of mortality (Newton, Valim et al. 2005). In order to fulfill a useful clinical role, laboratory tests will need to provide useful prognostic information above and beyond clinical observation. Costs associated with laboratory tests will need to be justified by improvements in reliability (consistency) or validity (accuracy) of prediction of mortality in malaria, over and above readily available clinical information. Accordingly, in Chapters 4-7, we report on biomarkers of disease severity that outperform routine laboratory parameters for classifying fatal and non-fatal malaria cases.

Beyond routine laboratory testing, biomarkers that reflect activation of key pathways in malaria pathogenesis may provide valuable prognostic information. Studies that have investigated inflammatory pathways have shown that elevated TNF (Grau, Taylor et al. 1989; Kwiatkowski, Hill et al. 1990) and low IL-10 levels were associated with increased risk of death in severe malaria (Day, Hien et al. 1999). Higher concentrations of alanine and 5-nucleotidase are seen in fatal compared to non-fatal cases (Molyneux, Taylor et al. 1989). In Chapters 4-7 of the present thesis, we report on the prognostic value of biomarkers of endothelial activation, another critical host response to malaria infection.

In addition to the considerable burden of mortality, neuro-developmental morbidity following severe malaria is increasingly recognized as a public health concern. Recent studies have documented a significant rate of long-term neurologic sequelae in survivors of cerebral malaria (Brewster, Kwiatkowski et al. 1990; Newton and Krishna 1998). Approximately 10-30% of survivors experience neurologic deficits including hemiplegia, ataxia, and generalized motor deficits, with spasticity or hypotonia (Newton and Warrell 1998; Mung’Ala-Odera, Snow et al. 2004). The prevalence of epilepsy is more than twice that reported after complicated febrile seizures (Carter, Neville et al. 2004). Up to 24% of children with cerebral malaria have evidence of developmental impairment following cerebral malaria, suggesting that malaria is the cause of neurocognitive deficits in as many as 250,000 children annually (Carter, Ross et al. 2005). This substantial long-term burden of illness highlights the need for adjunctive neuroprotective therapies that could improve developmental outcomes. We hypothesize in Chapter 8 that inhaled nitric oxide may be neuroprotective in patients with severe malaria, based on evidence from
animal models (Pansiot, Loron et al. 2010) and follow-up developmental studies of neonates who received nitric oxide (Mestan, Marks et al. 2005).

1.1.6 Host-pathogen interactions

Having reviewed clinical features of malaria, we next discuss selected aspects of host response to *P. falciparum* infection relevant to the current thesis. In particular, we review the activation of inflammatory and endothelial pathways, and the pleiotropic role of nitric oxide. Finally, the effect of malaria on host immunity is discussed, with relevance to the impairment of antimycobacterial defenses discussed in Chapter 2. Translational aspects of these findings are emphasized. A deeper understanding the mechanisms of disease in severe malaria may potentially lead to improved diagnostic or prognostic tools, as in Chapters 4-7, where molecular markers are applied to the diagnosis of cerebral malaria and to the prognosis of severe malaria syndromes. Furthermore, new molecular targets for therapeutic intervention may emerge from the study of host response. Chapters 8 and 9 exemplify translational aspects of nitric oxide biology applied to the design of a randomized controlled trial for the adjunctive treatment of severe malaria in African children.

1.1.6.1 Innate and adaptive immune responses to malaria

Prior to the onset of humoral or cell-mediated immune responses, innate immunity is essential to limit exponential parasite replication and prevent overwhelming infection. Macrophages, DCs, NK cells, NKT cells and possibly γδ T cells are important effectors cells of the innate immune response (Stevenson and Riley 2004). Furthermore, these cell types produce regulatory cytokines and thereby modulate adaptive immunity.

Mononuclear phagocytes (macrophages and monocytes) play a central role in both innate and adaptive responses to malaria. At early stages of infection in non-immune individuals, monocytes are able to phagocytose infected erythrocytes through non-opsonic phagocytosis. This clearance mechanism occurs in the absence of malaria-specific antibody, and involves an interaction between the class B scavenger receptor CD36 and the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Serghides, Smith et al. 2003). Macrophages also act as antigen presenting cells for CD4+ T H1 lymphocytes that produce IFN-γ and mediate class switching to the cytophilic antibody subclasses IgG1 and IgG3 (Stevenson and Riley 2004). Finally,
macrophages act as effector cells of the specific immune response, clearing antibody-bound IRBCs through Fc receptor-mediated phagocytosis. Macrophages may also modify the cytokine milieu following activation by CD4+ T-cells through IFN-γ.

Murine models of blood-stage malaria indicate that a strong IL-12 response early during infection induces natural killer (NK) cells and possibly γδ T cells to produce IFN-γ (Stevenson, Su et al. 2001). IFN-γ produced early in infection is important in activating the appropriate CD4+ TΗ-cell subset (Mohan, Moulin et al. 1997; Choudhury, Sheikh et al. 2000; Stevenson, Su et al. 2001). A TΗ1-dominant cytokine milieu during the early stage of infection modulates Ab isotype switching, resulting in production of the protective Ab subclasses. Thus, control of acute infection requires a co-ordinated response including phagocytes, cytokines, and T-lymphocytes, culminating in a specific protective cytophilic antibody response that halts parasite replication.

Specific, adaptive immunity to malaria remains incompletely understood, despite decades of research attempting to produce an effective vaccine. Natural immunity to malaria is generally slow to develop, requires continuous re-infection to be maintained and never reaches a sterile level (Serghides, Smith et al. 2003). Given the complex life cycle of the Plasmodium parasite, antibody- and cell-mediated mechanisms may act at numerous points to limit parasite replication. Antibodies may: (1) interact with pre-erythrocytic stages (sporozoites) to prevent hepatocyte invasion; (2) bind merozoites to increase their clearance or block erythrocyte invasion; (3) prevent sequestration of infected erythrocytes by preventing binding to adhesion molecules on the vascular endothelium; (4) neutralize parasite inflammatory products such as GPI; or (5) target extracellular gametes to promote complement-dependent lysis or prevent fertilization. Empirical evidence supporting the importance of humoral immunity to pre-erythrocytic stages of the parasite comes from trials of RTS,S, the most promising malaria subunit vaccine to date, based on the circumsporozoite antigen of P. falciparum (Bojang, Milligan et al. 2001; Abdulla, Oberholzer et al. 2008; Bejon, Lusingu et al. 2008). Early demonstration of induction of immunity using irradiated sporozoites (Nussenzweig, Vanderberg et al. 1967), and a more recent experimental trial involving human volunteers repeatedly inoculated with P. falciparum via the bites of infected mosquitoes under chloroquine prophylaxis (Roestenberg, McCall et al. 2009), suggest that protective immunity can be generated by targeting pre-erythrocytic stages. Numerous vaccine attempts targeting merozoite surface proteins have yet to demonstrate clinical
utility. Vaccines directed to sexual forms, aiming to block transmission but not prevent disease, are also under investigation.

1.1.6.2 Inflammatory pathways

Activation of host inflammatory pathways promotes parasite clearance, but simultaneously contributes to the clinical manifestations of severe malaria. Excessive pro-inflammatory responses to infection are observed in malaria and other severe infections such as pneumonia and sepsis (Lyke, Burges et al. 2004). The levels of the pro-inflammatory cytokine TNF are increased in children with severe compared to uncomplicated malaria and in fatal compared to non-fatal cases (Grau, Piguet et al. 1989; Grau, Taylor et al. 1989; Kwiatkowski, Hill et al. 1990). TNF increases the expression of ICAM-1 and thereby facilitates increased adherence of parasitized cells within the cerebral circulation (Clark, Chaudhri et al. 1989; Johnson, Swerlick et al. 1993). Although TNF appears to play a central role in malaria pathogenesis, this knowledge has not yet translated into useful therapeutic strategies. Anti-TNF therapy in childhood cerebral malaria does not improve survival and results in increased incidence of neurologic sequelae, possibly by prolonging TNF persistence in the bloodstream (van Hensbroek, Palmer et al. 1996).

Another more recently recognized mediator of the inflammatory response is the triggering receptor expressed on myeloid cells (TREM)-1 (Bouchon, Dietrich et al. 2000). TREM-1 is up-regulated on the cell surface of neutrophils and a subset of monocytes by inflammatory stimuli such as LPS and lipoteichoic acid. Engagement of TREM-1 on monocytes using an agonist monoclonal antibody results in production of the proinflammatory cytokines and chemokines (Bouchon, Facchetti et al. 2001). Conversely, interruption of TREM-1 signaling in an animal model of LPS-induced septic shock reduced excessive inflammatory responses and reduced mortality (Bouchon, Facchetti et al. 2001). Soluble TREM-1 (sTREM-1) is generated by cleavage of membrane TREM-1 upon myeloid cell activation (Gomez-Pina, Soares-Schanoski et al. 2007), thereby serving as peripheral blood marker of leukocyte stimulation. Both membrane and sTREM-1 are increased in inflammatory pathologies in humans (Ford and McVicar 2009). Furthermore, previous reports have documented upregulation of TREM-1 on the cell surface of peripheral monocytes in patients with uncomplicated malaria (Chimma, Roussilhon et al. 2009). Given the role of TREM-1 in leukocyte activation and the association of sTREM-1 with
inflammatory states, we hypothesized that sTREM-1 would serve as a useful biomarker of malaria severity, as described in Chapter 6.

Assaying inflammatory responses may have utility in clinical practice. As an example, the acute-phase reactants C-reactive protein (CRP) and procalcitonin (PCT) are elevated in the setting of bacterial infection and malaria (Chiwakata, Manegold et al. 2001) and have been used in clinical practice to distinguish between severe bacterial infection and benign causes of fever in children. Other potentially informative inflammatory biomarkers include the 10 kDa interferon gamma-induced protein (IP-10), a chemokine elevated in fatal malaria (Armah, Wilson et al. 2007), endoglin, a component of the tumor necrosis factor-beta (TGF-β) receptor complex which participates in inflammatory signaling pathways, and soluble triggering receptor expressed on myeloid cells-1 (sTREM-1). In Chapter 6, we assess these host proteins among children with uncomplicated and severe malaria to determine their clinical utility in differentiating between malaria syndromes and predicting fatal cases.

1.1.6.3 Endothelial activation

The vascular endothelium forms a continuous structure that controls the entry and egress of plasma and leukocytes from the blood stream to surrounding tissues. As one of the largest internal surfaces of the body (~4000-6000 m²), it is conceptually a systemically disseminated organ that is maintained in a quiescent state under normal conditions. Activation of the endothelium results in increased expression of luminal adhesion molecules, leukocyte recruitment, altered vasomotor tone, microvascular thrombosis and diffuse capillary leak (Aird 2003; Hein, Misterek et al. 2005). Widespread endothelial activation leads to impaired perfusion, capillary leak, and multi-system organ failure in severe sepsis (Ricciuto, dos Santos et al. 2011). Endothelial activation in the brain vasculature may account in part for the manifestations of cerebral malaria (Erdman, Dhabangi et al. 2011).

One of the principal mechanisms regulating the state of endothelial cell activation is the angiopoietin-tyrosine kinase with immunoglobulin-like loop epidermal growth factor domain (Tie) ligand-receptor system (Fiedler, Reiss et al. 2005; Fiedler and Augustin 2006). Angiopoietins (Angs) represent a distinct family of vascular growth factors, within which four molecules have been characterized: Ang-1, Ang-2, Ang-3, and Ang-4. Angiopoietins (Ang-1 and
-2) and Tie-2 were initially described in the context of embryonic vasculogenesis and angiogenesis (Sato, Tozawa et al. 1995; Davis, Aldrich et al. 1996; Suri, Jones et al. 1996; Maisonpierre, Suri et al. 1997; Hayes, Huang et al. 1999), but also play an important role in mediating inflammation and quiescence in mature vascular beds (Fiedler and Augustin 2006; Novotny, Lahm et al. 2008). Ang-1 is secreted from pericytes within the vascular intima, whereas Ang-2 is stored with von Willebrand factor in Wiebel-Palade bodies within endothelial cells. Ang-1 and Ang-2 are antagonistic ligands that bind to the extracellular domain of the Tie-2 receptor, expressed primarily on the surface of endothelial cells. Ang-1 stabilizes the endothelium, inhibits vascular leakage, and suppresses gene expression associated with inflammation and coagulation through the constitutive activation of Tie-2 (Gamble, Drew et al. 2000; Kim, Kim et al. 2000; Thurston, Rudge et al. 2000; Kim, Moon et al. 2001; Injune, Oh et al. 2002; Hughes, Marron et al. 2003; Witzenbichler, Westermnn et al. 2004; Baffert, Le et al. 2005; Fiedler and Augustin 2006). Ang-1 also increases levels of the protective signaling sphingolipid sphingosine-1-phosphate (Finney, Hawkes et al.). In contrast to Ang-1, Ang-2 has proinflammatory and proangiogenic effects and mediates vascular leak (Fiedler, Reiss et al. 2005; Roviezzo, Tsigkos et al. 2005; Parikh, Mammoto et al. 2006). Its mechanism of action may involve the antagonism of Tie-2, prohibiting its phosphorylation by Ang-1 (Maisonpierre, Suri et al. 1997). Ang-2 also appears to function as an autocrine regulator by sensitizing the endothelium to the effects of TNF, resulting in increased adhesion receptor expression (Fiedler, Reiss et al. 2006). In experimental systems, Ang-2 has been implicated in vascular leak, acute lung injury and cell death in response to a variety of stimuli including inflammatory cytokines (Mandriota and Pepper 1998; Fiedler, Reiss et al. 2005), hyperoxia (Bhandari, Choo-Wing et al. 2006), hypoxia (Mandriota and Pepper 1998), and LPS (Mofarrahi, Nouth et al. 2008). It is likely that Ang-2 has multiple downstream effects, depending on the local microenvironment (Lobov, Brooks et al. 2002; Daly, Pasnikowski et al. 2006).

As key regulators of endothelial activation, plasma levels of Ang-1 and Ang-2 may provide valuable information regarding the state of vascular endothelial dysfunction and associated organ damage (Parikh, Mammoto et al. 2006; Orfanos, Kotanidou et al. 2007; Lukasz, Hellpap et al. 2008; Van Der Heijden, Van Nieuw Amerongen et al. 2008; Kumpers, van Meurs et al. 2009; van der Heijden, Pickkers et al. 2009). Low levels of circulating Ang-1 in septic patients compared to normal controls have been documented, although these have not consistently
correlated with severity of illness (Parikh, Mammoto et al. 2006; Gallagher, Parikh et al. 2008; Van Der Heijden, Van Nieuw Amerongen et al. 2008; Kumpers, van Meurs et al. 2009; van der Heijden, Pickkers et al. 2009). Ang-2 levels correlate with markers of inflammation and coagulation (Orfanos, Kotanidou et al. 2007; Siner, Bhandri et al. 2008; Van Der Heijden, Van Nieuw Amerongen et al. 2008), and are elevated in patients with acute lung injury and sepsis (Parikh, Mammoto et al. 2006; Giuliano, Lahni et al. 2007; Orfanos, Kotanidou et al. 2007; Siner, Bhandri et al. 2008; Van Der Heijden, Van Nieuw Amerongen et al. 2008), trauma (Ganter, Cohen et al. 2008; Giamarellos-Bourboulis, Kanellakapoulou et al. 2008) or major surgery (Gallagher, Parikh et al. 2008). In addition, the endothelial cell surface angiopoietin receptor Tie-2 is released into the circulation in severe illness and may be a marker of disease severity. Chapters 4-7 extend these findings, describing the clinical utility of peripheral blood angiopoietins and sTie-2 for prognosis in malaria (Conroy, Phiri et al.; Erdman, Dhabangi et al.; Conroy, Lafferty et al. 2009).

Another critical regulator of endothelial stability is the vascular endothelial growth factor (VEGF). VEGF is a homodimeric glycoprotein with a central role in promoting angiogenesis and vascular permeability. The biological activity of VEGF is regulated by several related molecules. Tyrosine kinase receptors for VEGF, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), located on endothelial cells, play opposing roles in VEGF signaling. VEGFR-2 mediates most of the endothelial growth and survival signals, whereas VEGFR-1 acts as a negative regulator, binding with 10-fold higher affinity to VEGF than VEGFR-2, yet producing only weak autophosphorylation of its intracellular domains in endothelial cells. A naturally occurring splice variant of VEGFR-1 occurs in soluble form in the serum (sVEGFR-1 or sFlt-1), where it acts as a “decoy receptor,” binding with high affinity to VEGF and neutralizing its activity (Shibuya 2001; Malecki, Trembacz et al. 2005). sFlt-1 is elevated in children with severe malaria as well as sepsis patients (Shapiro, Yano et al. 2008). Data from murine models of sepsis suggest that sFlt-1 may play a modulatory role (Yano, Liaw et al. 2006), counterbalancing the increased tissue expression and plasma levels of VEGF (Casals-Pascual, Idro et al. 2008).

The sequestration of parasitized erythrocytes in the microvasculature of vital organs appears to be a central process in the pathogenesis of severe malaria. Cytoadherence of pRBCs to endothelial cells in vivo under natural flow conditions likely involves multiple steps including
tethering, rolling, followed by more static binding. The endothelial receptors involved include intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin, and CD36. The ligand for these endothelial receptors is PfEMP-1, which is expressed on mature stage parasitized erythrocytes. Sequestration of parasites in the microvasculature of skin and muscle may contribute to parasite evasion of phagocytic clearance in the spleen, and sequestration in the microvasculature of major organs is associated with severe disease manifestations, including CM (v'Turner, Morrison et al. 1994).

Following activation by pro-inflammatory stimuli, soluble endothelial cell receptors are released into the circulation via ectodomain shedding or alternative splicing, such that peripheral blood levels of key regulatory proteins reflect the state of whole body endothelial activation. Moreover, molecular regulators of endothelial stability may be altered in disease states characterized by endothelial activation such as severe malaria. In Chapter 6, we examine several critical molecules involved in the regulation of endothelial quiescence and activation.

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin supergene family. ICAM-1 acts as a ligand for the β2 leukocyte integrins, such as lymphocyte function-associated antigen (LFA)-1 and Mac-1 (CD11b/CD18) (Makgoba, Sanders et al. 1988; Diamond, Staunton et al. 1990). ICAM-1 is expressed on cells of multiple lineages, and is upregulated at sites of inflammation. Antibody blockade of ICAM-1 inhibits leukocyte adhesion to endothelial cells and translocation of granulocytes across the endothelium (Smith, Marlin et al. 1989) and inhibits neutrophil trafficking to the injury site in an animal model of lung inflammation (Barton, Rothlein et al. 1989). ICAM-1 is shed from endothelial cells after exposure to the pro-inflammatory cytokines TNF and IL-1. In the setting of malaria, ICAM-1 mediates the sequestration of parasitized erythrocytes in the brain microvasculature, leading to vessel occlusion (Beare, Harding et al. 2009) and blood-brain barrier dysfunction (Medana and Turner 2006). The soluble form of ICAM-1 (sICAM-1) is increased in sepsis and severe malaria (Turner, Ly et al. 1998; Dietmann, Helbok et al. 2009), and thus represents a candidate biomarker of malaria severity.

The selectin family of adhesion molecules consists of three distinct carbohydrate receptors expressed by leukocytes (L-selectin), endothelial cells (E-selectin), or platelets and endothelium
(P-selectin) (Mukae, Ashitani et al. 2003). Selectins play a critical role at early stages of the adhesion cascade, the ordered series of steps whereby leukocytes migrate from the intravascular space into inflamed tissue. Circulating white blood cells extravasate at loci of inflammation first by transient attachment to the endothelial wall (tethering and rolling), followed by firm adhesion and diapedesis (Adams and Shaw 1994). The selectins and their respective ligands are important in the early transient ‘rolling’ phase of cell migration. Soluble circulating forms of P-selectin (sP-selectin) in human serum and other body fluids, and elevated levels of these soluble forms have been found in numerous inflammatory diseases (Lai, Wong et al. 1993; Seki, Higashiyama et al. 2000) (Mukae, Kadota et al. 1997). It is likely that these soluble adhesion molecules originate from adhesion molecules expressed on activated cells, and that their presence in the circulation may reflect the severity and activity of inflammation. In Chapter 6, we investigate the predictive utility of circulating sP-selectin in pediatric malaria.

Coagulopathy is a well-recognized manifestation of several severe infectious syndromes such as meningococcemia, sepsis and severe malaria. Regulation of the coagulation cascade is intimately linked to the vascular endothelium, such that alterations in the profile of coagulation proteins also reflect activation and/or dysfunction of the endothelium. Like Ang-2, vWF and its precursor, vWF propeptide, are stored within WP bodies and released into the circulation in response to inflammatory stimuli. In severe malaria, vWF may help tether parasitized erythrocytes to endothelial cells via platelets (Bridges, Bunn et al. 2009). In Chapter 6, we evaluate vWF and its precursor, vWF propeptide, as potential markers of severe disease in hospitalized African children.

1.1.6.4  Nitric oxide

Because of its involvement in multiple biological pathways, the role of nitric oxide (NO) in malaria is complex. NO has a well-recognized antimicrobial function, coupling with hydrogen peroxide to form peroxynitrite. However, a direct antiparasitic role of NO against *P. falciparum* has not been observed *in vitro* (Rockett, Awburn et al. 1991). NO-derived reactive nitrogen intermediates released by phagocytes may also mediate bystander tissue injury (Clark and Rockett 1996). NO plays a role in neurotransmission, and some authors have postulated that excessive local NO might contribute to the coma of CM (Clark, Rockett et al. 1991); however, more recent data challenge this hypothesis (Sobolewski, Gramaglia et al. 2005). Experimental
cerebral malaria is associated with reduced NO bioavailability, and mortality was reduced by the administration of a NO donor or inhaled NO (Gramaglia, Sobolewski et al. 2006). Human observational studies have shown that severe malaria is associated with impaired NO bioavailability, reduced iNOS expression, low plasma concentrations of L-arginine (the substrate for NO synthesis), and increased levels of ADMA (an inhibitor of NO synthase (NOS)) (Hobbs, Udhayakumar et al. 2002; Lopansri, Anstey et al. 2003; Yeo, Lampah et al. 2007; Yeo, Lampah et al. 2010). Moreover, population genetic studies have linked polymorphisms in NOS genes that affect endogenous NO production to disease severity (Hobbs, Udhayakumar et al. 2002; Ohashi, Naka et al. 2002; Cramer, Mockenhaupt et al. 2004; Dhangadamajhi, Mohapatra et al. 2009; Dhangadamajhi, Mohapatra et al. 2009; Dhangadamajhi, Mohapatra et al. 2009).

NO functions in several ways that may be host-beneficial in malaria infection. NO regulates vasomotor tone, platelet and endothelial cell activation, immune and inflammatory responses, and apoptosis. In vitro, NO decreases the expression of cell adhesion molecules and PE adhesion (Bogdan 2001; Sobolewski, Gramaglia et al. 2005). NO inhibits the exocytosis of Weibel-Palade bodies containing Ang-2 and vWF, which promote a cascade of inflammation and coagulopathy that may contribute to the immunopathology of severe malaria (Matsushita, Morrell et al. 2003). Exogenous inhaled nitric oxide improves survival in a murine model of severe malaria and L-arginine (Gramaglia, Sobolewski et al. 2006), a nitric oxide donor molecule improves outcomes in Indonesian adults with severe malaria (Yeo, Lampah et al. 2007). We hypothesize in Chapter 8 that inhaled nitric oxide may benefit children with severe malaria, and describe an experimental design to test this hypothesis in Chapter 9.

1.1.6.5 Immunomodulatory effects of malaria

In the context of *P. falciparum* infection, immune effector mechanisms to clear parasites may be circumvented by evasion strategies and parasite countermeasures to neutralize host defenses. *P. falciparum* has immunomodulatory effects that may function to promote its own survival in the host, but may simultaneously alter responses to other co-infecting pathogens as a secondary consequence. Furthermore, in the setting of repeated inoculation as occurs in endemic areas, *P. falciparum* strains regulate the development of co-infecting strains, through a mechanism dependent on the host iron regulatory hormone hepcidin (Portugal, Carret et al.). Malaria infection induces macrophage dysfunction, immune effector cells which play a central role in
innate and adaptive defenses against *P. falciparum* as well as a wide array of other pathogens. As phagocytes internalize the parasite byproduct hemozoin, either in a cell-free form or through the phagocytosis of IRBCs, the hemozoin accumulates within phagocytic vacuoles, evidenced by dark brown birefringent malaria pigment. Hemozoin loading of macrophages alters several key functions, including the ability to repeat phagocytosis (Serghides, Smith et al. 2003), generate an oxidative burst upon stimulation (Schwarzer and Arese 1996), express MHC class II surface molecules (Schwarzer, Alessio et al. 1998) and activate protein kinase C (Schwarzer, Turrini et al. 1993). The mechanism underlying the toxicity of hemozoin to macrophages may be due to lipoperoxides formed from polyunsaturated fatty acids associated with hemozoin which have been shown to inhibit the phorbol-ester-induced oxidative burst of human monocytes (Schwarzer, Kuhn et al. 2003).

Dendritic cells (DCs) are a second cellular target for *P. falciparum* immunomodulation. DCs are important antigen presenting cells, linking innate and adaptive immune responses to malaria and other infections. Dendritic cell sample their environment by avidly phagocytosing particles through a variety of receptors, including the scavenger receptor CD36. Uptake pathways involving CD36 tend to be non-inflammatory, resulting in the secretion of IL-10 and dampening of inflammatory response (Voll, Herrmann et al. 1997). Although controversial, non-opsonic phagocytosis of *P. falciparum* through the interaction of CD36 with PfEMP on the IRBC surface has been reported to alter DC function. DCs internalizing CD36 binding parasites were unable to mature upon stimulation by lipopolysaccharide (LPS) or TNF-a (Urban, Ferguson et al. 1999; Urban, Willcox et al. 2001). Specifically, production of MHC class II and surface expression of co-stimulatory molecules were inhibited, secretion of IL-12 and IFN-g was downregulated, and secretion of IL-10 was enhanced (Urban, Ferguson et al. 1999; Urban, Willcox et al. 2001).

Thus, malaria infection results in a state of relative immune deficiency. Functional consequences include the exacerbation of viral (Thursz, Kwiatkowski et al. 1995; Moormann, Chelimo et al. 2005), parasitic (Phillips, Selby et al. 1974), and intracellular bacterial (Murphy and Lefford 1979; Mabey, Brown et al. 1987) infections, as well as alterations in the humoral and cell-mediated immune responses to heterologous vaccines and other antigens (Williamson and Greenwood 1978; Greenwood, Bradley et al. 1980; Theander, Svenson et al. 1987; Hviid, Theander et al. 1991). In a murine model, experimental malaria co-infection has been shown to
increase bacillary load following challenge with *M. tuberculosis* (Scott, Kumar et al. 2004). Chapter 2 extends these findings, demonstrating that malaria-induced immunomodulation exacerbates the course of both acute and re-activation mycobacterial infection.

### 1.1.7 Animal models of malaria

Experimental models of human malaria have employed various combinations of host and *Plasmodium* parasite. While no animal model precisely reproduces the clinical and pathological features of human malaria, similarities between of animal models and human disease have allowed investigators to draw important insights into the host response to malaria in experimental systems.

Non-human primates are the phylogenetically closest host to humans to model malaria. *Aotus* and *Saimiri* monkeys and chimpanzees can be infected with the human pathogen *P. falciparum*, providing a useful tool for vaccine development and drug testing (Siddiqui, Tam et al. 1987; Crandall, Collins et al. 1993; Herrera, Perlaza et al. 2002). Macaque monkeys infected with *Plasmodium coatneyi* and *Plasmodium fragile* demonstrate sequestration of parasitized RBCs in the microvasculature, as in human disease (Kawai, Aikawa et al. 1993; Tongren, Yang et al. 2000).

Rodent models of malaria were first described in 1974 (Yoeli and Hargreaves 1974). Given their convenience as a research tool, availability of immunologic reagents, and amenability to genetic manipulation, murine models of malaria have been extensively and increasingly studied (White, Turner et al.). Inbred murine strains differ widely in their susceptibility to malaria and outcomes following infection are heterogeneous, a feature that has been used to dissect genetic determinants of susceptibility to infection (Malo, Vogan et al. 1994; Fortin, Cardon et al. 2001; Min-Oo, Willemetz et al.). Four species of murine parasites are commonly used: *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium yoelii*, and *Plasmodium vinckei*. *P. berghei* infection of C57/BL6 mice is a widely used experimental model of cerebral malaria, although controversy surrounds its representativeness of human disease (Stevenson, Gros et al.; White, Turner et al.; Langhorne, Buffet et al.). *P. chabaudi* provides a useful model of blood-stage malaria. Similarities to infection with the human pathogen *P. falciparum* include analogous blood-stage antigens, invasion of both immature and mature erythrocytes, suppression of B- and
T-cell responses, and parasite sequestration in liver and spleen (Stevenson and Riley 2004). Two subspecies of *P. chabaudi* have been defined: *P. chabaudi chabaudi* and *P. chabaudi adami*, both of which have been extensively used to study the immunological basis of the pathologies observed in human malaria (De Souza, Williamson et al. 1997; Mohan, Moulin et al. 1997; Favre, Ryffel et al. 1999; Su and Stevenson 2000; Su and Stevenson 2002). The course of *P. chabaudi* infection is characterized by rapid multiplication of the parasite during the first week after infection with infected erythrocytes, leading to death in susceptible mice, or induction of adaptive immune responses and elimination of parasites in resistant strains (Stevenson and Riley 2004). The *P. chabaudi* model of blood stage malaria in a resistant C57/BL6 mouse host is used in Chapters 2 to dissect biological interactions between malaria and mycobacteria.

In contrast to traditional murine models of malaria that make use of rodent *Plasmodium* species, NOD.CB17-*Pkrd*<sup>−/−</sup> immunocompromized mice will tolerate a xenograft of human erythrocytes, permitting *P. falciparum* (human malaria) infection in a murine host (Moreno, Badell et al. 2001; Angulo-Barturen, Jimenez-Diaz et al. 2008). Thus, NOD CB17-*Pkrd*<sup>−/−</sup> immunocompromised mice provide an attractive *in vivo* model for testing the effect of anti-parasitic compounds against human malaria.

### 1.2 Tuberculosis

#### 1.2.1 Etiology

The *M. tuberculosis* complex is composed of 5 biochemically and immunologically related species: *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, and *Mycobacterium canetti*. Among these, *M. tuberculosis* is the most important cause of tuberculosis in man. *M. tuberculosis* is an acid-fast bacillus 2–4 μm long. The organism is a weakly gram-positive, non-spore-forming, non-motile obligate aerobe that may appear as beaded or clumped aggregates in clinical specimens or culture media. Distinguishing microbiological properties of mycobacteria include acid fastness: mycolic acids in the cell wall form stable complexes with dyes such as crystal violet, carbolfuchsin, auramine, and rhodamine and resist decoloration with ethanol and hydrochloric or other acids. The lipid-rich cell wall also confers resistance to the cidal effects of antibody and complement. The organism is slow-growing, with a doubling time of 12 to 24 hours.
Bacille Calmette-Guérin (BCG) is commonly used as a model organism for the study of host response to mycobacteria, as in Chapters 2 and 3 of the present thesis and in previous reports (Shands and Senterfitt 1972; Ferluga 1981; Nau, Liaw et al. 1999; Kremer, Estaquier et al. 2000; Jacobs, Fick et al. 2002; Heldwein, Liang et al. 2003; Nicolle, Fremond et al. 2004; Cosma, Humbert et al. 2008; Leisewitz, Rockett et al. 2008). The organism is non-pathogenic in immunocompetent humans, and is administered worldwide in infancy as a tuberculosis vaccine. The original BCG vaccine was a strain of *M. bovis* attenuated by subculture every 3 weeks for 13 years, and thus a phylogenetically closely related species within the *M. tuberculosis* complex. This strain was subsequently subcultured in multiple laboratories around the world under various conditions, resulting in BCG vaccines that differ significantly in microscopic appearance, growth characteristics, immunostimulatory potency, and animal virulence. While their genomes are closely related, several chromosomal regions, present in *M. tuberculosis* but absent in the avirulent BCG, have been identified (Behr, Wilson et al. 1999). Among these regions of deletion (RDs), RD1 is the only region absent in all strains of BCG and appears to be an important mycobacterial virulence factor, inducing the recruitment of new macrophages to nascent granulomas for the expansion of the mycobacterial pool early in granuloma formation. (Davis, Aldrich et al. 1996; Volkman, Clay et al. 2004; Tan, Lee et al. 2006). It comprises nine genes, including two genes that encode small, secreted proteins, the culture filtrate protein 10 (CFP-10) and the early secretory antigenic 6 kDa (ESAT-6) protein (Tan, Lee et al. 2006). Detection of these proteins is the basis of the interferon-gamma release assay, now commercialized and used in clinical practice for the diagnosis of tuberculosis.

Non-tuberculous mycobacteria are less common than *M. tuberculosis* as agents of human disease, but also serve as useful model organisms in the study of host response to mycobacteria, as in Chapters 2 and 3 as well as previous publications (Cosma, Humbert et al. 2004; Cosma, Swaim et al. 2006). They are classified into 4 Runyon groups based on their growth properties (fast or slow growing) and colony appearance (presence of pigment under light and dark growth conditions). *Mycobacterium marinum*, discussed in Chapters 2 and 3 is a cause of peripheral granulomatous disease in humans (fishtank granuloma), and belongs to Runyon group I (slow-growing photochromogen).
1.2.2 Transmission

Infection with *M. tuberculosis* is acquired through inhalation into the peripheral alveoli of droplet nuclei containing tubercle bacilli discharged into the atmosphere by an individual with active infection. By virtue of cell wall lipids that resist desiccation, viable mycobacteria can persist within aerosol droplets in the atmosphere for several hours. Resistance to desiccation, coupled with the low infectious dose in the range of one to ten bacilli (Russell, Cardona et al. 2009), makes transmission efficient. A recent epidemiologic study in a TB endemic zone in sub-Saharan Africa demonstrated that incident active infection occurs in 3% of household contacts within 24 months of exposure (Whalen, Zalwango et al. 2011). It is estimated that an individual with active smear-positive tuberculosis infects 10-15 others per year (Brady, Coronel et al. 2008).

1.2.3 Clinical features

Most infections caused by *M. tuberculosis* are asymptomatic. Over 50% of individuals exposed to *M. tuberculosis* become infected (Rouillon, Perdrizet et al. 1976), yet only 10% of those who become infected will develop clinical disease over their lifetime (Murray, Styblo et al. 1990). When primary tuberculosis disease occurs, it usually follows a subacute or chronic tempo, with symptoms appearing 1 to 6 months after exposure. Symptoms are non-specific and include fever, weight loss (poor weight gain or failure to thrive in children), cough, night sweats and chills. Radiographic findings include hilar, subcarinal, paratracheal or mediastinal adenopathy; segmental or lobar atelectasis or infiltrate; pleural effusion; cavitary lesions; or a miliary pattern. Extrapulmonary manifestations include meningitis and CNS disease, granulomatous inflammation of the lymph nodes, gastrointestinal, genitourinary, musculoskeletal, skin, middle ear or mastoid infection.

Recrudescence of infection from sites of latency in the lung apices causes “chronic” or “adult-type” pulmonary tuberculosis, which accounts for the majority of adult tuberculous infection. Apical lung scarring is a common feature in elderly infected individuals before the development of clinical manifestations (Stead 1965). These apical lesions contain cultivable tubercle bacilli, and these areas of pulmonary fibrosis can progress to active disease. Tissue necrosis and
cavitation may lead to the discharge of heavy loads of mycobacteria into the airways, producing cough and airborne dissemination of *M. tuberculosis*.

Extra-pulmonary tuberculosis may occur at virtually any anatomical site. Common locations include the pleura, lymph nodes, bone and joint, genitourinary tract, and peritoneum (Mehta, Dutt et al. 1991). In addition, a miliary pattern on chest x-ray signals disseminated and poorly controlled infection and is associated with a mortality rate of approximately 20% (Kim, Langston et al. 1990). Meningitis is another life-threatening or disabling complication of tuberculosis. In young children, tuberculous meningitis develops in the context of uncontrolled primary infection and thus is accompanied by other manifestation of tuberculosis, including miliary tuberculosis in 20% to 30% and non-miliary pulmonary disease in 85% of cases (Yaramis, Gurkan et al. 1998; van den Bos, Terken et al. 2004). Other patterns of CNS disease include tuberculoma, tuberculous encephalopathy and tuberculous radiculomyelitis. Tuberculomas typically presenting with symptoms and signs of a space occupying lesion; seizure, which may be focal or generalized, are the most common manifestation.

Disease manifestations differ between children and adults infected with *M. tuberculosis*. Children are more likely to have paucibacillary disease, without cavitation, which can make the microbiologic diagnosis difficult. Microbiologic confirmation is obtained in less than 50% of pediatric sputum or gastric aspirates done in the presence of significant exposure history radiographic findings suggestive of active disease. Pulmonary tuberculosis with cavitation is more common in adults, and represents the major source for transmission of *M. tuberculosis* in the population, whereas children transmit infection less frequently to others. Extra-pulmonary manifestations are more common in children, including miliary tuberculosis and meningitis. Miliary tuberculosis accounts for 8.3% of pediatric, compared to and 1.3% of adult hospitalizations for TB (Hussey, Chisholm et al. 1991). Tuberculous meningitis occurs in approximately 0.5% of primary *Mycobacterium tuberculosis* infections in childhood and occurs most commonly in children between 6 months and 4 years of age (Berman, Kibel et al. 1992). In contrast, in teenagers and adults, tuberculous meningitis more often develops in the context of latent *M. tuberculosis* infection (LTBI) and results from rupture of subependymal tubercles into the subarachnoid space. On the other hand, renal tuberculosis is more common in adults. Treatment outcomes in children are generally favorable, even in young and
immunocompromised children who are at higher risk of disease progression and disseminated disease, provided that treatment starts promptly.

1.2.4 Diagnosis

A definitive diagnosis requires the isolation of \textit{M. tuberculosis} complex by culture from clinical specimens including sputum, bronchial washings, gastric aspirates, pleural fluid, CSF, urine, or biopsy specimens. However, in many areas of tuberculosis endemicity, laboratory facilities are not adequate for mycobacterial culture and susceptibility testing. Culture techniques are challenging because of the biohazard of cultivating pathogenic airborne mycobacteria. Furthermore, isolation from clinical specimens on solid media usually takes 3–6 wk, with an additional 4 wk required for drug susceptibility testing. Faster turn-around times can be achieved using liquid medium and radiolabeled nutrients (the BACTEC radiometric system).

Latent \textit{M. tuberculosis} infection is diagnosed by a positive tuberculin skin test (Mantoux test), representing a delayed type hypersensitivity reaction to the tuberculous antigen purified protein derivative (PPD), in the absence of clinical or radiographic evidence of active disease. The tuberculin skin test has limited sensitivity and specificity. Recent BCG vaccination or past exposure to non-tuberculous mycobacteria can result in a false positive test. Anergy to the purified protein derivative (PPD) has been observed in 5-50\% of children with tuberculous meningitis (Lincoln, Sordillo et al. 1960; Molavi and LeFrock 1985; Waecker and Connor 1990; van den Bos, Terken et al. 2004) and others with advanced tuberculosis (false negative test). Severely debilitated, malnourished, and immunocompromised hosts, and those with recent viral infections such as measles, may also fail to mount a cellular immune response to the intradermal PPD. Interferon gamma release assays (IGRAs) represent a recent advance in TB diagnostics. The principle of the diagnostic test is the detection of IFN-\(\gamma\) released by memory or effector T-cells in response to mycobacterial antigens. One advantage of IGRA over the tuberculin skin test is that it employs fewer \textit{M. tuberculosis}-specific antigens (ESAT-6 and CFP-10) while the tuberculin of the TST is a mix of about 200 non-specific antigens that are shared with nontuberculous mycobacteria as well as BCG (Nahid, Pai et al. 2006). Thus IGRAs have the potential of being more specific, but their reliability in infants and young children whose Th1-type T-cell immune responses are relatively immature remains to be defined (Lewinsohn, Gennaro et al. 2004). Discordant results between IGRA and TST occur in 21-29\% of low-risk
patients, mostly TST-positive and IGRA-negative combinations consistent with increased specificity of IGRA (Menzies, Pai et al. 2007).

Advances in low-cost methods for TB diagnosis in resource poor settings include the microscopic-observation drug-susceptibility (MODS) assay. The underlying principles of the diagnostic test are: (1) faster growth of *M. tuberculosis* in liquid than on solid media; (2) early detection of growth using light microscopy for characteristic aggregate forms (cording) characteristic of *M. tuberculosis*; and (3) incorporation of isoniazid and rifampicin for the detection of resistant organisms. MODS offers improved sensitivity over sputum smear microscopy, shorter time turnaround time relative to culture on solid media, and lower cost than liquid (radiometric) culture assays. MODS is therefore potentially accessible to resource-limited settings, costing under USD$3 for testing for TB and MDRTB (Brady, Coronel et al. 2008).

High-pressure liquid chromatography analysis can be used for the speciation of culture isolates since each mycobacterial species has a unique profile of cell wall mycolic acids. Nucleic acid amplification (NAA) tests, including polymerase chain reaction, can also be used to amplify mycobacterial DNA in clinical samples to arrive at a rapid diagnosis. NAA tools to date have sensitivity for pulmonary tuberculosis between that of smear and culture. Restriction fragment length polymorphism (RFLP) profiling of mycobacteria can differentiate between strains and thus distinguish between recrudescence and re-infection, and thus is a helpful epidemiologic tool.

### 1.2.5 Treatment

The goals of TB treatment are to prevent death from active TB or its late effects, to rapidly reduce the burden of mycobacteria and thereby cure active infection, to prevent relapse of TB by eliminating latent foci, to prevent the development of drug resistance by using a combination of drugs, and to decrease TB transmission to others. Thus, typical anti-TB regimens consist of two phases: an intensive phase with multiple drugs to eradicate the majority of actively replicating bacilli while preventing the emergence of drug resistant clones, and continuation phase with fewer drugs targeting the smaller number of dormant organisms. Standard first line medications are isoniazid, rifampin, ethambutol, and pyrazinamide (American Thoracic Society, Centers for Disease Control and Prevention et al. 2003; American Academy of Pediatrics 2006). Other classes of antibiotics effective against *M. tuberculosis* include aminoglycosides (streptomycin,
amikacin, kanamycin and capreomycin), ethionamide, fluoroquinolones, and linezolid. After 2 months of treatment with the 3-4 drug regimen, stepdown to dual therapy with isoniazid and rifampin, administered daily or twice weekly, is recommended, usually for a total of 6 months in uncomplicated pulmonary tuberculosis. In some cases more prolonged courses of therapy may be required (Girgis, Sultan et al. 1998). Regimens are thus prolonged and complex, involving multiple medications. Failure to adhere strictly to combination therapy can result in the emergence of antibiotic resistant clones.

Drug resistant tuberculosis, including multidrug resistant (MDR) and extensively drug resistant (XDR) *M. tuberculosis* (TB), represents an emerging threat to the effective treatment of tuberculosis. MDR TB refers to strains resistant to at least isoniazid and rifampin and TB strains that also exhibit resistance to one or more fluoroquinolone antibiotics and any of the second line injectable agents (amikacin, kanamycin, and capreomycin) is classified as extensively-drug-resistant (XDR) TB. Illustrating its potential severity, pulmonary tuberculosis due to XDR TB was almost uniformly fatal within 2 weeks of diagnosis in adults with advanced HIV disease in South Africa (Gandhi, Moll et al. 2006). Treatment is often unsuccessful despite prolonged, complicated and toxic drug regimens: a cocktail of 5 or more drugs for 24 months was associated with treatment failure in 40% of cases in one series (Mitnick, Shin et al. 2008).

Adjunctive corticosteroid therapy is recommended for certain life-threatening complications of tuberculosis including tuberculous meningitis and pericarditis. A meta-analysis of randomized controlled trials found that corticosteroids reduced the risk of death or disabling residual neurological sequellae in tuberculous meningitis (Prasad and Singh 2008).

### 1.2.6 Prognosis

While the majority of infections are asymptomatic, and most cases are curable with antimicrobials (albeit prolonged courses with multiple drugs), severe manifestations of tuberculosis continue to cause death and disability. Mortality in miliary tuberculosis was 21% in one series involving elderly adults (Kim, Langston et al. 1990) and 14% in another pediatric series (Hussey, Chisholm et al. 1991). Untreated tuberculous meningitis is uniformly fatal; with treatment, mortality is approximately 20-30%. Major neurologic sequellae occur in many survivors of tuberculous meningitis, including hydrocephalus, spastic pareses, seizures,
paraplegia, and sensory disturbances of the extremities (Molavi and LeFrock 1985). Sequellae are more prevalent and severe in those with advanced disease at presentation (Delage and Dusseault 1979; Humphries, Teoh et al. 1990; Yaramis, Gurkan et al. 1998; Mahadevan, Mahadevan et al. 2002). The prognosis for children under 4 years of age is worse than that for older children and adults, in part because the diagnosis is more difficult to make in young children, leading to delays in instituting anti-TB therapy (Delage and Dusseault 1979). Malnutrition, presence of underlying debilitating diseases, and associated miliary disease have also been associated with more severe outcome.

1.2.7 Host response to tuberculosis

Selected aspects of the immune response to tuberculosis are reviewed here in relation to Chapter 2, which deals with the effect of murine malaria infection on the course of experimental mycobacterial challenge, and Chapter 3, which explores the role of the macrophage cell surface molecule CD36 on antituberculous defenses. The complex chain of cellular events leading to granuloma formation and containment of mycobacteria is susceptible to disruption by co-infecting pathogens at multiple stages. Furthermore, early innate mechanisms of defense rely on common cellular pathways that are governed at least in part by the cell surface glycoprotein CD36.

1.2.7.1 Innate and adaptive immune responses to TB

Inhaled bacilli are internalized by alveolar macrophages, initiating an inflammatory response and cell migration. Mycobacteria resist microbicidal programs of macrophages and are carried to regional lymph nodes as intracellular cargo, where ongoing replication or persistence of organisms within macrophages takes place. Lymphohematogenous seeding throughout the body occurs during this phase, establishing distant foci in the lung apices, as well as tissues at virtually any anatomical site. Cell-mediated immunity (delayed-type hypersensitivity) specific to \textit{M. tuberculosis} develops within 3-8 weeks after initial infection, through antigen presentation by dendritic cells and macrophages to CD4\(^+\) T-cells. The central role of the T-cell in the adaptive immune response to \textit{M. tuberculosis} is illustrated by the exquisite susceptibility of CD4-lymphopenic HIV-infected patients to tuberculosis disease (Chaisson and Martinson 2008). Cytokines play a key role in intercellular signaling in antituberculous defenses, including TNF,
INF-γ, IL-1 and IL-12 (Abel, Thieblemont et al. 2002; Drennan, Nicolle et al. 2004; Bafica, Scanga et al. 2005). In the vast majority of cases, the infection is controlled by the immune system and the infected individual remains asymptomatic. If this does not occur, primary progressive *M. tuberculosis* disease ensues. This occurs more commonly in young children than adolescents and adults.

Latent foci harboring dormant but viable *M. tuberculosis* may re-activate months to years later (Flynn and Chan 2001). Host pathways are actively engaged to keep mycobacterial replication in check at sites of latency. Nitric oxide, involved in RNI-mediated killing by macrophages, appears to play an important role, as demonstrated by the re-activation of latent tuberculosis using the NOS inhibitor aminoguanidine in two mouse models (Flynn, Scanga et al. 1998). TNF also plays a critical role to the maintenance of granulomas, and blockade of TNF signaling in animals with established mycobacterial granulomas results in a loss in granuloma cellularity and protective function (Kindler, Sappino et al. 1989). Loss of immunologic control results in recrudescence from sites of latency, most commonly in the lung apices. Liquefaction and cavitation of the necrotic center of the granuloma results in the discharge of thousands of infectious organisms into the airways and triggers a productive cough, generating the infectious aerosol and completing the organism's life cycle (Russell, Cardona et al. 2009).

### 1.2.7.2 Pattern recognition receptors in TB

At early stages of infection, pattern recognition receptors, such as TLR2, TLR4, and TLR9, have been implicated in the sensing and innate response to *M. tuberculosis* (Drennan, Nicolle et al. 2004). Chapter 3 reports on experiments showing that the macrophage cell surface receptor CD36 also participates in innate host defenses against mycobacteria. Here we introduce CD36 in the larger context of pathogen recognition receptors.

Macrophages bind and internalize pathogens through specific interactions between molecules on their cell surface and their prey. Pattern recognition receptors (PRRs) are molecular components of the innate immune system which act as sensors of a wide array of microbial products, termed pathogen-associated molecular patterns (PAMPs). These include carbohydrates such as lipopolysaccharide (LPS) and mannose, nucleic acids, peptides such as flagellin, peptidoglycans and lipids including lipoteichoic acids. Of note, although PRRs primarily function to identify
“non-self” biomolecules associated with invading pathogens, endogenous motifs may also act as immunostimulatory signals in the setting of tissue damage. This class of signaling molecules has been variously termed danger-associated molecular patterns (DAMPs), “alarmins” or “endokines”. Examples include high mobility group box 1 protein (HMGB-1), heat-shock proteins (HSPs), and uric acid (Foell, Wittkowski et al. 2007), which promote a marked inflammatory response.

Functionally, PRRs may be endocytic, promoting the binding, internalization and destruction of microorganisms by phagocytes, or may transduce signals and trigger downstream cellular pathways. Examples of endocytic receptors include macrophage mannose receptors, glucan receptors on phagocytes, and scavenger receptors. Signaling PRRs include the large family of membrane-bound surface or endosomal Toll-like receptors (TLRs) and the cytoplasmic NOD-like receptors.

Among the endocytic PRRs, scavenger receptors (SRs) play an important role in uptake and clearance of effete components, such as modified host molecules and apoptotic cells (Peiser, Mukhopadhyay et al. 2002). SRs are expressed on the surface of myeloid cells (macrophages and dendritic cells) and certain endothelial cells. SRs are classified into subtypes A, B, and C according to structural characteristics. Class A SRs include the macrophage receptor with collagenous structure (MARCO) and class A SR types I and II. MARCO and other subclass A SRs have a collagenous structure, bind modified low-density lipoprotein and bacteria, and promote the uptake and clearance of particles and bacteria. Class B SRs have two transmembrane regions and are concentrated in caveolae, microdomains of the plasma membrane. Class C scavenger receptors are transmembrane proteins whose N-terminus is located extracellularly.

CD36 is a 88-kDa class B scavenger receptor, expressed on the surface of innate immune cells including macrophages and dendritic cells, as well as on platelets, endothelial cells, adipocytes, striated muscle cells, and hematopoietic cells (Rac, Safranow et al. 2007). Its multiple functions include angiogenesis, phagocytosis, inflammation, cell adhesion, and lipid and glucose metabolism. The CD36 gene is located on chromosome 7 q11.2 (Fernandez-Ruiz, Armesilla et al. 1993) and contains 15 exons, of which exons 1, 2, and 15 are noncoding (Armesilla and Vega
1994; Armesilla, Calvo et al. 1996). Different tissues express varying levels of CD36 and gene expression is primarily controlled by the transcription heterodimer PPAR-γ-RXR (peroxisome proliferator-activated receptor-γ-retinoid-X-receptor). Two short intracellular domains may act to transduce signals in association with Src-family kinases. CD36 on phagocytes participates in the binding and internalization of apoptotic cells. Specific endogenous ligands include thrombospondin and collagen. CD36 has also been reported to bind diacylglycerides on gram positive bacteria (Hoebe, Georgel et al. 2005), and PfEMP1, sequestrin, modified band 3 and cytoadherence-linked asexual gene 9 (CLAG9) on *P. falciparum* parasitized RBCs (Serghides, Smith et al. 2003).

CD36 participates in the innate immune response to multiple pathogens, including *Staphylococcus aureus* (Hoebe, Georgel et al. 2005; Stuart, Deng et al. 2005), *P. falciparum* (Patel, Lu et al. 2007), *Escherichia coli* and *Enterococcus faecalis* (Baranova, Kurlander et al. 2008). A common theme across this diverse range of pathogens is the non-inflammatory uptake of microbes by phagocytes through the CD36 scavenger receptor. A genome-wide RNA interference screen in *Drosophila melanogaster* identified the CD36 homologue Pes as a key determinant of mycobacterial entry into phagocytes (Philips, Rubin et al. 2005). Chapter 3 explores the role of CD36 in innate immune responses to mycobacteria *in vitro* and *in vivo*.

### 1.2.7.3 The granuloma

The tuberculoid granuloma is the histological hallmark of tuberculosis, and traditionally believed to be essential to the control of tuberculosis in murine models and humans (Kaufmann and Hess 2000; Flynn and Chan 2001). Longstanding host-mycobacterial interaction results in an organized inflammatory lesion consisting of a complex network of immune cells at the interface of pathogen and host. Classically, granuloma formation has long been viewed as a host-driven mechanism to contain invading mycobacteria. Functional adaptive immunity is essential for granuloma maintenance and the breakdown of adaptive immunity, as in advanced HIV infection, is associated with the loss of granuloma integrity, disseminated infection, and severe disease (Muller and Takeshita 1991). Similarly, athymic mice with defective T-cell responses have poor granulomas and succumb to disseminated infection (Sher, Chaparas et al. 1975). On the other hand, more recent studies have demonstrated that granuloma formation is enhanced by
mycobacterial virulence factors, suggesting that granuloma formation is in part driven by the pathogen and contributes to disease manifestations (Volkman, Clay et al. 2004; Davis and Ramakrishnan 2009). Early events in granuloma formation promote the expansion of mycobacteria, through the senescence of infected macrophages and recruitment of new phagocytes which act as fresh cellular host for mycobacterial persistence and replication (Davis and Ramakrishnan 2009).

The granuloma is a tightly aggregated structure containing myeloid (macrophage) and lymphoid (B- and T-cell) populations. This architecture serves a protective function, by creating a physical barrier to bacterial dissemination, and by promoting interactions between key cell types such as antigen-specific T cells and macrophages. The fully-developed granuloma consists of a relatively static myeloid scaffold around which a highly dynamic effector T cell population circulates (Egen, Rothfuchs et al. 2008). Bacteria are found both within macrophages and extracellularly, in central areas of caseating necrosis.

Multiple coordinated steps culminate in the formation of this elaborate cellular complex. Initially, infected phagocytes nucleate granuloma formation, serving as an early source of chemoattractant for the recruitment of new mononuclear cells from surrounding tissue (resident tissue macrophages) and neighboring blood vessels (monocytes). A critical cytokine during granuloma formation is TNF, secreted by infected macrophages in the microenvironment of the developing granuloma. TNF is involved in a process of autoamplification, acting in an autocrine or paracrine fashion to enhance its own synthesis and release, thus favoring further macrophage accumulation and differentiation (Kindler, Sappino et al. 1989). Mice lacking TNF or its receptor have a severely impaired granulomatous response (Lin, Plessner et al. 2007). TNF is also required to maintain a stable and protective granuloma environment (Egen, Rothfuchs et al. 2008), the clinical significance of which is illustrated by recrudescence of latent TB with the therapeutic use of monoclonal antibodies to TNF (Keane, Gershon et al. 2001). Granuloma macrophages undergo morphological changes over time, from round or stellate cells to large, compact epithelioid forms (Dannenberg 1993; Cosma, Humbert et al. 2004). Despite temporal stability, the granuloma is porous to the influx of lymphocytes and macrophages (Cosma, Humbert et al. 2004; Cosma, Humbert et al. 2008). Lymphocytes recruited to the granuloma include T-cells, which play a critical role by secreting IFN-γ that activates adjacent macrophages,
priming them for intracellular killing. T-cells localize to the granuloma border, enter and accumulate within the granuloma while maintaining rapid motility, highly restricted migration, and limited egress into the surrounding tissue (Egen, Rothfuchs et al. 2008). With time, the granuloma develops a fibrous capsule that encases the macrophage core. Lymphocytes appear to be excluded from the center of the structure and the number of blood vessels penetrating the granuloma decreases. Foamy macrophages appear in the fibrous capsule and caseous debris accumulates in the center of the granuloma. This transition heralds the progression to active secondary disease, with necrosis at center of the granuloma and unchecked bacterial replication (Russell, Cardona et al. 2009).

1.2.8 Animal models of tuberculosis

As in studies of malaria, several host-pathogen combinations have been used to model aspects of tuberculosis. The most widely used is the mouse model, given pragmatic considerations including ease of manipulation and housing, inbred strains, availability of congenic or genetically engineered strains, and the wide range of available reagents. In addition, guinea pigs (Flynn 2006), rabbits (Dannenberg 2003), and non-human primates (Langermans, Andersen et al. 2001) serve as additional mammalian hosts. Mycobacterial infection in more distantly related ectotherms, including frogs and transparent zebrafish, has also provided important insights into mycobacterial pathology (Cosma, Humbert et al. 2004; Cosma, Swaim et al. 2006). *M. marinum*, discussed in Chapters 2 and 3, is a natural pathogen of fish and produces granulomas with caseous necrosis.

Several disease mechanisms are shared in mice and humans. Consistent with human disease (Keane, Gershon et al. 2001; Casanova and Abel 2002), cytokines including TNF and IFN-γ are critical regulators of susceptibility and pathology in murine infection with *M. tuberculosis* (Flynn, Chan et al. 1993; Flynn, Goldstein et al. 1995), and intracellular killing mechanisms including reactive nitrogen intermediates are conserved in both mice and humans (Chan, Tanaka et al. 1995; Flynn, Scanga et al. 1998; Chan, Chan et al. 2001). However, murine infection with *M. tuberculosis* remains an imperfect model of human tuberculosis. Unlike the distinctive period of latency following primary infection in humans, *M. tuberculosis* infection is progressive in mice. While resistant strains may control mycobacterial replication, the organism burden remains high in infected organs, unlike in human disease where bacilli are reduced to very low levels by
the host, and evidence of disease is absent until reactivation occurs. Two models of latent TB in mice have been proposed: (1) “low-dose” challenge, resulting in partial control of the initial infection for a period of 15 to 18 mo, at which time recrudescence occurs (Orme 1988); and (2) the “Cornell” model, in which antibiotics are used to reduce the infectious burden after an initial period mirroring primary infection, and subsequently withdrawn, allowing the organisms to resume replication (Radaeva, Nikonenko et al. 2005). In the “low dose” model, mycobacteria are contained by host mechanisms alone, but the number of organisms is several orders of magnitude higher than would be seen in latent human infection. In the “Cornell” model, the infectious burden is similar to that in human disease, but the control of bacterial replication depends on antimicrobials rather than host immunity. Furthermore, the pathology of tuberculosis in mouse lungs differs significantly from humans. Granulomas lack a caseating necrotic core as seen in human disease, which may be an important feature of mycobacterial latency (Flynn 2006).

Alternative mycobacterial species also mimic aspects of tuberculous disease in humans and serve as useful model organisms. Infection of mice with *M. bovis* BCG represents a well-established model of cellular immunity and granuloma formation, in Chapters 2 and 3 of the present thesis, as elsewhere (Kaufmann, Ladel et al. 1995; Egen, Rothfuchs et al. 2008). In C57BL/6 mice, BCG inoculation via the intraperitoneal route results in lymphohematogenous dissemination of mycobacteria, with large numbers of organisms cultivatable from the spleen, and a histologically discernible granulomatous response in the liver as early as 2 weeks after challenge (Co, Hogan et al. 2004). Mycobacterial replication is controlled by adaptive host immune responses after 3 weeks post-infection, but viable bacilli persist at low levels in infected organs, mirroring the course of latent tuberculosis in humans (Blanden, Lefford et al. 1969; Leisewitz, Rockett et al. 2008). In this respect, murine BCG infection parallels human tuberculosis more closely than the relentlessly progressive course of murine infection with *M. tuberculosis* itself. Intraperitoneal challenge with BCG is used to model acute and latent tuberculous infection in Chapter 2.

Furthermore, this model is used to demonstrate a role for CD36 in the innate immune response to mycobacteria in Chapter 3.
Chapter 2
Malaria exacerbates experimental mycobacterial infection *in vitro* and *in vivo*

2.1 Abstract

Tuberculosis (Mt) and malaria are among the most important infectious causes of morbidity and mortality worldwide, causing an estimated 1.5 million and 1 million deaths every year, respectively. Here we demonstrate a biological interaction between malaria and mycobacteria *in vitro* and *in vivo*. Murine macrophages co-incubated with *Plasmodium falciparum* parasitized erythrocytes demonstrated impaired control of intracellular Mt replication, and reduced production of reactive nitrogen species in response to mycobacteria. Infection of C57BL/6 mice with *Plasmodium* species exacerbated the course of acute mycobacterial infection (57% increase in peak splenic CFU, p=0.043 for difference over time course of infection), induced disruption of the structural integrity of established granulomas, and caused reactivation of latent mycobacterial infection (2.6-fold increase in peak splenic CFU, p=0.016 for difference over time course of reactivation). Malaria pigment deposition within the granulomas of co-infected mice suggested that the influx of dysfunctional hemozoin-laden monocytes into the locus of mycobacterial control may contribute to impaired containment of mycobacteria. Collectively, these results point to malaria-induced dysregulation of innate and adaptive anti-mycobacterial defences, and suggest that the interaction of these globally important pathogens may potentiate Mt infection and transmission.
2.2 Introduction

Tuberculosis and malaria are leading causes of infectious disease associated morbidity and mortality globally. *Mycobacterium tuberculosis* (Mtb) infects one third of the world’s population and accounts for 1.5 million deaths annually, more than any other bacterial pathogen (Egen, Rothfuchs et al. 2008). *Plasmodium falciparum* is the leading parasitic cause of mortality worldwide, causing approximately 300 million new infections and one million deaths per year (Davis and Ramakrishnan 2009). Both diseases are endemic in tropical and impoverished areas of the world, and co-infection is likely to occur in individuals in these zones of intense transmission (Page, Jedlicka et al. 2005). In addition to the socio-economic health determinants that account in part for the significant geographic overlap of both pathogens, biological interactions within the host may play a role in malaria-tuberculosis co-infection. Establishing a role for *P. falciparum* in exacerbating the course of mycobacterial infection, analogous to its potentiating effect on the spread of HIV in Africa (Abu-Raddad, Patnaik et al. 2006), could have important public health implications.

Control of mycobacterial infection requires a highly co-ordinated host response that may be vulnerable to dysregulation by co-infecting pathogens. In human tuberculosis, following inhalation of aerosolized tubercle bacilli, alveolar macrophages internalize Mtb, initiating a cascade of cellular migratory events that lead to lymphohematogenous dissemination. In the majority of immunocompetent hosts, cell mediated immunity results in the containment of mycobacteria; however, acute infection may occasionally progress to miliary or meningeal disease. Risk factors for disseminated disease are incompletely understood, but include factors associated with varying degrees of immune compromise such as young age, HIV-infection, and corticosteroid use (Yang, Kong et al. 2004). Moreover, the protective effect of BCG vaccination against infant and childhood miliary and meningeal disease suggests a role for adaptive immunity in limiting distal spread of mycobacteria after primary exposure (Rodrigues, Diwan et al. 1993; Colditz, Berkey et al. 1995). The histological hallmark of mycobacterial infection is the granuloma, a highly structured yet dynamic unit, comprised of a wide array of immune effector cells including macrophages, CD4+ and CD8+ T-lymphocytes, and B-lymphocytes acting in concert to restrict mycobacterial replication, while allowing viable mycobacteria to persist for decades (Co, Hogan et al. 2004). Reactivation of bacterial replication from foci of latent
infection is associated with disruption of the granuloma structure (Chakravarty, Zhu et al. 2008), and occurs in approximately 10% of infected hosts over their lifetime. Determinants of reactivation have not been completely defined, although recrudescence of chronic mycobacterial infection can be induced through depletion of CD4+ lymphocytes or blockade of the T\textsubscript{H}1 cytokine tumour necrosis factor (TNF) in experimental animal models (Scanga, Mohan et al. 2000; Mohan, Scanga et al. 2001; Chakravarty, Zhu et al. 2008; Egen, Rothfuchs et al. 2008), or by administration of corticosteroids or TNF neutralizing agents in humans (Keane, Gershon et al. 2001). Thus, challenges to host defences may affect the course of both acute disseminated mycobacterial infection and chronic or reactivation disease.

Malaria has known immunomodulatory effects (Williamson and Greenwood 1978; Theander, Svenson et al. 1987; Theander, Hviid et al. 1990; Hviid, Theander et al. 1991), which may impair host responses to mycobacterial infection. The macrophage, which plays a central role in the internalization and intracellular control of mycobacteria, is also the primary phagocyte for clearance of parasitized erythrocytes (PEs). Circulating monocytes as well as tissue macrophages and dendritic cells that have phagocytosed PEs accumulate toxic parasite hemozoin, with consequent functional impairment (Schwarzer, Skorokhod et al. 2008). Furthermore, malaria alters the balance of circulating cytokines that are instrumental to the control of mycobacterial infection (Langhorne, Quin et al. 2002). Given that malaria modulates elements of host immune response that play an important role in the control of mycobacterial infection, we hypothesized that malaria co-infection would exacerbate the course of acute and reactivation mycobacterial infection, and tested this hypothesis using murine infection models \textit{in vitro} and \textit{in vivo}.

Several experimental systems have been used to study the pathogenesis and immunology of tuberculosis and malaria, each of which has its advantages and limitations. One well-established murine model of disseminated tuberculosis utilizes the closely related mycobacterial species \textit{M. bovis} BCG. C57BL/6 mice develop systemic infection following BCG inoculation via the intraperitoneal route, developing the characteristic granulomatous lesions of human tuberculosis in multiple organs including the liver and spleen (Co, Hogan et al. 2004). Mycobacterial replication is ultimately controlled by adaptive host immune responses, but viable bacilli persist at low levels in infected organs, mirroring the course of latent tuberculosis in humans (Blanden, Lefford et al. 1969; Leisewitz, Rockett et al. 2008). Thus, both acute and latent tuberculous
infection can be modeled with a single mycobacterial species in the same murine host. In keeping with clinical observations (Keane, Gershon et al. 2001; Casanova and Abel 2002), cytokines including TNF and IFN-γ are critical regulators of susceptibility and pathology in murine models of Mtb (Flynn, Chan et al. 1993; Flynn, Goldstein et al. 1995), and intracellular killing mechanisms including reactive nitrogen intermediates are conserved in both mice and humans (Chan, Tanaka et al. 1995; Flynn, Scanga et al. 1998; Chan, Chan et al. 2001). In addition, infection of C57BL/6 mice with PccAS produces a transient parasitemia that recapitulates human blood stage malaria infection (Langhorne, Quin et al. 2002; Leisewitz, Rockett et al. 2008) and the immune dysregulation observed in humans is paralleled in murine malaria (Phillips, Selby et al. 1974; Murphy and Lefford 1979; Prada, Malinowski et al. 1996). Thus, murine BCG and PccAS effectively simulate important immunological aspects of human tuberculosis and malaria, respectively.

Here we show a deleterious effect of malaria co-infection on the course of experimental acute and reactivation mycobacterial infection in an established murine model of disseminated disease (Blanden, Lefford et al. 1969; Egen, Rothfuchs et al. 2008), and present in vitro and histopathological evidence that granuloma disruption and macrophage dysfunction secondary to ingested parasite hemozoin may contribute to this observation.
2.3 Materials and methods

2.3.1 Mice, mycobacteria and *Plasmodium* parasites

C57BL/6 mice were bred and kept in the animal facility at the University of Toronto. Animal protocols were approved by the Animal Care Committee of the University of Toronto. *M. tuberculosis* strain H37Rv (TMC no. 102), *M. bovis* BCG-Pasteur strain, and *Mycobacterium marinum* type strain 1218R (ATCC 927) were grown at 37 °C or 30 °C in Middlebrook 7H9 broth (BD Biosciences; Franklin Lakes, NJ USA) supplemented with 0.2% glycerol and 10% OADC (Oleic Acid, Albumin, Dextrose, Catalase; BD Bioscience) or on Middlebrook 7H11 agar (BD Biosciences) supplemented with 0.5% glycerol and 10% OADC. Infections in experimental animals were initiated by intraperitoneal injection of $1.5 \times 10^7$ *M. bovis* BCG. *Plasmodium chabaudi chabaudi* AS (PccAS) parasites from stock frozen at -80 °C were passaged through wild type C57BL/6 mice prior to intraperitoneal injection in experimental animals. *P. falciparum* (ITG and 3D7 strains) used for *in vitro* studies was cultured as previously described (Trager and Jensen 1976). Mature stage schizonts were isolated by density gradient centrifugation using 80% Percoll. A ratio of 20 PEs to each macrophage was used in all *in vitro* experiments.

2.3.2 *In vivo* infection and histopathology

Mice (C57BL/6) were inoculated intra-peritoneally with $1.5 \times 10^7$ CFU of *M. bovis* BCG (day 0). For the acute co-infection model, mice were inoculated intra-peritoneally with $1.0 \times 10^6$ PccAS PEs 7 days later, such that the peak microbial burdens of mycobacteria and malaria would coincide temporally. For the re-activation experiments, latent mycobacterial infection was established by 84 days after IP inoculation of BCG, at which time mice were challenged with $1.0 \times 10^6$ PccAS PEs IP. Blood was collected by cardiac puncture and spleens, livers and lungs were harvested from mice after euthanasia by CO$_2$ inhalation at various times over a course of infection. Half of each organ was homogenized and plated on 7H11 agar (BD Biosciences) at appropriate dilutions, and incubated for 21 days at 37 °C in order to determine BCG CFUs. The remaining half was preserved in 10% formalin, embedded in paraffin, processed in 5μm sections, and stained with H&E for histopathological analysis. Immunohistochemistry for the murine macrophage marker F4/80 was performed on paraffin embedded liver sections using a rat anti-mouse F4/80 IgG primary antibody (AbD Serotec; Raleigh, NC), followed by vector-blue
conjugated goat anti-rat IgG secondary antibody (AbD Serotec), as previously described (Chakravarty, Zhu et al. 2008). Image analysis was performed by enumeration of malaria pigment (Adobe Photoshop) expressed as a proportion of the total pixel area (Mulrane, Rexhepaj et al. 2008).

2.3.3 Infection of macrophages

Thioglycollate-elicited macrophages from C57BL/6 mice were co-incubated with mycobacteria at a MOI of 10:1 (*M. tuberculosis*), 10:1 (*M. bovis* BCG) or 1:1 (*M. marinum*) for 3 hours. Non-adherent cells were washed away. Following overnight incubation, cells were co-cultivated with parasitized erythrocytes (PEs) or uninfected erythrocytes (as controls). Co-infected macrophages were incubated in medium containing gentamicin (RPMI 1640, with 10% fetal bovine serum, and 2.5 mg/L gentamicin) at 37 ºC for 1, 3, 5 or 7 days. Cell lysates were plated on 7H11 medium (BD Biosciences) and mycobacterial colony forming units (CFUs) were quantified after incubation at 37 ºC for 21 days (*M. tuberculosis* and *M. bovis* BCG) or at 32 ºC for 7 days (*M. marinum*).

2.3.4 Cytokines and nitric oxide assay

Blood was collected from euthanized mice by cardiac puncture, allowed to clot, and cleared by centrifugation. Serum was stored at -80 ºC and later assayed for cytokines using a cytometric bead array assay (Mouse Inflammation Kit, BD Biosciences) according to manufacturer’s instructions.

Murine thioglycollate-elicited peritoneal macrophages were isolated from C57BL/6 mice, and plated at a density of 200,000 cells per well in 200 μL of media (RPMI 1640 supplemented with 10% fetal bovine serum). Macrophages were exposed to PEs or control conditions for 12-16 hours, primed with interferon-gamma (IFN-γ), then co-incubated with BCG or *M. marinum* (MOI of 50:1). Culture supernatants were collected 24 hours later and assayed for nitrite concentration as an index of nitric oxide production using the Greiss reaction (Greiss Reaction Kit, Promega) as well as TNF by ELISA (eBioscience; San Diego, CA).
2.4 Results

2.4.1 Co-infection with malaria exacerbates acute mycobacterial infection *in vivo*

Given the known immunomodulatory effects of malaria infection, we hypothesized that malaria would exacerbate mycobacterial infection in a mammalian host. Using a well characterized experimental model of disseminated mycobacterial infection (Scott, Kumar et al. 2004; Page, Jedlicka et al. 2005; Leisewitz, Rockett et al. 2008), we challenged C57BL/6 mice with BCG and quantified the burden of mycobacteria in the liver, spleen and lung over a time course of infection (63 days). Co-infection with PccAS was initiated intraperitoneally 7 days after BCG infection such that the peak pathogen burden of mycobacteria and malaria parasites were co-incident. Control groups consisted of mice infected with BCG alone or PccAS alone. All mice survived the infection with one or both pathogens. As previously described (Page, Jedlicka et al. 2005), BCG counts in the liver and spleen rose to peak levels two weeks after infection, declining thereafter to low but non-sterile levels (Figure 2.1A-B). In mice co-infected with PccAS, higher mycobacterial counts were observed over the time course of infection in the spleen (Figure 2.1A, p=0.043) and the liver (Figure 2.11B, p=0.010).

In addition, clinically relevant correlates of disease severity including weight loss (Figure 2.1C, p<0.0001) and splenomegaly (Figure 2.1D, p=0.0034) were more pronounced in co-infected mice than mice infected with BCG alone. Liver mass was equivalent in both groups (Figure 2.1E).

T_H1 cytokine responses are critical for granuloma formation and control of mycobacterial infection, with TNF and IFN-γ playing a central role, as evidenced by the severity of disease observed in TNF- and IFN-γ-deficient mice (Flynn, Chan et al. 1993; Kamijo, Le et al. 1993; Flynn, Goldstein et al. 1995), in humans with inherited disorders of the IL-12-IFN-γ axis (Casanova and Abel 2002) and individuals receiving therapeutic TNF antagonists (Keane, Gershon et al. 2001). We measured a panel of pro-and anti-inflammatory cytokines in the sera of co-infected mice and observed that TNF and IFN-γ levels rose and declined in parallel to the mycobacterial burden (Figure 2.1F and G). Co-infected mice had circulating TNF levels higher than mice infected with PccAS alone (Figure 2.1F, p=0.036), but similar to BCG infected mice
(Figure 2.1F, p=0.31). The levels of IFN-γ (Figure 2.1G) as well as IL-6, IL-10, IL-12p70, or MCP-1 (data not shown) were not significantly different in co-infected mice and controls.
Figure 2.1. Co-infection with murine malaria exacerbates acute mycobacterial infection in vivo.

A and B. Bacillary loads in spleen (A; p=0.043, 2-way ANOVA) and liver (B; p=0.010, 2-way ANOVA) homogenates were significantly higher in C57BL/6 co-infected with BCG (1.5×10⁷ CFU intraperitoneally) and *P. chabaudi* chabaudi AS (PccAS; 1×10⁶ parasites IP, 7 days later) than control mice infected with BCG alone. Data are shown as boxplots (median, inter-quartile range and range) with 4 mice per group at each time point. One representative experiment of two is shown. C. Weight loss was exacerbated in co-infected mice at peak of infection (day 14; 4 mice per group, p<0.0001). D and E. Splenomegaly was more pronounced in co-infected mice at day 14 (D, *p=0.0034), but differences in liver weights were not statistically significant (E, p=0.068). F. Serum levels of TNF rose and declined in parallel with the mycobacterial burden, reaching a local maximum at day 14 to 21. TNF levels were higher in co-infected mice (grey boxplots) than in mice infected with PccAS (black boxplots, p=0.036, 2-way ANOVA), but similar to mice infected with BCG alone (white boxplots, p=0.31). Data are shown as boxplots (median, inter-quartile range, range) with 3 to 4 mice at each time point in each group. G. IFN-γ levels during acute BCG infection and BCG+PccAS co-infection also appear to be maximal at day 21, but no significant differences between groups were observed. *p<0.05; TNF: tumor necrosis factor; IFN-γ: interferon-gamma; BCG: *M. bovis* BCG; PccAS: *Plasmodium chabaudi chabaudi* AS
2.4.2 Parasite hemozoin co-localizes with macrophages within mycobacterial granulomas

Intra-erythrocytic *Plasmodium* catabolizes host hemoglobin and sequesters the released toxic heme as crystalline hemozoin, which appears as a brown pigment under light microscopy (Schwarzer, Skorokhod et al. 2008). PccAS infection led to hemozoin accumulation in reticulo-endothelial organs, resulting in darkly pigmented livers and spleens on gross examination, compared to the (normal) tan appearance of formalin-fixed livers from mice infected with BCG alone (Figure 2.2A-C). Histopathological examination of the livers of mice infected with PccAS demonstrated scattered deposition of pigment throughout the liver parenchyma (Figure 2.2D). On the other hand, granulomas, the histological hallmark of mycobacterial infection, were apparent in liver sections of mice infected with BCG (Figure 2.2E). Interestingly, in co-infected mice, enhanced pigment deposition within granulomas was evident (Figure 2.2F). The micro-architecture and size of granulomas was not affected by PccAS co-infection (Figure 2.2G-I). Immunohistochemical staining for murine macrophages (F4/80 antigen) demonstrated co-localization of macrophage aggregates and malaria pigment in liver sections of co-infected mice (Figure 2.2J-L). Quantitative morphometric analysis demonstrated similar granuloma size (Figure 2.2M) but increased levels of pigment, concentrated within the granulomas of co-infected animals, above background levels in the surrounding liver parenchyma (Figure 2.2N, p<0.0001), and well above the negligible level in control images of granulomas of BCG infected mice (Figure 2.2N, p<0.0001). This result suggests that the influx of dysfunctional hemozoin-laden monocytes into granulomas may contribute to impaired containment of mycobacteria.
Figure 2.2. Malaria pigment co-localizes with macrophages within mycobacterial granulomas.

A to C. Livers harvested at day 35 post-infection were darkly pigmented in PccAS infection (A), and BCG+PccAS co-infection (C), but not in BCG infected mice (B). D to I. Histopathological examination of liver sections (day 35, H&E stain) revealed scattered pigment deposition (black arrowhead), representing parasite hemozoin, associated with PccAS infection (D and G), and granulomas associated with BCG infection (E and H). Of note, in co-infected animals, malaria pigment (black arrowhead) was observed within the granuloma, the locus of mycobacterial containment (F and I), although granuloma micro-architecture was not altered. J to L. Labeling the murine macrophage F4/80 antigen (blue) identified tissue resident macrophages (Kupffer cells) in association with pigment in PccAS infection (J), and macrophage aggregates (granulomas) in BCG infection (K) and BCG+PccAS co-infection (L) which co-localized with malaria pigment (L). M. Granuloma size was equivalent in BCG and BCG+PccAS co-infected mice (p=0.66). N. Quantification of pigment using image analysis demonstrated that pigment density, expressed as a fraction of cross-sectional area, was higher within the granuloma than surrounding liver parenchyma, and well above the negligible levels in granulomas of mice infected with BCG alone (p<0.0001). Each point represents one granuloma or surrounding parenchyma, bars represent median; a total of 100 granulomas from 4 mice in each group were analysed. *p<0.05; BCG: M. bovis BCG; PccAS: Plasmodium chabaudi chabaudi AS
2.4.3 Malaria reactivates latent mycobacterial infection in a murine model

Up to one third of the world’s population is latently infected with *M. tuberculosis*, which can resume replication from a dormant state to cause active disease in approximately 10% of infected persons over their lifetime. Reactivation of *Mtb* can be experimentally triggered in mice through depletion of CD4+ lymphocytes or TNF blockade (Scanga, Mohan et al. 2000; Mohan, Scanga et al. 2001; Chakravarty, Zhu et al. 2008); however, natural precipitating factors of reactivation are not well defined. We examined whether malaria, a prevalent infection in many TB endemic areas, can trigger re-activation of latent mycobacterial infection *in vivo*. Latent infection with *M. bovis* BCG was established in C57BL/6 mice by 49 days after intraperitoneal inoculation, as evidenced by stably low levels of viable mycobacteria in the homogenized organs of infected mice (Figure 2.1A-B). Latently infected animals were challenged with PccAS at day 84, resulting in a significant rise in the mycobacterial counts in the spleen relative to control mice over 4 weeks following infection with PccAS (Figure 2.3A, p=0.036). All mice survived and ultimately controlled the reactivation mycobacterial infection, and the secondary (reactivation) peak mycobacterial burden was more than 10-fold lower than the primary (acute) peak mycobacterial burden. Body mass was reduced (Figure 2.3B), spleen mass was increased (Figure 2.3C), and liver mass was reduced (Figure 2.3D) in mice challenged with PccAS.
Figure 2.3. Malaria co-infection induces re-activation of latent mycobacterial infection in vivo.

A. In a murine model of latent mycobacterial re-activation, mice were infected with *M. bovis* BCG, and followed for 84 days, at which time mycobacteria are controlled, but not eliminated, by adaptive immune mechanisms. Mice were then infected with PccAS by IP injection (1×10^6 parasites), resulting in a transient rise in viable mycobacteria isolated from the spleens of co-infected mice (p=0.016). One representative experiment of two is shown. Data are displayed as boxplots (median, inter-quartile range, range).

B to D. Weight loss (B, p=0.0039) and splenomegaly (C, p<0.0001) were more pronounced, and liver mass was reduced (D, p=0.0006) in mice challenged with PccAS at peak of reactivation (day 98). *p<0.05; BCG: *M. bovis* BCG; PccAS: *Plasmodium chabaudi chabaudi* AS
2.4.4 Malaria-induced re-activation of latent mycobacterial infection is characterized by hepatic and splenic inflammation and disorganization of granulomas

Reactivation tuberculosis is associated with disruption of the ordered architecture of the granuloma (Chakravarty, Zhu et al. 2008). We examined histopathological liver sections during malaria-induced re-activation of BCG infection and observed diffuse mononuclear cell infiltration, as previously described with other forms of inflammatory challenge in BCG infected mice (Shands and Senterfitt 1972; Ferluga 1981) (Figure 2.4A-D). Poorly organized, loose collections of mononuclear cells were observed, often in association with malaria pigment, compared to the compact pigment-free granulomas of mice with latent BCG (Figure 2.4C-D). In spleens of mice with latent BCG infection, typical histological features were observed including lymphoid follicles and intervening red pulp (Figure 2.4E) in contrast to mice challenged with PccAS, where intense mononuclear cell infiltrate obscured the splenic micro-architecture (Figure 2.4F). Morphometric analysis revealed more numerous (Figure 2.4G, p=0.0079) and smaller (Figure 2.4H-I, p<0.0001) mononuclear cell aggregates in co-infected mice, reflecting a generalized inflammatory infiltrate (Ferluga 1981) rather than the quiescent chronic granulomatous inflammation observed in mice latently infected with BCG alone.
Figure 2.4. Malaria-induced re-activation of latent mycobacterial infection is characterized by granuloma disruption.

A to D. The compact, organized granulomas in livers of mice with latent BCG (A and C) are contrasted with mice challenged with PccAS (B and D). Disordered aggregates of mononuclear cells were observed in association with malaria pigment (black arrowhead), suggesting a breakdown of granuloma architecture. E and F. The spleens of mice with latent BCG show typical lymphoid follicles (arrowhead) and red pulp (E) compared with the intense mononuclear infiltrate in spleens of mice challenged with PccAS (F). G to I. In contrast to the compact granulomas in livers of mice latently infected with BCG, mononuclear cell aggregates in mice challenged with PccAS were scattered, ill-defined and variable in size. Morphometric analysis demonstrated more numerous (G, p=0.0079), and smaller (H, p<0.0001 and I, p<0.0001) mononuclear cell aggregates in contrast to the structured granulomas of latent BCG infection. All mononuclear cell aggregates (collection of >10 mononuclear cells) from eighty random low-power (10×) fields of liver sections from 4 mice per group were counted (>250 aggregates).

* p<0.05; BCG: M. bovis BCG; PccAS: Plasmodium chabaudi chabaudi AS
2.4.5 Co-cultivation with *P. falciparum* parasitized erythrocytes impairs the control of *M. tuberculosis* replication by macrophages

Having observed that malaria exacerbates mycobacterial infection *in vivo*, we next explored the mechanism underlying this phenomenon using macrophage co-cultivation experiments in vitro. The macrophage is a principal effector cell for the control of both malaria and mycobacteria. We hypothesized that internalization of malaria parasitized erythrocytes by macrophages would impair their ability to restrict intracellular mycobacterial replication. After 10 days co-cultivation *in vitro*, Mtb proliferated to levels approximately two-fold higher in macrophages co-cultivated with either of two laboratory clones of *P. falciparum* than control macrophages co-incubated with uninfected erythrocytes (Figure 2.5, 3D7 and ITG, p=0.0097 and 0.0073, respectively). To examine whether these findings were generalizable to other mycobacterial species, we repeated co-cultivation experiments with *M. marinum* and observed similar results (data not shown).
Figure 2.5. Co-incubation with *P. falciparum* inhibits innate control of *M. tuberculosis* by macrophages *in vitro*.

A. Co-incubation with *P. falciparum* parasitized erythrocytes (PEs; laboratory strains 3D7 and ITG, grey bars) led to increased growth of *M. tuberculosis* (MOI=10:1) within murine macrophages. Significant differences (p=0.0097 and p=0.0073 for 3D7 and ITG, respectively) were observed in the mycobacterial counts 10 days after infection, compared with control macrophages infected with Mtb and cultivated with uninfected erythrocytes (white bar). Data are shown as mean +/- SEM (4 replicate wells at each time point). *p<0.05; PE: Plasmodium falciparum parasitized erythrocytes; RBC: red blood cell
2.4.6 *P. falciparum* alters macrophage-mediated anti-mycobacterial defences.

We next investigated possible mechanisms for macrophage dysfunction that might account for the impaired mycobacterial control. We hypothesized that intracellular killing mechanisms may be impaired, and examined the production of nitric oxide, an important effector molecule for macrophage antimicrobial activity (Chan, Chan et al. 2001). *In vitro*, murine macrophages co-cultivated with BCG (MOI 50:1) produced higher levels of nitric oxide than macrophages treated with media alone (Figure 2.6A). Nitric oxide production was diminished in macrophages co-incubated with PEs but not uninfected erythrocytes (Figure 2.6A). Similar results were observed using *M. marinum* (MOI 50:1) as the mycobacterial stimulus (data not shown). Under similar conditions, TNF production in response to BCG was not affected by co-incubation with PEs (Figure 2.6B).
Figure 2.6. *P. falciparum* alters anti-mycobacterial defences in murine macrophages *in vitro*.

A. Defective nitric oxide production by murine macrophages in malaria-BCG co-infection. Reactive nitrogen species were assayed in macrophage culture supernatants (Greiss reaction) in response to uninfected erythrocytes (RBC, light grey), *P. falciparum* PEs (black) (MOI=20:1), BCG (MOI=50:1), individually and together (dark grey). Nitric oxide synthesis in response to BCG was diminished in macrophages co-incubated with PEs (p<0.0001 for BCG+PE vs BCG+RBC). B. Levels of the pro-inflammatory cytokine TNF assayed in culture supernatants (ELISA) were unchanged during co-infection. *p<0.05; PEs: Plasmodium falciparum parasitized erythrocytes; RBC: red blood cell; BCG: M. bovis BCG
2.5 Discussion

Here we show that malaria co-infection exacerbates the course of mycobacterial infection in vitro and in vivo. We provide histopathological evidence that malaria pigment co-localizes with macrophages within the granuloma, and that macrophages ingesting malaria parasites or hemozoin have defects in nitric oxide production and in their ability to restrict intracellular mycobacterial growth. Taken together, these findings provide a cohesive description of innate and adaptive immune dysregulation in the setting of malaria infection with consequences for the pathogenesis of experimental mycobacterial infection.

Malaria co-infection is known to exacerbate viral (Thursz, Kwiatkowski et al. 1995; Moormann, Chelimo et al. 2005), parasitic (Phillips, Selby et al. 1974), and intracellular bacterial (Murphy and Lefford 1979; Mabey, Brown et al. 1987) infections in experimental animals and humans. Malaria impairs humoral and cell-mediated immune responses to heterologous vaccines (Williamson and Greenwood 1978; Greenwood, Bradley et al. 1980) and other antigens, including the mycobacterial purified protein derivative (Theander, Svenson et al. 1987; Hviid, Theander et al. 1991). P. yoelii 17XL co-infection has been previously shown to increase bacillary load in experimental murine infection with Mtb (Scott, Kumar et al. 2004). Our results extend these findings in a distinct model system using the malaria species PccAS and the mycobacterial strain M. bovis BCG, with intraperitoneal initiation of systemic infection.

The histopathological observation of pigmented granulomas in co-infected mice led us to postulate that trafficking of hemozoin-laden monocytes or macrophages to the granuloma may result in loss of mycobacterial control. The granuloma is a highly dynamic unit, as demonstrated by [³H]thymidine labelling of macrophage bone marrow precursors to track their flux in and out of rabbit skin granulomas (Dannenberg 2003) and intravital microscopy of nascent granulomas in transparent zebrafish embryos (Davis and Ramakrishnan 2009) as well as BCG-induced murine liver granulomas (Egen, Rothfuchs et al. 2008). Previous investigators have shown that super-infecting mycobacteria home to existing granulomas in zebrafish (Cosma, Humbert et al. 2004), amphibian (Cosma, Humbert et al. 2004) and mammalian (Cosma, Humbert et al. 2008) models, transported as intracellular cargo within macrophages. Although macrophages harbouring mycobacteria seem preferentially directed to the granuloma, antigenically heterologous latex beads and Salmonella enterica subsp. arizonae were also carried to pre-
formed granulomas (Cosma, Humbert et al. 2004). By extension, we propose that circulating monocytes and/or tissue resident macrophages bind and internalize PEs, acquiring and accumulating hemozoin, and subsequently transit to established foci of chronic inflammation (Cosma, Humbert et al. 2004; Cosma, Humbert et al. 2008; Davis and Ramakrishnan 2009). Influx into the granuloma of hemozoin-containing monocytes may thus disturb the host-pathogen equilibrium that maintains mycobacterial quiescence.

In our model of mycobacterial reactivation following malaria challenge, we observed increased mycobacterial burden in infected organs, in association with hepatic and splenic mononuclear infiltrates, which contrasted markedly with the structured granulomas seen in latent BCG. Previous studies have documented hypersensitivity hepatitis and splenic injury in mice infected with BCG and subsequently challenged with various inflammatory stimuli, in a process that is dependent on IFN-γ and associated with intense TNF production (Ferluga 1981; Kamijo, Le et al. 1993; Guler, Olleros et al. 2004). Given the critical role of tightly coordinated local cytokine and cellular networks in maintaining granuloma integrity and mycobacterial dormancy (Egen, Rothfuchs et al. 2008), we postulate that the generalized inflammation associated with inflammatory challenge may disrupt ordered host defences, allowing mycobacterial replication to resume, albeit temporarily, as reflected by disorganized granulomas and transiently elevated burden of viable mycobacteria in our study. This finding contrasts with the effect of malaria on acute mycobacterial infection, where we hypothesize that influx of dysfunctional hemozoin-laden macrophages leads to impaired mycobacterial control without alterations in granuloma microarchitecture.

We demonstrated that macrophages phagocytes ingesting parasitized erythrocytes are dysfunctional in their ability to control replication of Mtb in vitro. Similarly, previous investigators have shown that macrophages internalizing parasitized erythrocytes have reduced microbicidal activity against bacterial and fungal pathogens (Fiori, Rappelli et al. 1993). The parasite by-product hemozoin alters macrophage function in vitro, including inhibitory effects on respiratory burst, NADPH oxidase activity, protein kinase C activity, expression of ICAM-1, integrin CD11c, MHC class-II expression, and differentiation and maturation into functional dendritic cells (Schwarzer, Skorokhod et al. 2008). Furthermore, hemozoin and its synthetic analog β-hematin inhibit nitric oxide synthesis by murine macrophages in response to
inflammatory stimuli (Morakote and Justus 1988; Taramelli, Basilico et al. 1995; Prada, Malinowski et al. 1996; Taramelli, Recalcati et al. 2000). We extend these findings to mycobacteria-malaria co-infection in vitro, showing that PEs inhibit nitric oxide synthesis by murine macrophages in response to stimulation with BCG. This may contribute to the impaired macrophage anti-mycobacterial defences observed in vitro and possibly in vivo, particularly within the granuloma, where hemozoin-laden macrophages were noted. Despite speculation based on previous work (Scott, Kumar et al. 2004), we did not find differences in TNF production by co-infected macrophages in vitro, or serum TNF or IFN-γ in co-infected mice. Thus, differences in mycobacterial control in the context of malaria co-infection do not appear to be due to major alterations in the Th1 cytokine profile. Despite the well-recognized role of TNF in host defence against mycobacteria (Keane, Gershon et al. 2001; Mohan, Scanga et al. 2001; Wallis, Broder et al. 2004; Chakravarty, Zhu et al. 2008), other independently-regulated processes including nitric oxide synthesis may account for deficiencies in anti-mycobacterial defences in the setting of malaria co-infection.

Our experimental models have several limitations. In attempting to recapitulate human tuberculous disease, murine models are imperfect. Relevant examples include the non-caseating BCG granuloma in the mouse, whereas human granulomas form necrotic caseum, which may play a role in bacterial persistence (Saunders and Britton 2007). The aerogenic route is the mode common mode of transmission for tuberculosis in humans whereas we used an intraperitoneal route to simulate systemic disease. Parasitemias in excess of those seen in typical human disease may limit the ability to generalize findings in PccAS infection to human malaria.

Nonetheless, our findings of a biologically plausible malaria-mycobacterial interaction suggest that malaria may play an underappreciated role in the course of acute and re-activation infection with M. tuberculosis. Given that 300 million clinical episodes of malaria occur annually, in areas of the world where tuberculosis prevalence frequently exceeds one in three individuals, these findings may have important implications for public health globally.
Chapter 3
CD36 deficiency attenuates experimental mycobacterial infection

3.1 Abstract

Members of the CD36 scavenger receptor family have been implicated as sensors of microbial products that mediate phagocytosis and inflammation in response to a broad range of pathogens. We investigated the role of CD36 in host response to mycobacterial infection. Methods to address this question included experimental *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) infection in *Cd36*<sup>+/+</sup> and *Cd36*<sup>−/−</sup> mice, and *in vitro* co-cultivation of *M. tuberculosis*, BCG and *M. marinum* with *Cd36*<sup>+/+</sup> and *Cd36*<sup>−/−</sup> murine macrophages. Using an *in vivo* model of BCG infection in *Cd36*<sup>+/+</sup> and *Cd36*<sup>−/−</sup> mice, we found that mycobacterial burden in liver and spleen is reduced (83% lower peak splenic colony forming units, p<0.001), as well as the density of granulomas, and circulating tumor necrosis factor (TNF) levels in *Cd36*<sup>−/−</sup> animals. Intracellular growth of all three mycobacterial species was reduced in *Cd36*<sup>−/−</sup> relative to wild type *Cd36*<sup>+/+</sup> macrophages *in vitro*. This difference was not attributable to alterations in mycobacterial uptake, macrophage viability, rate of macrophage apoptosis, production of reactive oxygen and/or nitrogen species, TNF or interleukin-10. Using an *in vitro* model designed to recapitulate cellular events implicated in mycobacterial infection and dissemination *in vivo* (i.e., phagocytosis of apoptotic macrophages containing mycobacteria), we demonstrated reduced recovery of viable mycobacteria within *Cd36*<sup>−/−</sup> macrophages. Together, these data indicate that CD36 deficiency confers resistance to mycobacterial infection. This observation is best explained by reduced intracellular survival of mycobacteria in the *Cd36*<sup>−/−</sup> macrophage and a role for CD36 in the cellular events involved in granuloma formation that promote early bacterial expansion and dissemination.
3.2 Introduction

*Mycobacterium tuberculosis* (*M. tb*) infects an estimated 2 billion people worldwide and is responsible for the most deaths annually (1.6 million/year) of any single bacterial pathogen (Noss, Pai et al. 2001). However, only 5 to 7% of infected immunocompetent individuals develop disease during their lifetime (Saunders and Britton 2007), demonstrating the critical role of host factors in the control of *M. tb*. The histological hallmark of tuberculosis is the granuloma, composed of an inner core of activated macrophages primed for intracellular killing by surrounding T-lymphocytes (Saunders and Britton 2007). Cellular dynamics within the granuloma foster interactions between the innate and adaptive immune systems (Egen, Rothfuchs et al. 2008), but granuloma formation may also promote bacterial expansion and dissemination during initial stages of tuberculosis infection (Davis and Ramakrishnan 2009). Recent work using quantitative intravital imaging of early granuloma formation in zebrafish embryos has demonstrated that macrophages internalizing mycobacteria undergo apoptosis and are phagocytosed by previously uninfected macrophages recruited to the granuloma, which then become infected. Granuloma formation may therefore promote mycobacterial infection by allowing for intracellular persistence and expansion of bacteria as well as systemic dissemination through egress of infected cells to generate new granulomas (Davis and Ramakrishnan 2009). Thus, macrophages play a central role in host-pathogen interactions during tuberculosis, acting as both the primary phagocytic line of defense against *M. tb* as well as the intracellular niche for bacterial replication.

Alterations in macrophage function have been implicated as risk factors for mycobacterial infection, including defects in NADPH oxidase (Lau, Chan et al. 1998; Winkelstein, Marino et al. 2000), the interleukin (IL)-12-interferon (IFN)-γ axis (Jouanguy, Lamhamedi-Cherradi et al. 1997; Altare, Durandy et al. 1998; Dupuis, Dargemont et al. 2001; Picard, Fieschi et al. 2002), natural resistance-associated macrophage protein-1 (NRAMP1) (Bellamy, Ruwende et al. 1998; Bellamy 2002), and the vitamin D receptor (Bellamy, Ruwende et al. 1999). However, variability in host susceptibility to tuberculosis is not fully explained by alterations in these molecular determinants, and other host factors are likely to play an important role (Bellamy, Beyers et al. 2000; Casanova and Abel 2002).
Model systems using both *M. bovis* Bacillus Calmette-Guérin (BCG) and *M. marinum* have been extensively used to study the pathogenesis and immunology of tuberculosis, each of which has its advantages and limitations (Flynn 2006). Murine infection with *M. bovis* BCG is a well-established experimental model system of disseminated tuberculosis (Costello, Izumi et al. 1971; Cosma, Humbert et al. 2008; Egen, Rothfuchs et al. 2008). Mice of the BCG-sensitive C57BL/6 genetic background serve as permissive hosts for mycobacteria and develop systemic infection following inoculation via the intraperitoneal route. Mycobacterial replication occurs in multiple organs and is ultimately controlled by adaptive host immune responses (Kremer, Estaquier et al. 2000), mimicking the course of primary human tuberculosis. *M. marinum*, a relatively rapidly growing mycobacterial species, is a close genetic relative of *M. tuberculosis* (Tonjum, Welty et al. 1998) that has been used to study the pathogenesis of tuberculosis (Cosma, Sherman et al. 2003; Cosma, Humbert et al. 2004; Cosma, Swaim et al. 2006; Davis and Ramakrishnan 2009). *M. marinum* causes systemic granulomatous disease in ectotherms such as frogs and fish and peripheral granulomatous disease (fishtank granulomas) in humans (Decostere, Hermans et al. 2004). *M. marinum* shares genetic determinants of pathogenicity with *M. tuberculosis*, such as the ESX-1/RD1 locus, which induces recruitment of new macrophages to nascent granulomas (Volkman, Clay et al. 2004; Davis and Ramakrishnan 2009), and modulates phagolysosome maturation and the intracellular fate of mycobacteria (Tan, Lee et al. 2006; van der Wel, Hava et al. 2007). As such, *M. marinum* has provided valuable insights into tuberculous disease using *in vivo* and *in vitro* model systems. We used BCG *in vivo* and BCG, *M. marinum*, and *M. tb* *in vitro* to model aspects of tuberculosis in order to dissect the role of CD36 in disease pathogenesis.

The cell surface glycoprotein CD36, present in many cell types including macrophages, has been implicated in a variety of cellular processes including fatty acid transport, regulation of angiogenesis, atherosclerosis, inflammation, and as a pattern recognition receptor mediating innate immune responses to a range of pathogens, including mycobacteria (Febbraio, Hajjar et al. 2001). CD36 belongs to the class B scavenger receptor family, a group of phylogenetically conserved molecules involved in sensing a variety of microbial products and endogenous ligands. CD36 plays a physiological role in the recognition and clearance of apoptotic cells by professional phagocytes (Voll, Herrmann et al. 1997). CD36 also acts as a co-receptor with the Toll-like receptor (TLR) 2/6 complex that binds diacylglycerides, such as lipoteichoic acids, and
participates in innate sensing and the phagocytic clearance of Staphylococcus aureus (Hoebe, Georgel et al. 2005; Stuart, Deng et al. 2005). Compared to wild type (Cd36+/+) mice, CD36-deficient (Cd36−/−) mice are more susceptible to experimental S. aureus infection, exhibiting higher mortality, increased levels of bacteremia, and multiple renal and cardiac abscesses (Stuart, Deng et al. 2005). CD36 participates in the uptake of and inflammatory response to other bacterial species including Escherichia coli and Enterococcus faecalis in model cell systems in vitro (Baranova, Kurlander et al. 2008). CD36 contributes to macrophage-mediated clearance of Plasmodium falciparum parasitized erythrocytes (Serghides, Smith et al. 2003; Patel, Lu et al. 2007), and CD36 deficiency is associated with a dysregulated cytokine response and increased mortality in experimental animal models of severe malaria (Patel, Lu et al. 2007).

Recently, a genome-wide RNA interference screen of Drosophila melanogaster macrophage-like cells identified the CD36 homologue Peste (Pes) and the related mammalian class B scavenger receptors as important factors in the uptake of mycobacteria (Philips, Rubin et al. 2005). Furthermore, reversible alterations in the expression of CD36 on peripheral monocytes/macrophages have been observed in patients with active tuberculosis (Sanchez, Garcia et al. 2006). Based on the hypothesis that CD36 deficiency may alter host susceptibility to tuberculosis, we examined the role of CD36 in mycobacterial infection in vitro and in an experimental model in vivo. We show that Cd36−/− mice have decreased mycobacterial burdens and reduced granulomatous responses after challenge with BCG. Furthermore, macrophages deficient in CD36 restrict the growth of multiple mycobacterial species in vitro. Taken together, our results suggest that CD36 deficiency confers relative protection against mycobacterial infection.

### 3.3 Methods

#### 3.3.1 Mice strains and mycobacteria isolates

Cd36−/−, Tlr2−/−, Tlr4−/−, and Irak4−/− and wild type control (Cd36+/+) C57BL/6 mice were bred and kept in the animal facility at the University of Toronto. Animal protocols were approved by the Animal Care Committee of the University of Toronto, and all experiments involving animals were performed in compliance with current University of Toronto guidelines. Mice 8–12 wk of age were used in all experiments. M. tb strain H37Rv (TMC no. 102), M. bovis BCG-Pasteur
strain, and *Mycobacterium marinum* type strain 1218R (ATCC 927) were routinely grown at 30°C or 37°C in Middlebrook 7H9 broth (BD Biosciences; Franklin Lakes, NJ USA) supplemented with 0.2% glycerol and 10% OADC (Oleic Acid, Albumin, Dextrose, Catalase; BD Bioscience; Franklin Lakes, NJ USA) or on Middlebrook 7H11 agar (BD Biosciences) supplemented with 0.5% glycerol and 10% OADC. Infections in experimental animals were initiated by intraperitoneal injection of 1.5×10⁷ BCG.

### 3.3.2 Determination of mycobacterial density in infected mice

At different points during infection, mice were euthanized by CO₂ inhalation and spleens, livers and lungs were collected from infected mice. Half of each organ was homogenized and plated on 7H11 agar (BD Biosciences; Franklin Lakes, NJ USA), and incubated for 21 days at 37°C for BCG colony counts.

### 3.3.3 Histopathologic examination

After collection of spleens, livers and lungs from infected mice, half of each organ was preserved in 10% formalin, embedded in paraffin and processed in 5μm sections. Sections were stained with H&E for histopathology and with Ziehl–Neelsen stain for acid-fast bacilli.

### 3.3.4 Intracellular survival of mycobacteria in murine macrophages

Thioglycolate-elicited macrophages from *Cd36*⁺⁺ and *Cd36*⁻⁻ mice were seeded in 12-well polystyrene plates (300,000 cells/well) and allowed to adhere for 24 hr. They were then co-incubated with mycobacteria at a MOI of 10:1 (*M. tb*), 10:1 (*M. bovis* BCG) or 1:1 (*M. marinum*) for 3 hr. Cells were washed and incubated in medium containing gentamicin (RPMI 1640, with 10% fetal bovine serum, and 2.5 mg/L gentamicin) at 37°C for *M. tb* and *M. bovis* BCG, or at 30°C for *M. marinum*. At different times (1, 3, 5 or 7 days) after infections, cell lysates of macrophages were prepared and plated on 7H11 medium (BD Biosciences; Franklin Lakes, NJ USA) and bacterial colonies were counted after incubation at 37°C for 21 days (*M. tb* and *M. bovis* BCG) or at 30°C for 7 days (*M. marinum*).
3.3.5 Uptake of mycobacteria by macrophages

Differentially labeled intracellular and extracellular mycobacteria were imaged using fluorescent confocal microscopy following phagocytosis by wild type (Cd36+/+) and Cd36−/− murine macrophages. Thioglycolate-elicited macrophages from Cd36+/+ and Cd36−/− mice were seeded on glass cover slips at a density of 125,000 cells per cover slip. *M. marinum* was incubated for 10 minutes with sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin, Thermo Fisher Scientific; Rockford, IL) at pH 8.0 in order to biotinylate the bacterial surface. Biotinylated *M. marinum* was then co-incubated with murine macrophages for 3 hr at a multiplicity of infection (MOI) of 100:1. Extracellular *M. marinum* were labeled using streptavidin-conjugated tetramethylrhodamine (streptavidin-TMR, Invitrogen; Carlsbad, CA). Macrophages were fixed and permeabilized (4% paraformaldehyde for 20 min followed by 0.1% Triton X-100 in 5% milk for 20 min) and intracellular *M. marinum* were labeled with a second fluorophore, streptavidin-conjugated Alexa Fluor® 488 (Invitrogen). Images were obtained using spinning disk confocal microscopy (Zeiss Axiovert 200 equipped with a Hamamatsu Orca AG CCD camera and spinning disk confocal scan head, Volocity acquisition software). Control conditions included non-biotinylated *M. marinum* (negative control for fluorescent labeling) and 10 µM cytochalasin D (Calbiochem, Gibbstown, NJ) to inhibit phagocytosis (Sulahian, Imrich et al. 2008).

Internalization of *M. marinum* by wild type (Cd36+/+) and Cd36−/− murine macrophages was quantified with a flow cytometric technique. Biotinylated *M. marinum* (MOI=100:1) was co-incubated with murine macrophages in suspension at a concentration of 10^6 cells/ml in RPMI 1640 with 10% fetal bovine serum for 3 hr. Extracellular mycobacteria were labeled with streptavidin-conjugated allophyco-cyanin (eBioscience; San Diego, CA), cells were fixed and permeabilized according to manufacturer’s protocol using BD CytoFix/CytoPerm™ (BD Biosciences; Franklin Lakes, NJ USA), and intracellular *M. marinum* was labeled with streptavidin-conjugated Alexa Fluor® 488 (Invitrogen). Flow cytometric analysis was performed using FACSCalibur (BD Biosciences; Franklin Lakes, NJ USA) acquired with CellQuest (BD, San Jose, CA) software and analysed with FlowJo 8.7.3 (Tree Star Inc., Ashland, OR).

Quantitative measurement of viable internalized mycobacteria was performed *in vitro* following 3 hr co-incubation of bacteria and macrophages, allowing for phagocytosis without significant intracellular replication. Thioglycolate-elicited macrophages from Cd36+/+ and Cd36−/− mice were
plated and co-incubated with mycobacteria at MOI of 100:1 for 3 hr. Extracellular mycobacteria and non-adherent macrophages were removed by repeated washing (3 times) with media containing gentamicin (2.5 mg/l) and remaining intracellular mycobacteria were harvested by scraping and lysis using 1% Triton X-100 (Sigma; St. Louis, MO). Cell lysate was plated for mycobacterial counts as described above.

3.3.6 Electron microscopy

Cd36<sup>+/+</sup> and Cd36<sup>-/-</sup> thioglycollate-elicited peritoneal macrophages were plated on glass coverslips, then incubated with M. marinum (MOI 100:1) for 6 hr at 37°C. Cells were washed and fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, dehydrated with alcohol, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate–lead citrate, then examined with a FEI Tecnai 20 transmission electron microscope with EDX, Gatan image filter, and 1k by 1k digital camera.

3.3.7 Cell viability

Cd36<sup>+/+</sup> and Cd36<sup>-/-</sup> peritoneal macrophages were plated in 96-well polystyrene plates (50,000 cells/well), and co-incubated with mycobacteria as above. After incubation for 1, 3, 5 or 7 days, 10% (v/v) MTS reagent (CellTitre 96 ® AQ<sub>ueous</sub> One Solution Assay, Promega; Madison, WI) was added directly to culture wells, incubated for 2 hours, and the absorbance measured at 490 nm.

3.3.8 Apoptosis assay

Fragmented DNA of apoptotic cells was end-labeled by a modified TUNEL assay (DeadEnd<sup>™</sup> Colorimetric TUNEL system, Promega; Madison, WI) according to manufacturer’s instructions and nuclei of apoptotic cells were identified on the basis of their darkly stained pyknotic nuclei. Caspase-3 activity was determined in cultures of macrophages co-incubated according to manufacturer’s instructions (Colorimetric CaspACE<sup>™</sup> Assay, Promega; Madison, WI).

3.3.9 Genetic polymorphisms in the vicinity of Nramp1

Genomic DNA was isolated from macrophages of Cd36<sup>+/+</sup> and Cd36<sup>-/-</sup> using column purification according to manufacturer’ instructions (QIAamp DNA blood mini kit, Qiagen, Valencia, CA).
Five markers in the vicinity of *Nramp1* (D1Mcg2, D1Mcg3, D1Mcg5, D1Mit19, D1Mit23) (Malo, Vogan et al. 1994) were tested in *Cd36*+/+, *Cd36*−/− mice (C57BL/6 genetic background) and in two reference strains (C57BL/6, BCG sensitive; and 129/Sv, BCG resistant). Four markers were polymorphic between the reference strains and all 4 showed that *Cd36*+/+ and *Cd36*−/− mice carried the C57BL/6 alleles.

### 3.3.10 Production of reactive nitrogen and reactive oxygen intermediates

The Griess reaction (Griess Reagent System, Promega; Madison, WI) was used according to manufacturer’s instructions to quantify the nitrite concentration in the supernatant of thioglycollate-elicited peritoneal macrophages (plated at a density of 200,000 cells per well in 96-well plates) co-incubated with mycobacterial (MOI=10:1). Nitrite is a stable, non-volatile breakdown product of nitric oxide (NO), produced by activated macrophages as a mechanism for intracellular killing of mycobacteria.

Oxidative burst was assessed using a chemiluminescence assay. Adherent macrophages (200,000 per well in opaque 96-well plates) were stimulated with BCG (MOI=100:1), 10 μM phorbol myristate acetate (PMA, Sigma; St. Louis, MO), or media alone in the presence of 100 μM luminol (Sigma; St. Louis, MO). Chemiluminescence was detected using a MonoLight 2010C luminometer (Analytical Luminescence Laboratory, San Diego, CA).

### 3.3.11 Measurement of cytokine production

Blood was collected from euthanized mice by cardiac puncture, allowed to clot, and cleared by centrifugation. Serum was stored at -80°C and later assayed for cytokines using a cytometric bead array assay (Mouse Inflammation Kit, BD Biosciences) according to manufacturer’s instructions.

For assays of cytokine production *in vitro*, macrophages were plated in 96 well plates at a density of 200,000 cells/well. Cells were washed, pre-incubated with IFN-γ (10ng/mL) for 24 hours, then co-incubated with *M. marinum* or BCG over a time course of infection, at various multiplicities of infection. TNF and IL-10 concentrations in the culture supernatant were determined by commercial ELISA according to manufacturer’s instructions (eBiosciences; San Diego, CA).
3.3.12 *In vitro* model of cellular events in granuloma formation

Thioglycolate-elicited peritoneal macrophages from *Cd36*+/+ and *Cd36*−/− mice were seeded in 6-well polystyrene plates (1×10^6 cells/well) and allowed to adhere for 24 hr. Macrophages were then co-incubated with *M. marinum* (MOI=10:1) for 3hr at 37°C, and washed three times to eliminate extracellular bacteria. Cells were incubated overnight in RPMI 1640 and gentamicin (2.5 mg/l) to induce apoptosis by serum starvation. Control macrophages were incubated in RPMI 1640 and gentamicin (2.5 mg/l) with 10% fetal bovine serum. Apoptosis was confirmed by elevated capase-3/7 activity (Kumar and Lavin 1996), determined using a commercially available kit according to manufacturer’s instructions (Apo-ONE Homogeneous Caspase-3/7 Assay, Promega). Apoptotic cells containing mycobacteria were scraped and co-incubated with fresh macrophages, plated at a density of 1×10^6 cells/well in 6-well plates for 3hr at 37°C. Macrophages were washed three times with media containing gentamicin (2.5 mg/l) to eliminate extracellular apoptotic cells and bacteria. Macrophages were then harvested by scraping and lysis using 1% TrotonX-100 (Sigma). Cell lysate was plated for mycobacterial counts as described above.

3.4 Results

3.4.1 *Cd36*−/− mice restrict mycobacterial growth relative to wild type (*Cd36*+/+) mice.

Based on observations implicating class B scavenger receptors in the uptake of *Mycobacterium fortuitum* (Philips, Rubin et al. 2005), we hypothesized that disruption of the *Cd36* gene would result in an altered host response to mycobacterial infection. To test this, we experimentally infected *Cd36*−/− and *Cd36*+/+ mice with *M. bovis* BCG and examined the burden of mycobacteria and histopathology over the course of infection. All mice survived BCG infection. Consistent with previous reports (Costello, Izumi et al. 1971; Nau, Liaw et al. 1999), we observed an initial rise in the BCG counts in the spleen, reaching a local maximum at 2-3 weeks after infection, with subsequent continuous decline over 9 weeks of infection (Figure 3.1). Of note, *Cd36*−/− mice had lower BCG CFU counts in the spleen overall (*p*<0.001) and at the peak of infection (day 14 post infection; mean ± SEM 5.4 ± 1.1×10^4 vs 3.3 ± 0.5×10^5 CFU; 83% lower; *p*<0.001, Figure 3.1A and D). Differences in total spleen bacillary load were attributable to both relative splenomegaly (i.e., greater average spleen weight, *p*<0.001), as well as a higher density of BCG.
per gram of splenic tissue \( (p=0.02) \) in \( Cd36^{+/+} \) mice compared to \( Cd36^{-/-} \) mice. Significantly lower bacillary loads at peak (day 14) were observed in the livers of infected mice \( (p=0.005) \), with a similar trend in the lungs \( (p=0.054) \). Consistently, mice with higher mycobacterial counts in the spleen had correspondingly higher counts in the liver (Spearman’s \( \rho = 0.818, p<0.001 \)).
Figure 3.1. *Cd36*<sup>−/−</sup> mice have lower bacillary burden relative to *Cd36*<sup>+/+</sup> controls after challenge with *M. bovis* BCG.

A. Intraperitoneal infection (day 0) with BCG (1.5×10<sup>7</sup> organisms) resulted in a rise in mycobacterial counts in the spleens of *Cd36*<sup>+/+</sup> (wild type) mice to a maximum at day 14 with subsequent decline. The mycobacterial counts in *Cd36*<sup>−/−</sup> mice (white box, dashed line) were lower overall (*p*<0.001), and at specific time points (day 7, *p*<0.01; day 14, *p*<0.001). B and C. Differences between genotypes were less pronounced in the liver (B) and lung (C). Results are displayed as mean ± SEM, with 8 mice per group at each time point, representing two pooled independent experiments.
On histopathological examination, there were fewer granulomas in the livers of Cd36\(^{-/-}\) mice compared to the Cd36\(^{+/-}\) mice (Figure 3.2A and B), but the microarchitecture of the granulomas appeared unchanged (Figure 3.2C and D). In addition, fewer acid-fast bacilli were visible in splenic tissue sections of Cd36\(^{-/-}\) mice (Figure 3.2E and F), consistent with the lower BCG bacillary loads observed in Cd36\(^{-/-}\) mice. Quantitative assessment of liver sections demonstrated a 68% reduction in cross-sectional granuloma density in Cd36\(^{-/-}\) mice (median 116 (range 46 to 124) vs 359 (239 to 489) granulomas/cm\(^2\); \(p=0.029\); Figure 3.2G).
Figure 3.2. Histopathological sections of organs of Cd36+/+ (upper row) and Cd36-/- (lower row) mice 14 days after IP infection with M. bovis BCG.

A and B. Liver sections (H&E stain, 10× magnification) demonstrate more numerous granulomas in Cd36+/+ mice (arrowheads, A) compared to Cd36-/- mice (arrowhead, B). C and D. Liver sections (H&E stain, 100× magnification) demonstrate similar microarchitecture of individual granulomas. E and F. Splenic sections (Ziehl–Neelsen stain, 100× magnification) show multiple acid fast bacilli (AFB) in a single field for Cd36+/+ mice (E), and no visible AFB in Cd36-/- mice (F). G. Liver granuloma cross-sectional density at day 14 post-infection was lower in Cd36-/- mice (*p=0.0038). All granulomas in each histopathological section were counted, with observer blinding, and normalized to the liver cross-sectional area. Data represent mean ± SEM, 4 mice per group.
3.4.2 \textit{Cd36}/- macrophages restrict mycobacterial growth \textit{in vitro} relative to \textit{Cd36}+/+ macrophages.

The macrophage plays a central role in host defense against \textit{M. tb}, and several molecular determinants of host susceptibility to mycobacterial infection involve alterations in macrophage function (Bhatt and Salgame 2007). In order to examine the mechanisms by which \textit{Cd36}/- mice restrict mycobacterial infection, we exposed peritoneal macrophages derived from \textit{Cd36}/- and \textit{Cd36}+/+ mice to \textit{M. tb} \textit{in vitro}. After infection with \textit{M. tb}, intracellular mycobacterial counts progressively increased over 7 days, with fewer \textit{M. tb} inside \textit{Cd36}/- macrophages than \textit{Cd36}+/+ macrophages ($p<0.0001$, Figure 3.3A). Mycobacterial counts were also lower overall in \textit{Cd36}/- compared to \textit{Cd36}+/+ macrophages infected with \textit{M. marinum} ($p<0.0001$, Figure 3.3B) and BCG ($p<0.0001$, data not shown). Because the intracellular growth restriction pattern of \textit{M. marinum} was similar to \textit{M. tb}, we used \textit{M. marinum} as a model organism in subsequent \textit{in vitro} experiments, replicating our findings with BCG where feasible.
Figure 3.3. Co-cultivation of mycobacteria with Cd36−/− murine macrophages.

*In vitro*, mycobacterial infection of thioglycolate-elicited peritoneal macrophages resulted in lower mycobacterial loads in cultures of Cd36−/− macrophages (white box, dashed line) compared with wild type controls (black box, solid line). **A.** Infection with *M. tuberculosis* (MOI=10:1) produced a progressive rise in mycobacterial counts over 7 days, with significant difference (*p*<0.0001) between groups. **B.** Similar results were seen after infection with the rapidly growing *M. marinum* (MOI=1:1, *p*<0.001). Data are shown as mean +/- 95% CI with 4 to 6 replicates at each time.
3.4.3 Mycobacterial internalization is similar in Cd36\(^{-/-}\) and Cd36\(^{+/+}\) macrophages.

Previously, the CD36 homologue Peste (Pes) in D. melanogaster as well as mammalian class B scavenger receptors have been implicated in the uptake of M. fortuitum (Philips, Rubin et al. 2005). Therefore, we tested the hypothesis that differences in the initial uptake of mycobacteria by macrophages might account for the differences observed in the mycobacterial bacterial loads following in vitro infection. However, using several experimental approaches including confocal fluorescent microscopy, flow cytometry, in vitro co-cultivation, and electron microscopy, we found no significant difference in uptake of mycobacteria by Cd36\(^{-/-}\) and Cd36\(^{+/+}\) murine macrophages.

Using differential labeling of biotinylated intracellular and extracellular M. marinum with distinct streptavidin-conjugated fluorophores before and after cell permeabilization (see Methods section), we qualitatively observed that Cd36\(^{+/+}\) and Cd36\(^{-/-}\) macrophages internalize M. marinum to a similar extent (Figure 3.4A-F). Image analysis (Figure 3.4G and H) and flow cytometric analysis (Figure 3.4I and J) both showed that the uptake of M. marinum was quantitatively similar between Cd36\(^{+/+}\) and Cd36\(^{-/-}\) murine macrophages.

Next, viable intracellular mycobacteria were enumerated after 3 hours incubation with macrophages and after repeated washing with media containing gentamicin to eliminate extracellular mycobacteria. Using three mycobacterial species, M. tb, M. bovis BCG and M. marinum, we found no significant difference in the mycobacterial counts between Cd36\(^{-/-}\) and Cd36\(^{+/+}\) macrophages (Figure 3.4K), indicating that uptake of mycobacteria was similar in the presence or absence of CD36. We exposed macrophages to different innocula of M. tb and again observed no difference between Cd36\(^{-/-}\) and Cd36\(^{+/+}\) macrophages in the uptake of mycobacteria at various multiplicities of infection (MOI) (Figure 3.4L). Experiments with M. tb at MOI=10:1 were repeated four times to confirm this observation.
Figure 3.4. No difference in uptake of mycobacteria between Cd36+/+ and Cd36-/- macrophages. A to F.

*M. marinum* was biotinylated and incubated with *Cd36*+/+ (top row) and *Cd36*-/ (bottom row) macrophages for 3 hours to allow phagocytosis. Extracellular *M. marinum* was labeled using streptavidin-conjugated tetramethylrhodamine (TMR) and appears red. Macrophages were fixed and permeabilized and a second streptavidin-conjugated fluorophore (AlexFluor488©) was used to label intracellular *M. marinum* (appears green). A and B. Intracellular (green) *M. marinum* is readily visible within *Cd36*-/ macrophages, with uptake qualitatively equivalent to *Cd36*+/+ macrophages. C and D. Control conditions using cytochalasin D (10 µM) to inhibit phagocytosis, demonstrating decreased intracellular (green) mycobacteria in both *Cd36*+/+ and *Cd36*-/ macrophages. E and F. Control conditions using unbiotinylated mycobacteria, demonstrating the specificity of fluorescent labeling for mycobacteria. G and H. Image analysis was used to quantify the intracellular (G, green) and extracellular (H, red) signal in 10 random high power fields for each condition. *Cd36*+/+ and *Cd36*-/ macrophages contain similar quantities of intracellular (green) *M. marinum* (p=0.29) and internalization is inhibited to a similar extent by cytochalasin D. I and J. Flow cytometry was used as a second quantitative technique, and showed no significant difference in the fluorescence intensity associated with intracellular (green) or extracellular (red) *M. marinum* between *Cd36*+/+ (solid line) and *Cd36*-/ (dashed line). Shown for comparison are negative control conditions (unbiotinylated *M. marinum*, no fill color). K and L. *In vitro* uptake of mycobacteria by *Cd36*+/+ and *Cd36*-/ macrophages. Adherent macrophages (plated in 96-well polystyrene plates at a density of 50,000 cells/well) were exposed to mycobacteria for 3 hours, and wells were washed 3 times with media containing gentamicin to eliminate extracellular mycobacteria. Similar uptake of three mycobacterial species, *M. tuberculosis* (MOI=10:1), *M. bovis* BCG (MOI=10:1) and *M. marinum* (MOI=1:1) was seen in *Cd36*-/ and *Cd36*+/+ macrophages (p>0.05 for all species). Similar uptake was observed at different multiplicities of infection with *M. tuberculosis* (p>0.05 for each MOI).
Finally, *Cd36*+/+ and *Cd36*−/− macrophages were co-incubated with *M. marinum* (MOI=100:1) for 6 hr and visualized by electron microscopy. Numerous electron-dense bacilli were evident within cells of either genotype, and were contained within a membrane-bound phagolysosome (Figure 3.5A-D). Quantification of mycobacteria within a total of 293 random macrophages (3 pooled experiments) revealed no difference in the number of internalized *M. marinum* (Figure 3.5E; median (range) 14 (0-93) vs 17 (0-81); p=0.19) between *Cd36*+/+ and *Cd36*−/− macrophages.
Figure 3.5. Electron micrographs demonstrating similar numbers of internalized M. marinum bacilli after 6 hours co-incubation with Cd36+/+ and Cd36-/- macrophages. A and B. Full cell view demonstrating electron-dense bacilli (arrowheads) within macrophages. C and D. In both cell types, bacilli are contained within a membrane- (black arrowhead) bound phagolysosome (white arrowhead, electron-luscent phagolysosome lumen). E. Enumeration of internalized bacilli demonstrated no significant difference in uptake of M. marinum between Cd36+/+ and Cd36-/- macrophages (p=0.19). Bacilli were counted in a total of 293 macrophages from 3 independent experiments, taking a minimum of 30 random EM fields for each genotype in each experiment.
3.4.4 Cell viability and rate of apoptosis of \( Cd36^- \) and \( Cd36^{+/+} \) macrophages are similar.

CD36 has been implicated in macrophage apoptosis (Wintergerst, Jelk et al. 2000; Sanchez, Garcia et al. 2006), which may be an important host defense strategy for the containment of intracellular mycobacteria (Balcewicz-Sablinska, Keane et al. 1998; Loeuillet, Martinon et al. 2006), and for subsequent priming of adaptive immune effector cells (Winau, Weber et al. 2006). Therefore, we investigated whether an increased rate of apoptosis in \( Cd36^- \) macrophages might explain the lower mycobacterial counts in these cells. By terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, we found no differences in apoptosis between \( Cd36^- \) and \( Cd36^{+/+} \) macrophages after 7 days of co-cultivation with \( M. tb \) or BCG (data not shown). Similarly, no difference was observed in caspase-3 activity between cultures of \( Cd36^- \) and \( Cd36^{+/+} \) macrophages co-incubated with \( M. tb \) or BCG (data not shown). Furthermore, examination of spleen and liver tissue sections from \textit{in vivo} infection experiments with BCG did not reveal any differences in the density of apoptotic cells (data not shown).

We also examined if there were differences in viability of infected \( Cd36^- \) macrophages compared to \( Cd36^{+/+} \), which could account for the observed lower mycobacterial counts in cultures of \( Cd36^- \) macrophages. Viability of macrophages, as determined by quantitative MTS assay, was equivalent over 7 days after infection with \( M. tb \) between \( Cd36^- \) and \( Cd36^{+/+} \) macrophages (\( p=0.578 \); data not shown).

3.4.5 Production of reactive nitrogen and reactive oxygen intermediates is similar in \( Cd36^{+/+} \) and \( Cd36^- \) macrophages

The production of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) are effective intracellular killing mechanisms against microbial pathogens including mycobacteria (Moncada, Palmer et al. 1991; Chan, Chan et al. 2001). We examined whether differences in nitric oxide production and/or oxidative burst in response to infection with mycobacteria might account for the restriction of mycobacterial growth in \( Cd36^- \) macrophages. Nitrite concentration was similar in the supernatant of interferon-\( \gamma \) (IFN-\( \gamma \)) activated \( Cd36^{+/+} \) and \( Cd36^- \) macrophages stimulated with BCG for 24 hours (Figure 3.6A). Consistent with previous reports (Thoma-Uszynski, Stenger et al. 2001; Heldwein, Liang et al. 2003; Sugawara, Yamada et al. 2003), nitric oxide production in response to mycobacterial challenge was toll-like receptor
2 (TLR2)- and interleukin-1 receptor-associated kinase 4 (IRAK-4)-dependent, but TLR4-independent in our experimental system (Figure 3.6A). Nitric oxide production in response to live and heat-killed BCG and *M. marinum*, as well as TLR2 and TLR4 specific ligands, was similar in *Cd36*+/+ and *Cd36*−/− (but not *Tlr2*−/−) macrophages (Figure 3.6B) and followed similar kinetics in both cell types (Figure 3.6C and D). Oxidative burst of *Cd36*+/+ and *Cd36*−/− macrophages, as determined by luminol-enhanced chemiluminescence assay, was similar in response to phorbol myristate acetate, and neither cell type produced significant reactive oxygen species above baseline in response to stimulation with BCG (Figure 3.6E and F), as previously described (Gordon and Hart 1994; Rojas-Espinosa, Wek-Rodriguez et al. 2002).
Figure 3.6. Production of reactive nitrogen and reactive oxygen intermediates is similar in 
$Cd^{36^{+/+}}$ and $Cd^{36^{-/-}}$ macrophages.

A. Nitric oxide production was measured using the Griess reaction in culture supernatant. In 
response to stimulation with BCG, nitric oxide production is TLR2 ($p<0.0001$) and IRAK-4 ($p<0.0001$) dependent, but independent of TLR4 ($p=0.16$) and CD36 ($p=0.83$). B. Similar nitric oxide production by $Cd^{36^{+/+}}$ and $Cd^{36^{-/-}}$ macrophages in response to live and heat-killed BCG 
and $M. marinum$, as well as specific toll-like receptor ligands (Pam$_3$CSK$_4$ (PAM), a TLR2 
ligand, and lipopolysaccharide (LPS); $p>0.05$ for all comparisons $Cd^{36^{+/+}}$ vs $Cd^{36^{-/-}}$). C and D. 
Similar kinetics of nitric oxide production over 36 hours in response to both BCG (C) and $M. marinum$ (D). E and F. Similar oxidative burst in response to phorbol myristate acetate (PMA), 
and no significant response to BCG above baseline among $Cd^{36^{+/+}}$ and $Cd^{36^{-/-}}$ macrophages.
3.4.6 Differences in mycobacterial susceptibility are not explained by polymorphisms of \textit{Nramp1}.

Because polymorphisms in the \textit{Nramp1} gene are known to affect host susceptibility to mycobacterial infection (Bellamy 1999), we analyzed genomic DNA from \textit{Cd36}^{+/+} and \textit{Cd36}^{-/-} mice for polymorphisms at this genetic locus. Comparing to C57BL/6 (BCG-sensitive) and 129/Sv (BCG-resistant) controls, both \textit{Cd36}^{+/+} and \textit{Cd36}^{-/-} mice were found to carry characteristic C57BL/6 alleles (data not shown). Therefore, differences in mycobacterial susceptibility between the \textit{Cd36}^{+/+} and \textit{Cd36}^{-/-} mice do not appear to be attributable to variation in the \textit{Nramp1} gene.

3.4.7 Levels of TNF correlate with bacillary burden.

\textit{T}H1 cytokine responses are critical for granuloma formation and control of mycobacterial infection (Saunders and Britton 2007), with TNF playing a central role (Bhatt and Salgame 2007). We examined levels of TNF, IFN-\gamma, IL-10, IL-6, IL-12p70 and MCP-1 in the sera of mice infected with BCG to determine whether differential cytokine responses could explain the reduced BCG loads in \textit{Cd36}^{-/-} mice. TNF concentrations rose to a maximum 2-3 weeks after infection and subsequently decreased (Figure 3.7A), in parallel with BCG counts in the spleen and liver. \textit{Cd36}^{-/-} mice had lower TNF levels overall (\textit{p}<0.001). TNF levels were positively correlated with the BCG counts in the spleen (\textit{p}=0.596, \textit{p}<0.001, Figure 3.7B). Although it might be predicted that higher levels of \textit{T}H1 cytokines would be associated with improved control of mycobacterial infection, \textit{Cd36}^{-/-} mice exhibited lower levels of TNF, which in turn correlated with lower mycobacterial loads in infected organs. Rather than determining the course of infection, TNF levels appear to reflect the burden of infected macrophages in our mouse model.

In support of this explanation, we observed similar levels of TNF in the supernatant of \textit{Cd36}^{+/+} and \textit{Cd36}^{-/-} thioglycollate-elicited peritoneal macrophages infected with mycobacteria \textit{in vitro} (Figure 3.7C), which increased in a dose-dependent manner with increasing multiplicity of infection with both \textit{M. marinum} (Figure 3.7E) and BCG (Figure 3.7F). These findings were replicated with bone-marrow derived murine macrophages (data not shown). In contrast, TNF production was reduced in \textit{Tlr2}^{-/-} and \textit{Irak4}^{-/-} macrophages compared to their wild type counterparts (Figure 3.7C).
3.4.8 Altered IL-10 production does not account for enhanced antimycobacterial defenses in Cd36<sup>−/−</sup> mice and macrophages.

IL-10 is an immunomodulatory cytokine, known to inhibit macrophage antimycobacterial activity <i>in vitro</i> (Flesch, Hess et al. 1994) and <i>in vivo</i> (Murray, Wang et al. 1997), as exemplified by the enhanced clearance of mycobacteria in IL-10 deficient mice (Murray and Young 1999; Jacobs, Brown et al. 2000; Jacobs, Fick et al. 2002). Mycobacteria and their products, including the glycolipid AraLAM, induce IL-10 production by macrophages (Roach, Barton et al. 1995). IL-10 production by macrophages in response to apoptotic cells is mediated by CD36 to a large extent (Voll, Herrmann et al. 1997; Chung, Liu et al. 2007). Therefore, we hypothesized that the enhanced antimycobacterial activity of Cd36<sup>−/−</sup> macrophages may be related to reduced production of IL-10. However, we observed significantly higher levels of IL-10 by Cd36<sup>−/−</sup> macrophages over a time course of infection (Figure 3.7D) and over a range of multiplicities of infection with both <i>M. marinum</i> (Figure 3.7G) and BCG (Figure 3.7H). This finding was replicated using both thioglycollate-elicited peritoneal macrophages (Figure 3.7D, G and H) and bone-marrow derived macrophages (data not shown). In contrast, IL-10 production by Tlr2<sup>−/−</sup> and Irak4<sup>−/−</sup> macrophages was significantly reduced (Figure 3.7D). Furthermore, no difference in IL-10 levels was detected in the sera of Cd36<sup>−/−</sup> mice relative to wild type controls over the course of experimental BCG infection (data not shown). Therefore, improved mycobacterial defenses in Cd36<sup>−/−</sup> mice do not appear to be attributable to reduced production of IL-10 by macrophages.

Additionally, there were no significant differences observed in serum IFN-γ, IL-6, IL-10, IL-12p70 and MCP-1 levels in Cd36<sup>−/−</sup> vs Cd36<sup>+/+</sup> mice up to 63 days after infection (data not shown).
Figure 3.7. Alterations in TNF and IL-10 production do not explain relative resistance of Cd36−/− mice to mycobacterial infection.

A. Serum levels of TNF in Cd36+/+ and Cd36−/− mice rose after intraperitoneal infection (day 0) with M. bovis BCG, reaching a maximum after 14-21 days, then decreased. This trend paralleled the mycobacterial counts in organs of infected mice. TNF levels were higher overall (p<0.001) in Cd36+/+ mice (solid line) compared to Cd36−/− mice (dashed line). Results are displayed as mean ± SEM, with each point representing 4 replicate mice. Data from one representative experiment of two are shown. B. Significant correlation (ρ=0.596, p<0.001) between splenic mycobacterial counts and serum level of TNF. C. Time course of TNF production by Cd36+/+ and Cd36−/− thioglycollate-elicited peritoneal macrophages co-incubated with M. marinum in vitro shows no difference between genotypes (p=0.33). In contrast, TNF production by Tlr2−/− (p<0.0001) and Irak4−/− (p<0.0001) macrophages is markedly deficient. D. Cd36−/− macrophages are not deficient in IL-10 production, and produce higher levels of IL-10 at some time points (* p=0.040) following infection with M. marinum. E and F. TNF production in response to M. marinum (E) and BCG (F) is similar (p=0.54 for M. marinum, p=0.96 for BCG) between Cd36+/+ and Cd36−/− macrophages over a range of multiplicities of infection. G and H. IL-10 production in response to M. marinum (G) and BCG (H) was dose-dependent (p<0.0001 for both) and was higher in Cd36−/− macrophages (p<0.0001 for M. marinum, p=0.0003 for BCG). Cytokine levels were assayed in supernatant of macrophages in 96-well plates (200,000 adherent cells and 250 µl media per well) after 24 hours incubation. These findings were confirmed using bone-marrow derived macrophages (data not shown).
3.4.9 Reduced recovery of viable mycobacteria from Cd36\(^{-/-}\) macrophages using an in vitro model of cellular events involved in granuloma formation.

Based on studies using quantitative intravital imaging in transparent zebrafish infected with M. marinum, macrophage turnover within the granuloma contributes to early mycobacterial growth and dissemination (Davis and Ramakrishnan 2009). Arriving macrophages efficiently find and phagocytose infected macrophages undergoing apoptosis, leading to expansion of infected macrophages and bacterial numbers (Davis and Ramakrishnan 2009). Given the role of CD36 in the uptake of apoptotic cells by macrophages (Voll, Herrmann et al. 1997), this observation may account for the decreased mycobacteria loads observed in Cd36\(^{-/}\) mice. We therefore designed an in vitro system to recapitulate the cellular events of M. marinum internalization, apoptosis of infected macrophages, and phagocytosis by secondary uninfected macrophages. Peritoneal macrophages from Cd36\(^{+/}\) or Cd36\(^{-/-}\) mice were plated and infected with M. marinum (MOI=10:1). Apoptosis of the infected macrophages was induced by serum starvation, as demonstrated by elevated caspase-3/7 activity (Kumar and Lavin 1996) (data not shown). Then, the infected apoptotic “prey” macrophages were gently scraped and co-incubated with healthy uninfected “predator” macrophages. After incubation at \(37^\circ C\) for 3 hr to allow internalization of prey macrophages, cells were carefully washed to remove extracellular apoptotic bodies and bacteria. Cells were lysed and plated on mycobacterial culture media for CFU counts. The fraction of viable mycobacteria recovered was reduced in Cd36\(^{-/-}\) compared to Cd36\(^{+/}\) predator macrophages \((p=0.0026;\) Figure 3.8). These observations are consistent with the known role of CD36 as a receptor for apoptotic cell uptake on phagocytic cells (Voll, Herrmann et al. 1997), together with their bacterial contents. Collectively, these results suggest that CD36 participates in granuloma turnover, and contributes to the expansion of the intracellular mycobacterial pool.
Figure 3.8. Recovery of viable intracellular mycobacteria is reduced in Cd36<sup>−/−</sup> macrophages in an in vitro model recapitulating cellular events in early granuloma formation. Primary “prey” macrophages were incubated with *M. marinum* and washed to remove extracellular bacteria. Apoptosis was induced by overnight serum starvation, and was evidenced by increased caspase-3/7 activity (data not shown). Control macrophages (no apoptosis) were incubated in media containing 10% fetal bovine serum. Apoptotic primary prey macrophages containing *M. marinum* were gently scraped and co-incubated with uninfected secondary “predator” macrophages to allow phagocytosis of primary prey macrophages. After washing to remove extracellular apoptotic bodies and bacteria, cells were scraped and plated on mycobacteria culture media for CFU counts. The recovery of viable *M. marinum* was reduced in systems using *Cd36<sup>−/−</sup>* (white bar) relative to *Cd36<sup>+/+</sup>* (black bar) macrophages (*p=0.0026), suggesting a defect in the uptake of mycobacteria-laden apoptotic macrophages.
3.5 Discussion

Host genetic factors play a major role in influencing the severity and the ultimate outcome of tuberculosis. In this study, we have identified CD36 as a potentially important determinant of host susceptibility to mycobacterial infection. We demonstrated that CD36 deficiency confers relative resistance to mycobacterial infection. This conclusion was supported by both in vivo experimental model infections and in vitro cell culture infection approaches.

We used a well-established murine model of disseminated mycobacterial infection and demonstrated that disruption of the Cd36 gene confers an altered susceptibility phenotype. In this experimental murine model, systemic BCG infection is ultimately controlled with the induction of antigen-specific immunity, similar to human tuberculosis (Costello, Izumi et al. 1971; Nau, Liaw et al. 1999; Heldwein, Liang et al. 2003). In our studies, M. bovis BCG counts in the spleens and livers of infected mice rose to a maximum after 2 weeks, and subsequently declined to nearly undetectable levels with the induction of adaptive immunity (Figure 3.1). Splenomegaly and granulomatous infiltrates in the spleen and liver have been previously described in murine M. bovis BCG infection (Kremer, Estaquier et al. 2000), as observed in our experiments (Figure 3.2). We used three species of mycobacteria (M. tb, M. marinum and BCG) for in vitro experiments in order to model distinct aspects of tuberculosis pathogenesis and demonstrate the generalizability of these findings. In addition to sharing many known virulence factors with M. tb, M. marinum showed growth restriction similar to M. tb within Cd36−/− macrophages (Figure 3.3), and was therefore used in subsequent assays as a model organism. M. marinum offers several technical advantages including a rapid growth rate and minimal biohazard risk. Where feasible, we replicated our findings with BCG to demonstrate generalizability across different mycobacterial species. However, because BCG forms large aggregates when cultured in vitro (Devadoss, Klegerman et al. 1991), it was not suitable for all experiments, particularly imaging studies which required suspensions of single organisms.

Disruption of the Cd36 gene affected the early and peak mycobacterial burdens, but did not appear to impact the ultimate clearance of the organism (Figure 3.1), consistent with the role of CD36 as a receptor predominantly functioning in innate immunity (Gough and Gordon 2000). Likewise, a recent study demonstrated a limited role for CD36 in controlling the outcome of M. tuberculosis pulmonary infection, with no survival difference and a modest effect on lung
mycobacterial loads and granulomas only observed at early time points following intranasal challenge (Court, Vasseur et al.). Further evidence of intact adaptive immune mechanisms includes the universal survival of \( Cd36^{-/} \) mice, and the microscopic structure of granulomas, which were morphologically normal albeit reduced in number, commensurate with the reduced mycobacterial loads observed in these mice (Figure 3.2). As a macrophage cell surface receptor involved in the uptake of apoptotic cells (Voll, Herrmann et al. 1997), CD36 may participate in the cellular dynamics within the granuloma. Recent landmark studies have shown that mycobacteria promote and exploit granuloma formation for the establishment of infection (Davis and Ramakrishnan 2009). After internalization of mycobacteria, macrophages undergo apoptosis, and are phagocytosed by newly recruited macrophages followed by egress of these cells to seed new granuloma (Davis and Ramakrishnan 2009). We modeled this process \textit{in vitro} and found reduced recovery of viable intracellular \textit{M. marinum} from \( Cd36^{-/} \) macrophages exposed to apoptotic macrophages containing mycobacteria (Figure 3.8). These data link our \textit{in vivo} findings with recent insights into tuberculosis pathogenesis, suggesting that CD36 may play a role in the cellular events co-opted by mycobacteria during the establishment and dissemination of infection.

Consistent with results of \textit{in vivo} studies, cell culture infection experiments demonstrated that CD36 deficiency limits intracellular replication of mycobacteria in macrophages (Figure 3.3). The mechanism underlying the restricted replication of mycobacteria in \( Cd36^{-/} \) mice or macrophages is presently unclear; nevertheless, we have excluded differences in early mycobacterial uptake, macrophage cell viability or apoptosis, production of reactive nitrogen and oxygen species, \textit{Nramp1} gene, and selected cytokine responses among possible explanations. Our finding that CD36 was not required for mycobacterial internalization was similar to one recent report involving BCG uptake by murine peritoneal macrophages (Court, Vasseur et al.), but contrasts with an earlier study that implicated the \textit{Drosophila} homologue of CD36 in \textit{M. fortuitum} uptake (Philips, Rubin et al. 2005). Of note, however, transfection of murine \( Cd36 \) into HEK293 cells did not enhance uptake of \textit{M. fortuitum} in the latter study (Philips, Rubin et al. 2005), consistent with our and others’ findings using murine macrophages (Court, Vasseur et al.). Another possibility is that inherent differences in the properties of the mycobacterial species used in the earlier and the current studies may account for this discrepancy.
The restricted growth of mycobacteria within $Cd36^{−/−}$ macrophages might be explained by impairment of mycobacterial immune evasion strategies that take advantage of CD36. Detailed structural studies of the mycobacterial cell wall lipomannans (LMs) have demonstrated that diacylated LMs inhibit LPS-induced inflammation by murine macrophages (Doz, Rose et al. 2007). Intriguingly, CD36 is a sensor of diacylglycerides (Hoebe, Georgel et al. 2005) from a broad range of pathogens (Stuart, Deng et al. 2005; Gowda 2007; Patel, Lu et al. 2007) and may be the host receptor through which diacylated LMs suppress macrophage function. Alternatively, this observation may be explained by participation of CD36 in Toll-like receptor signaling.

CD36 is known to associate with the TLR2/6 heterodimer on the cell surface (Hoebe, Georgel et al. 2005; Stuart, Deng et al. 2005), and may participate in TLR2-dependent immunosuppressive signaling pathways in the context of mycobacterial infection (Noss, Pai et al. 2001; Fortune, Solache et al. 2004; Weiss, Souza et al. 2007). In our experiments, nitric oxide, TNF and IL-10 production in response to mycobacteria were TLR2 dependent; however, CD36 did not appear to participate in these processes. Nonetheless, a role for CD36 in other TLR2 mediated mycobacterial evasion mechanisms cannot be excluded.

Alterations in cytokine profile did not appear to explain CD36-mediated differences in mycobacterial control in vivo and in vitro. Reduced TNF in the sera of infected $Cd36^{−/−}$ mice around the peak of infection appears to reflect the reduced mycobacterial stimulus rather than acting as a mediator of antimycobacterial defenses. In vitro, TNF production in response to live BCG and $M. marinum$ was no different between $Cd36^{−/−}$ and $Cd36^{+/+}$ macrophages, consistent with one previous report (Court, Vasseur et al.). Other $T_{H1}$ cytokines, IFN-$γ$ and IL-12, were not significantly different in BCG-infected $Cd36^{−/−}$ and $Cd36^{+/+}$ mice. Likewise, anti-inflammatory IL-10 levels were not significantly different in vivo, and were increased in vitro in $Cd36^{−/−}$ murine macrophages pre-stimulated with IFN-$γ$. We speculate that the latter finding may suggest a role for CD36 in the previously described IFN-$γ$ inhibition of TLR2-induced IL-10 expression in the context of mycobacterial infection (Hu, Paik et al. 2006). However, elevated levels of IL-10 do not appear to explain the reduced mycobacterial counts in $Cd36^{−/−}$ macrophages, given the known inhibitory role of IL-10 on mycobacterial control (Murray, Wang et al. 1997; Jacobs, Fick et al. 2002).
Our observations that deficiency of CD36 reduces the susceptibility of mice in vivo and of murine macrophages in vitro to mycobacterial infection are novel and somewhat unexpected, given the roles of CD36 in host defense against pathogens such as S. aureus and other bacteria (Stuart, Deng et al. 2005; Baranova, Kurlander et al. 2008), as well as P. falciparum (Patel, Lu et al. 2007). Although the underlying molecular mechanisms remain unclear and require further investigation, our study nevertheless suggests a unique role of CD36 in host susceptibility to tuberculosis. Within this context, it is interesting to note that a CD36\(^{-}\) genotype occurs with relatively high frequency in African, Japanese and other Asian populations (Yamamoto, Ikeda et al. 1990; Curtis and Aster 1996), although the evolutionary advantage of this putative balanced polymorphism is unknown. First described in patients refractory to platelet transfusion (Tomiyama, Take et al. 1990), this genotype has subsequently been associated with susceptibility to a variety of metabolic diseases (Rac, Safranow et al. 2007). Recent population and family-based studies have not associated CD36 gene polymorphisms with severe malaria phenotypes and it has been suggested that CD36 deficiency alleles may be maintained in human populations through selection pressure via a prevalent infection other than malaria (Aitman, Cooper et al. 2000; Fry, Ghansa et al. 2009). If our observations are subsequently confirmed in human infection, it is conceivable that M. tb, being highly prevalent and virulent, could in part account for the persistence of CD36 deficiency in populations from tuberculosis-endemic regions.

3.6 Conclusions

In summary, our findings indicate a novel role for CD36 in host response to mycobacterial infection and suggest that future population-based studies to examine the relationship between CD36 deficiency and susceptibility to tuberculosis would be of interest.
Chapters 4-7

Biomarkers of disease severity in malaria

This group of chapters assembles a body of published work (4 manuscripts presented as 4 chapters) that collectively point to the alteration of circulating levels of endothelial regulatory proteins in the context of severe and fatal malaria. These results have important clinical implications for the development of new predictive tools that can be used to identify patients at risk of adverse outcomes.

Angiopoietin-2 (Ang-2), a key modulator of endothelial activation and vascular leak, is consistently elevated in patients with severe malaria, cerebral malaria, and fatal disease. Ang-2 therefore represents an objective and quantitative marker of disease severity. Other studies have examined the longitudinal course of Ang-2 levels during recovery from severe malaria, demonstrating a linear decrease to normal levels over a period of approximately 4 days (Yeo, Lampah et al. 2008). This demonstrates that Ang-2 can be used as a marker of recovery from severe disease.

We have leveraged this finding in the design of a randomized controlled trial of a novel adjunct for severe malaria in Chapters 8 and 9. Use of a quantitative and objective biomarker (Ang-2), measured serially during recovery from severe malaria, allowed for efficient study design and a modest sample size, while maintaining adequate statistical power.

The author’s role in these studies was to assist in the design of the case-control observational studies, and in the analysis and interpretation of results. The analysis includes numerous statistical techniques including receiver operator characteristic (ROC) curves, multivariable logistic regression, and classification and regression trees (CART).
Chapter 4
Serum angiopoietin-1 and -2 levels discriminate cerebral malaria from uncomplicated malaria and predict clinical outcome in African children

4.1 Abstract

**Background:** Validated biomarkers to identify *Plasmodium falciparum*-infected individuals at risk of developing serious complications such as cerebral malaria (CM) do not currently exist.

**Methods:** Based on the hypothesis that endothelial and blood-brain-barrier dysfunction contributes to CM pathogenesis, we examined the endothelial regulators, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), in serum samples from *P. falciparum*-infected patients with uncomplicated malaria or CM, from two diverse populations – Thai adults and Ugandan children. Ang-1 and Ang-2 levels were compared to tumour necrosis factor (TNF).

**Findings:** In both populations, Ang-1 levels were significantly decreased in CM versus uncomplicated malaria and healthy controls (Kruskal-Wallis (K-W) test, p<0.001). Additionally, Ang-2 levels were significantly increased in CM versus uncomplicated malaria and healthy controls (K-W test, p<0.001). TNF was only significantly elevated in CM compared to uncomplicated cases in the Thai adult population (p<0.001). Receiver operating characteristic curves (ROC) were used to compare how reliably each biomarker distinguished CM from uncomplicated malaria cases. Ang-1 and the ratio of Ang-2:Ang-1 were found to be highly accurate tests to discriminate CM patients in both populations (Area under the ROC) for Thai and Ugandan populations (Ang-1: 1.0 and 0.795; Ang-2:Ang-1: 1.0 and 0.782, respectively). TNF did not discriminate well between CM and uncomplicated malaria in African children. Furthermore, low Ang-1 levels at presentation predicted subsequent mortality in children with CM (p=0.01).

**Interpretation:** Ang-1 and the Ang-2/1 ratio are promising clinically informative biomarkers for CM. Additional studies should address their utility as prognostic biomarkers and as potential therapeutic targets in severe malaria.
Introduction

Although greater than 500 million *Plasmodium falciparum* malaria infections are estimated to occur each year, only a small proportion of affected individuals progress to severe and potentially fatal complications such as cerebral malaria (Snow, Guerra et al. 2005). Unfortunately, the mechanisms underlying cerebral malaria are poorly understood and limited diagnostic tools are available to determine which patients infected with *P. falciparum* will progress to cerebral complications (Marsh, Forster et al. 1995; Jaffar, Van Hensbroek et al. 1997; Dzeing-Ella, Nze Obiang et al. 2005). Additionally, the signs and symptoms of severe and cerebral malaria are non-specific, rendering definitive diagnosis challenging and resulting in frequent misdiagnoses and mistreatment of other life-threatening conditions (Reyburn, Mbatia et al. 2004). Even in research or tertiary care settings, it may be difficult to exclude other causes of coma in children believed to have cerebral malaria (CM). In prospective autopsy studies in Malawi, over 20% of children diagnosed with CM were shown to have an alternative cause for their neurological syndrome when examined at autopsy (Taylor, Fu et al. 2004).

The discovery of a reliable bedside or laboratory test that accurately identifies individuals with, or at risk of, CM could provide considerable benefit. The capacity for early detection and focused intervention for individuals at risk of adverse malaria outcomes would have considerable clinical and economic impact, particularly in resource-poor settings where effective allocation of limited health resources is vital. Fundoscopic examination demonstrating malarial retinopathy has been reported to be a useful pre-mortem discriminator of severe malaria and has been proposed as a diagnostic test for CM (Beare, Southern et al. 2004; Taylor, Fu et al. 2004). However, indirect fundoscopy has inherent limitations, including requirements for pupil dilation, specialized training and equipment (Beare, Southern et al. 2004). Furthermore, it is unclear whether fundoscopy can be used to predict which children will progress to CM, and retinopathy does not appear to be a consistent feature of CM in adults (Kochar, Shubhakaran et al. 1998; Kochar, Shubhakaran et al. 2000; Beare, Southern et al. 2004). Other studies have examined the correlation of serum markers, such as cytokines, with severe and complicated malaria. Elevated levels of the pro-inflammatory cytokine tumour necrosis factor (TNF) have been associated with severe malaria in a number of studies (Grau, Taylor et al. 1989; Kern, Hemmer et al. 1989; Kwiatkowski, Hill et al. 1990; Akanmori, Kurtzhals et al. 2000; Lyke, Burges et al. 2004;
Tchinda, Tadem et al. 2007) and were identified as a predictor of mortality in CM (Grau, Taylor et al. 1989; Kwiatkowski, Hill et al. 1990). However, others have reported that TNF levels do not correlate with disease severity (Gimenez, Barraud de Lagerie et al. 2003), and high serum TNF levels are also observed during *Plasmodium vivax* infection, which is not typically associated with neurological involvement (Kern, Hemmer et al. 1989).

Endothelial cell activation and dysfunction has been implicated in the pathogenesis of cerebral malaria, since the endothelium responds to parasite-induced inflammation and mediates parasitized erythrocyte sequestration, especially in vital organs such as the brain (Turner, Morrison et al. 1994). Endothelial activation markers, such as endothelial microparticles, vonWillebrand factor and soluble cell-adhesion molecules (sCAMs) including soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 and soluble endothelial leukocyte adhesion molecule-1 are increased in malarial infection and have been positively correlated with disease severity (Jakobsen, Morris-Jones et al. 1994; Turner, Ly et al. 1998; Combes, Taylor et al. 2004; Tchinda, Tadem et al. 2007). However the role of sCAMs in the pathophysiology of malaria is unclear and circulating levels of sCAMs may not reflect their true expression in vascular beds. Furthermore, it is unclear how robust these markers are in predicting disease progression or outcome.

In addition to systemic endothelial activation, recent work has focused on how malaria may compromise the structural and functional integrity of the blood-brain-barrier (BBB), leading to leakage of plasma proteins, perivascular edema and neuronal injury. Angiopoietins, a recently described distinct family of angiogenic molecules, have recently been shown to play fundamental physiological roles in maintenance of vascular integrity. Angiopoietin-1 (Ang-1) is constitutively expressed and acts to maintain vascular quiescence (Fiedler and Augustin 2006). The Ang-1 stabilizing effect is antagonized by angiopoietin-2 (Ang-2), which primes the endothelial activation response and promotes vascular permeability (Loughna and Sato 2001; Fiedler and Augustin 2006). In healthy individuals, serum Ang-1 levels are normally high, while serum Ang-2 levels are low. Consequently, an increase in Ang-2, or a dysregulation of the Ang-1/2 balance, may be associated with disease states that cause inflammation and vascular permeability (Loughna and Sato 2001; Fiedler and Augustin 2006). Elevated Ang-2 levels have
been reported in patients with severe sepsis and may contribute to sepsis-related vascular leak (Parikh, Mammoto et al. 2006).

A role for angiopoietins in malaria pathogenesis has not been previously described. Based on the hypothesis that dysregulation of angiopoietins may be associated with endothelial and BBB dysfunction during malaria infection, we examined whether Ang-1 and Ang-2 were informative biomarkers for cerebral malaria. We measured Ang levels in individuals with cerebral or uncomplicated malaria and compared them to serum levels of TNF. In two distinct patient populations, a decrease in Ang-1 serum levels and the dysregulation of the Ang-2/Ang-1 balance was superior to TNF as biomarkers of cerebral malaria. Furthermore, low Ang-1 levels at presentation predicted subsequent mortality in African children with CM. These data indicate that Ang-1 and the Ang-2/1 ratio may have clinical utility in the diagnosis and prognosis of severe malaria syndromes such as cerebral malaria.
4.2 Materials and methods

4.2.1 Thai Study population

Individuals (≥13 years of age) living in Thailand and admitted to the Hospital for Tropical Disease (Mahidol University, Bangkok, Thailand) for ongoing studies of anti-malarial drug efficacy were eligible for enrolment. The institutional review board of Mahidol University approved the study, and informed consent was obtained from all patients or their legal guardians. Venous blood samples were collected from 50 patients with *P. falciparum* malaria (25 consecutive cases of uncomplicated malaria and 25 consecutive cases of CM), and from 10 healthy controls, who had negative blood smears and no history of malaria infection in the previous 6 months. Uncomplicated malaria patients were defined based on a positive blood smear for *P. falciparum* without evidence for severe or complicated malaria as defined by the World Health Organization criteria (WHO) (1990). CM was defined as *P. falciparum* infection on blood smear, unrousable coma (Glasgow coma scale ≤8) not attributable to other causes (2). Patient demographic data, including sample size, age and peripheral parasitemia, are summarized in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adult (Thailand)</th>
<th>Pediatric (Uganda)</th>
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<tbody>
<tr>
<td></td>
<td>N</td>
<td>Age (years)</td>
</tr>
<tr>
<td>HC</td>
<td>10</td>
<td>32 (25-48)</td>
</tr>
<tr>
<td>UM</td>
<td>25</td>
<td>22 (14-63)</td>
</tr>
<tr>
<td>CM</td>
<td>25</td>
<td>25 (17-50)</td>
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</table>

Age and parasitemia are presented as median (range). *p*<0.05 vs. HC and *p*<0.05 vs. UM (Kruskal-Wallis test with Dunn’s multiple comparison post-test). doi:10.1371/journal.pone.0004912.1001

4.2.2 Ugandan Study population

The Ugandan study population has been previously described (John, Opika-Opoka et al. 2006). Ethics approval for the study was granted by the institutional review boards for human studies at
Makerere University, Faculty of Medicine (Kampala, Uganda) and written informed consent was obtained from the parents or guardians of study participants. Briefly, children 4–12 years old admitted to Mulago Hospital were eligible for enrolment if they had uncomplicated malaria or met the WHO criteria for CM: *P. falciparum* on blood smear and coma (Blantyre coma scale \( \leq 2 \) or Glasgow coma scale \( \leq 8 \)) not attributable to hypoglycemia, convulsions, meningitis or other identifiable cause (2). Lumbar punctures were performed to rule out meningitis and encephalitis. Children were considered to have uncomplicated malaria if they had fever (or a history of fever within 24 hours), *P. falciparum* infection on blood smear, but no evidence of severe or complicated malaria (e.g., seizures, respiratory distress, severe anemia, or altered level of consciousness [2]) or other acute illness. Healthy controls were recruited from the extended household areas of children with cerebral or uncomplicated malaria and were determined to be healthy by medical history (with no malaria history for the previous 6 months), physical examination and microscopic examination of blood smears. Healthy controls were excluded from serum cytokine testing. Patient demographic data, including sample size, age and peripheral parasitemia, are summarized in Table 4.1.

4.2.3 Quantification of serum cytokine and angiopoietin and cytokine levels

Serum concentrations of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and tumour necrosis factor (TNF) were measured using a standard sandwich enzyme immunoassay (EIA) according to the manufacturer’s instructions (Ang-1 and -2: R&D Systems, Minneapolis MN; TNF: eBioscience, San Diego CA). Serum samples were diluted 1:20 for Ang-1, 1:5 for Ang-2, and 1:2 for TNF in PBS/1%BSA. Concentrations were interpolated from 4-parameter-fit standard curves generated using a standard curve of recombinant human proteins. TNF values from Ugandan children were measured as previously described (John, Opika-Opoka et al. 2006).

4.2.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (v4.03). Serum protein levels were analyzed using a Kruskal-Wallis test, followed by Dunn’s multiple comparison tests. Receiver operating characteristic (ROC) curves and area under the ROC curves were generated using SPSS (v11). Cutoff values were derived mathematically from the ROC curves, using the point on
the ROC curve with the lowest value for the formula: $(1 \text{-sensitivity})^2 + (1 \text{-specificity})^2$.

Sensitivity, specificity, positive predictive value and negative predicted value were calculated using standard formulas. Angiopoietin levels and survival outcomes were analyzed using the Wilcoxon rank-sum test. Multivariable logistic regression modeling was used to examine the independent predictive value of biomarkers on outcome (CM vs. UM) in order to account for potential confounding effects of multiple covariates (SPSS 16.0). Hosmer Lemeshow test was used to verify model goodness of fit.
4.3 Results

4.3.1 Angiopoietin-1 levels are decreased and angiopoietin-2 levels increased in the serum of cerebral malaria patients compared to uncomplicated patients and healthy controls

In Thailand, serum Ang-1 levels were significantly lower in adults with CM compared to uncomplicated malaria or healthy controls, and in uncomplicated malaria compared to healthy controls (Figure 4.1A; Kruskal-Wallis: p<0.001). Moreover, serum Ang-2 levels were significantly increased in CM compared to uncomplicated malaria or healthy controls, and also in uncomplicated malaria compared to healthy controls (Figure 4.1A; Kruskal-Wallis: P<0.001). As an additional measure, the ratio of Ang-2 to Ang-1 for each patient was found to be significantly different between healthy controls and uncomplicated malaria patients (Figure 4.1A; Kruskal-Wallis: p<0.05) and between healthy controls or uncomplicated malaria and CM (p<0.001). To compare these novel biomarkers to an established biomarker of CM, serum TNF levels were also determined. TNF was significantly increased in CM compared to either uncomplicated malaria or healthy controls (Figure 4.1A; Kruskal-Wallis: p<0.001), however absolute levels of TNF were very low and required larger sample volumes to detect.
Figure 4.1. Comparison of Angiopoietin-1 and -2 with a current marker of cerebral malaria (TNF) in adult malaria patients from Thailand.

A. Serum concentrations of Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), the ratio of Angiopoietin 2 to 1 (RATIO, expressed as log base 10) and Tumour necrosis factor (TNF) were measured in 10 healthy controls (HC), 25 consecutive uncomplicated malaria (UM) patients, and in consecutive 25 cerebral malaria (CM) patients. B. Receiver operating characteristic curves (blue line) were generated for each test to compare CM with UM patients, with the null hypothesis (green line) that area under the curve equals 0.5.
The manifestations and outcomes of severe and CM may differ between adults and children and between varying genetic backgrounds of patient and parasite populations. Therefore the hypothesis that angiopoietin levels are informative biomarkers for CM was further examined in a larger cohort of African children. Similar to the observations in Thailand, serum Ang-1 levels were significantly decreased in Ugandan children with CM compared to uncomplicated malaria patients and healthy controls, and in uncomplicated malaria compared to healthy controls (Figure 4.2A; Kruskal-Wallis: p<0.001). Additionally, Ang-2 levels were significantly elevated in CM compared to uncomplicated malaria and healthy controls (Figure 4.2A; Kruskal-Wallis: p<0.001), and between uncomplicated malaria and healthy controls (p<0.01). Furthermore, as in the adult population, the Ang-2:Ang-1 ratio was significantly higher in CM than in healthy controls and uncomplicated malaria, and in uncomplicated malaria compared with healthy controls (Figure 4.2A; Kruskal-Wallis: p<0.001). While TNF levels were significantly lower in healthy controls compared to uncomplicated malaria and cerebral malaria patients (Figure 4.2A; Kruskal-Wallis: p<0.001), there was no significance difference differ between CM and uncomplicated malaria cases.
Figure 4.2. Comparison of Angiopoietins 1 and 2 with TNF in pediatric malaria patients from Uganda.

A. Serum concentrations of Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), the ratio of Angiopoietin 2 to 1 (RATIO, expressed as log base 10), and Tumour necrosis factor (TNF) were measured in 28 healthy controls (HC), 72 uncomplicated malaria (UM) patients, and in 69 cerebral malaria (CM) patients. B. Receiver operating characteristic curves (blue line) were generated for each test to compare CM with UM patients, with the null hypothesis (green line) that area under the curve equals 0.5.
Comparisons of the median and range of each serum biomarker concentration (Table 4.2), revealed no overlap in the ranges of Ang-1 and the Ang-2:Ang-1 ratio measures in the CM, uncomplicated malaria and healthy controls groups in Thai adults, indicating that these markers clearly discriminated the clinical malaria groups. However, there was some overlap in the concentration ranges in the Ugandan children with uncomplicated or CM.

Table 4.2. Biomarker levels in serum of healthy controls (HC), uncomplicated malaria patients (UM) and cerebral malaria patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>Adult (Thailand)</th>
<th>Pediatric (Uganda)</th>
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<tbody>
<tr>
<td></td>
<td>HC</td>
<td>UM</td>
</tr>
<tr>
<td>ANG-1 (ng/ml)</td>
<td>378 (151–946)</td>
<td>82.25 (27.3–379)</td>
</tr>
<tr>
<td>ANG-2 (ng/ml)</td>
<td>0.0089 (0.005–0.847)</td>
<td>1.84 (0.25–5.44)</td>
</tr>
<tr>
<td>Ratio (ANG-2/ANG-1)</td>
<td>0.00003 (0.00001–0.00021)</td>
<td>0.017 (0.03–0.44)</td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>0 (0–8)</td>
<td>0 (0–44.8)</td>
</tr>
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Values are presented as median (range).

4.3.2 Angiopoietin biomarkers do not correlate with peripheral parasite burden

Although higher parasite burdens are generally associated with an increased risk of severe or CM, these complications can occur in individuals with relatively low parasitemias. In the Thai population, patients with CM had significantly higher parasitemias than in uncomplicated malaria patients. However, this was not the case in Ugandan children. Arguably, increased serum cytokine levels may reflect the immune response to increased parasite burdens, rather than being indicative of a clinical syndrome such as CM. To address this, the correlation between biomarker levels and parasitemia was determined. In both patient populations, Ang-1, Ang-2 and the Ang-2:Ang-1 ratio show poor correlations with parasitemia in both uncomplicated and CM (Table 4.3). Conversely, TNF was positively correlated with parasitemia, especially in the Ugandan population (Table 4.3: r²=0.69 (uncomplicated malaria) and r²=0.36 (CM)), suggesting that TNF levels may relate more to parasitemia than to a clinical syndrome.
4.3.3 Receiver operating characteristic (ROC) curves indicate that angiopoietin levels discriminate between uncomplicated and cerebral malaria patients

The receiver operating characteristic (ROC) curves for the biomarkers, examining CM patients as “cases” and uncomplicated malaria patients as “controls”, were plotted and compared to assess the ability each marker to discriminate between patients with and without cerebral complications (Figure 4.1B, Figure 4.2B, Table 4.4).

Table 4.4. Test performance characteristics. Optimal cut-off values (95% CI) for each test and sensitivity (SEN), specificity (SPEC), positive likelihood ratio (LR(+)) and negative likelihood ratio (LR(−)) at the chosen cut-off value comparing uncomplicated malaria with cerebral malaria patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>Adult (Thailand)</th>
<th>Pediatric (Uganda)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cut-off</td>
<td>SEN</td>
</tr>
<tr>
<td></td>
<td>(ng/ml)</td>
<td></td>
</tr>
<tr>
<td>ANG-1</td>
<td>21.26</td>
<td>1 (0.87–1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.52–0.86)</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td>(1.8–11)*</td>
<td>(0.00–0.02)</td>
</tr>
<tr>
<td>ANG-2</td>
<td>3.04</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>(0.52–0.86)</td>
<td>(0.65–0.94)</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio</td>
<td>0.131</td>
<td>1 (0.87–1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.52–0.86)</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>1.46</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>(0.57–0.89)</td>
<td>(0.70–0.96)</td>
</tr>
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</tbody>
</table>

*significantly different from 1 (p<0.05).
In the Thai population, Ang-1 and the Ang-2:Ang-1 ratio have an area under the curve (AUC) of 1 (Figure 4.1B, Table 4.3) and differ significantly (p<0.001) from that of a chance result (AUC: 0.5). Therefore, in this population, Ang-1 levels and the Ang-2:Ang-1 ratio allows perfect discrimination between the CM and uncomplicated cases. While none of the biomarkers tested perfectly differentiated cerebral from uncomplicated malaria in the Ugandan pediatric population, Ang-1 (AUC: 0.795) and the ratio of Ang-2 to Ang-1 (AUC: 0.782) were still the best of the biomarkers examined (Figure 4.2B, Table 4.3; data not shown). Although Ang-2 did not have such large AUC values, it shows moderate accuracy as a discriminatory marker in both populations (Figure 4.1B - Thai: AUC=0.835, p<0.001; Figure 4.2B - Uganda: AUC=0.672, p<0.001).

Compared to Ang-1 and Ang-2 as biomarkers of CM, previously studied markers of severe and CM such as TNF (Figure 4.1B, Figure 4.2B, Table 4.3; data not shown) had moderate accuracy as a discriminating test (Figure 4.1B, AUC: 0.834, p<0.001) in Thai adults; however, TNF was a poor discriminator between CM and uncomplicated malaria in the Ugandan pediatric population (Figure 4.2B, AUC: 0.532, p=0.544).

**4.3.4 Ang-1 shows high sensitivity and specificity as a biomarker of cerebral malaria**

For each of the tests, a cut-off value to discriminate between CM uncomplicated malaria cases, was derived from the ROC curve (Table 4.5). Using this value, the data were re-examined to determine sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). In the Thai patients, Ang-1 had a sensitivity and specificity of 1, indicating that these tests correctly identified cerebral cases 100% of the time and equally correctly identified uncomplicated cases. Ang-2 and TNF had similar specificities (0.84 and 0.88, respectively), although showed much lower sensitivity than the other tests (Ang-2: 0.72, TNF: 0.76).

In African children, Ang-1 were again superior biomarkers of CM with sensitivity values of 0.7 and 0.72 respectively (Table 4.5). The specificity of Ang-1 as a diagnostic test was 0.756 As
with the Thai patients, both Ang-2 and TNF, despite having higher sensitivities (0.83 and 0.85 respectively), had lower specificity values (0.57 and 0.2 respectively).

**Table 4.5.** Results of a multivariate logistic regression model to predict CM (versus UM) in two diverse patient populations

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Adjusted OR (95%CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand</td>
<td>1.0*</td>
<td></td>
</tr>
<tr>
<td>Uganda</td>
<td>0.36 (0.029–4.7)</td>
<td>0.44</td>
</tr>
<tr>
<td>Age</td>
<td>0.96 (0.86–1.1)</td>
<td>0.53</td>
</tr>
<tr>
<td>Parasitemia (parasites/µL)</td>
<td>1.00 (1.00–1.00)</td>
<td>0.20</td>
</tr>
<tr>
<td>ANG-1 (ng/mL)</td>
<td>0.899 (0.864–0.934)**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ANG-2 (ng/mL)</td>
<td>1.10 (0.944–1.28)</td>
<td>0.22</td>
</tr>
<tr>
<td>Ratio (ANG-2/ANG-1)</td>
<td>1.01 (0.932–1.09)</td>
<td>0.82</td>
</tr>
<tr>
<td>TNF (pg/mL)</td>
<td>1.00 (0.994–1.003)</td>
<td>0.91</td>
</tr>
</tbody>
</table>

*baseline comparator group.
**Adjusted odds ratio represents the incremental odds of CM for every unit increase (1 ng/mL) in the ANG-1 level

4.3.5 Angiopoietin-1 levels and the angiopoietin-2/angiopoietin-1 ratio predict survival in African children with cerebral malaria

Mortality data were available for the Ugandan pediatric cohort. We examined angiopoietin levels at presentation and subsequent survival in children with CM and observed that Ang-1 levels and the ratio of Ang-2:Ang-1 were related to mortality. Higher Ang-1 levels at presentation were associated with protection from fatal CM (p=0.01; Figure 4.3) whereas Ang-2:Ang-1 ratios were higher in those who subsequently died of CM (median (range): non-fatal CM 0.13 (0.01 to 82) versus fatal CM 2.6 (1.4 to 13), p =0.013). No patients died in the Thai cohort.
Figure 4.3. Angiopoietins 1 levels are associated with outcome in pediatric cerebral malaria patients from Uganda.
Serum concentrations of Angiopoietin-1 (Ang-1) were measured in 69 cerebral malaria (CM) patients at presentation and compared to outcome. Higher Ang-1 levels at presentation were associated with protection from fatal cerebral malaria. *p=0.01, CM survivors versus CM fatalities (Wilcoxon rank-sum test). 1 = CM survivors. 2 = CM fatalities.
4.4 Discussion

This study provides the first evidence implicating dysregulation of angiopoietins in the pathogenesis of cerebral malaria, suggesting they may be informative diagnostic and prognostic biomarkers for this syndrome. Since the manifestations of severe malaria may differ between children and adults and in varying backgrounds, we measured serum angiopoietin levels in two geographically and genetically diverse patient and parasite populations and demonstrate that these endothelial regulators were highly accurate discriminators of cerebral malaria versus uncomplicated malaria in both settings. In both adults from Thailand (Figure 4.1, Table 4.2, Table 4.3) and children from Uganda (Figure 4.2, Table 4.2, Table 4.3) low Ang-1 levels or increased Ang-2:Ang-1 ratios were shown to be accurate biomarkers of cerebral malaria and superior to TNF. Furthermore Ang-1 levels and the Ang-2:Ang-1 ratios predicted survival in African children with cerebral malaria (Figure 4.3).

No laboratory tests are currently available to definitively confirm a diagnosis of cerebral malaria. Severe and cerebral malaria are frequently misdiagnosed contributing to the inappropriate treatment of other serious diseases and resulting in increased adverse outcomes (Reyburn BMJ 2004). The ability to accurately determine the presence of, or risk for progression to cerebral malaria would be of potential benefit in patient triage, appropriate management and efficient resource allocation. An ideal biomarker for cerebral malaria might be expected to meet a number of logistical, diagnostic, prognostic and therapeutic criteria. These include: the capacity to be easily and reproducibly measured in a readily available specimen such as serum or whole blood by a standardized assay that requires limited specialized equipment and can be performed with minimal training; the reliable detection of cerebral malaria with high sensitivity and specificity; predicting those at increased risk of progression from uncomplicated to severe disease; the detection of determinants likely to be involved in the underlying pathogenesis of the disorder, rather than bystander reactions/epiphenomena, and therefore providing a readout of the significance of the underlying disease process as well as representing potential therapeutic targets for intervention.

Despite the growing realization that cerebral malaria is a complex multisystem disorder with heterogenous manifestations, our data suggest that angiopoietins meet several of these criteria
and may represent clinically useful biomarkers for this syndrome. Angiopoietins appear to be robust and accessible targets, readily detectable by standard immunoassays in serum or whole blood even if stored for extended periods (up to years; data not shown). ROC curve analysis in both African pediatric and Thai adult populations indicated they were highly accurate tests for the detection of cerebral malaria and its discrimination from uncomplicated disease (areas under the curve of 0.795 to 1.0; Figure 4.1, Figure 4.2; Table 4.2), and superior to current markers such as TNF (Figure 4.1, Figure 4.2; Table 4.2) and sICAM-1 (data not shown). In this study, serum TNF levels were positively correlated with parasitemia suggesting higher levels may relate more to parasitemia than to a clinical syndrome such as CM. This may be an important distinction given the growing body of evidence supporting an essential role for host-mediated immunopathology and tissue injury in the pathogenesis of CM (reviewed in (Hunt, Golenser et al. 2006)).

Our observations that angiopoietins are dysregulated in patients with cerebral malaria, supports the hypothesis that they may be involved in the pathogenesis of this syndrome. As key regulators of endothelial integrity, there are several mechanisms by which angiopoietins may contribute to parasite sequestration to CAMs, dysregulated inflammation, and endothelial cell and BBB dysfunction that characterize cerebral malaria (reviewed in (Hunt, Golenser et al. 2006)). The endothelium is a large and continuous vascular organ whose state of activation is dependent upon the angiopoietin-Tie2 system. ANG-1 maintains endothelial quiescence and intact tight junctions important for preventing vascular permeability especially across the BBB. ANG-2, stored in endothelial cell granules, may be rapidly released resulting in endothelial activation, augmented inflammation, loosening of endothelial cell junctional complexes, and upregulation of cerebral endothelial adhesiso molecules such as ICAM-1 to which parasitised erythrocytes adhere. Increases in BBB permeability have been proposed to be one of the earliest events in the pathogenesis of cerebral malaria therefore dysregulation of angiopoietins, as occurs when ANG-2 levels rise and Ang-1 levels fall, may reflect one of the pivotal and initiating events in the syndrome (Hunt, Golenser et al. 2006).

It will be important to dissect the putative mechanisms by which angiopoietins may contribute or augment malaria pathogenesis in animal models where endothelial and BBB dysfunction and vascular leak are central features of disease (Medana and Turner 2006). If confirmed by
additional studies in humans and clinically relevant animal models, advanced therapies to preserve regulated angiogenic responses, for example by delivering recombinant Ang-1 to restore endothelial cell quiescence, can be examined to determine if they offer clinical benefit as they have in other models of life-threatening infectious disease (Mei, McCarter et al. 2007). It will also be of interest to determine if Ang-2/Ang-1 imbalance will predict outcome in other severe infectious and inflammatory disease states that impact vascular integrity and permeability such as dengue and other viral hemorrhagic fevers, rickettsial infections, toxic shock syndrome and sepsis.

Limitations of our study include relatively small sample sizes particularly in Southeast Asia. The sensitivity and specificity of Ang-1 levels and the Ang-2/Ang-1 ratio for the diagnosis of cerebral malaria was 100% in the Thai population and somewhat lower in the Ugandan pediatric cohort. While the reasons for these differences are not entirely clear, the lack of a reference standard for the definitive diagnosis of cerebral malaria, leading to misdiagnosis particularly in African children (Taylor, Fu et al. 2004) may account for some misclassification in this population and may be, in part, an explanation for these differences. It will be important to confirm and extend our observations and further assess performance and specificity in larger prospective clinical trials, especially those using malarial retinopathy with indirect ophthalmoscopy and autopsy studies with histopathologically confirmed cases of cerebral malaria (Beare, Southern et al. 2004; Taylor, Fu et al. 2004). An additional limitation is that we only focused on the utility of angiopoietin levels in the diagnosis and outcome of cerebral malaria and therefore future studies are required to assess the value of serum angiopoietin levels in predicting individuals at risk of progression from uncomplicated infection to severe and cerebral malaria. With respect to the specificity of angiopoietins for CM versus other life-threatening infections, it is important to note that biomarkers such as angiopoietins are more likely to provide clinically relevant information pertaining to the mechanism and severity of the underlying disease process and the need for critical care triage/referral, and are not expected to be pathogen-specific. Therefore they will be expected to complement, rather than replace, conventional pathogen diagnosis (for example, microscopy, HRPII or pLDH detection for malaria) and enhance triage and clinical management.
In summary, these data suggest that the dysregulation of angiogenic factors may be involved in the pathogenesis of cerebral malaria and that serum angiopoietin levels are accurate biomarkers to discriminate cerebral malaria from uncomplicated disease and predict survival in African children with cerebral involvement. Additional prospective studies will be required to further define diagnostic cut-off values, and assess their value in predicting progression and outcome of severe or cerebral disease.
Chapter 5
Endothelium-based biomarkers are associated with cerebral malaria in Malawian children

5.1 Abstract

Background

Differentiating cerebral malaria (CM) from other causes of serious illness in African children is problematic, owing to the non-specific nature of the clinical presentation and the high prevalence of incidental parasitaemia. CM is associated with microvascular endothelial activation and dysfunction. In this study we tested the hypothesis that endothelium-derived biomarkers are associated with the pathophysiology of severe malaria and may help identify children with CM.

Methods and Findings

We tested plasma samples from febrile children who had been prospectively recruited with uncomplicated malaria (UM; n=32), cerebral malaria with retinopathy (CM-R; defined by the presence of coma and malaria retinopathy; n=38), clinically defined CM without retinopathy (CM-N; n=29), or non-malaria febrile illness with decreased consciousness (CNS; n=24). We determined admission and follow-up levels of angiopoietin (Ang) -1, Ang-2, sTie-2, von Willebrand factor (VWF) and its propeptide (VWFpp), VEGF, sICAM-1 and IP-10. We generated receiver operator characteristic (ROC) curves to assess the diagnostic accuracy of individual biomarkers.

Compared to clinically defined CM without retinopathy, children with CM-R had significantly higher median levels of Ang-2, Ang-2:Ang-1, sTie-2, VWFpp and sICAM-1. Compared to children with UM, CM-R cases had a significantly lower median level of Ang-1 and significantly higher median concentrations of Ang-2, Ang-2:Ang-1, sTie-2, VWF, VWFpp, VEGF and sICAM-1. Compared to febrile children with altered consciousness due to other causes (CNS), children with CM-R had a significantly lower median level of Ang-1 and higher median levels of VWF and VWFpp. Ang-1 was the best discriminator between UM and CM-R and between CNS and CM-R with areas under the ROC curve (AUROC) of 0.96 (95% CI: 0.93-1.0) and 0.93 (95% CI: 0.88-0.99), respectively (p<0.0001 for each). The ratio of Ang-2:Ang-1 had the same
AUROC but an improved positive likelihood ratio (UM vs. CM-R: 7.1 for Ang-1 vs. 18 for Ang-2:Ang-1; CNS vs. CM-R: 6.7 for Ang-1 vs. 13 for Ang-2:Ang-1). A comparison of biomarker levels in CM-R between admission and recovery showed a significant and uniform increase in Ang-1 levels and a decrease in the Ang-2/Ang-1 ratio, suggesting that these biomarkers may have utility in monitoring clinical response.

Conclusions

These results indicate that endothelium-based proteins are clinically informative biomarkers of malarial disease severity. These results require validation in larger prospective studies to confirm that this group of biomarkers consistently improves diagnostic accuracy of CM from similar conditions causing fever and altered consciousness.
5.2 Introduction

Differentiating cerebral malaria (CM) from other conditions causing fever and altered consciousness is a clinical challenge, owing to the non-specific clinical presentations of CM (fever, coma, convulsions) and the high prevalence of incidental parasitaemia in malaria-endemic areas (Taylor, Fu et al. 2004). In a study of African children diagnosed with CM, approximately one quarter were shown to have alternative causes for their neurological syndrome at post-mortem examination (Taylor, Fu et al. 2004). These findings indicate that CM is over-diagnosed, a situation that is likely to have serious consequences for children in whom other treatable or life-threatening conditions are not identified (World Health Organization 2000; Taylor, Fu et al. 2004). There is a clear need for a diagnostic test that could distinguish CM from other conditions causing encephalopathy in malaria-endemic areas. In comatose African children, a distinctive retinopathy consisting of haemorrhages, patchy retinal whitening and vessel changes is strongly associated with malaria being the only identifiable cause of death (Taylor, Fu et al. 2004).

Features of severe *P. falciparum* malaria include the adhesion of mature parasitized erythrocytes to the microvasculature of vital organs and acute endothelial activation (reviewed in (Medana and Turner 2006); (Jakobsen, Morris-Jones et al. 1994)). Exocytosis of Weibel-Palade bodies (WPBs) occurs in association with endothelial activation and the products of WPBs have been identified as biomarkers of malarial disease severity (Holest elle, Donkor et al. 2006; Yeo, Lampah et al. 2008; Larkin, de Laat et al. 2009). WPBs release bioactive products, including von Willebrand factor (VWF), its propeptide (VWFpp), and angiopoietin-2 (Ang-2) into the systemic circulation. Together with vascular endothelial growth factor (VEGF), the angiogenic factors angiopoietin-1 (Ang-1) and Ang-2, are major regulators of vascular inflammatory response, endothelial activation and endothelial integrity (Fiedler and Augustin 2006; Findley, Cudmore et al. 2007). Ang-1 is constitutively released from perivascular cells including pericytes and smooth muscle cells and signals through the Tie-2 receptor to maintain vascular quiescence and stability. Ang-2 antagonizes Ang-1 function resulting in endothelial activation and increased vascular permeability. Ang-2 sensitizes the endothelium to sub-threshold levels of tumour necrosis factor, resulting in increased expression of adhesion molecules such as ICAM-1 to which parasitized erythrocytes bind (Fiedler, Reiss et al. 2006). VEGF induces WPB exocytosis, mediates Tie-2
shedding and regulates Ang-1 and Ang-2 function (Lobov, Brooks et al. 2002; Findley, Cudmore et al. 2007). WPBs are also an important source of VWF, particularly ultralarge multimers (ULVWF) that are considered biologically hyperactive with respect to their enhanced binding avidity for collagen and platelets (Sadler 1998). Severe malaria has been associated with increased levels of VWF and ULVWF multimers and decreased levels of the regulatory VWF-specific cleaving protease ADAMTS13 (A disintegrin and metalloprotease with thrombospondin type-1 repeats) (Larkin, de Laat et al. 2009). ICAM-1 is a receptor for the cytoadherence of mature parasitized erythrocytes in the cerebral microvasculature and its soluble form (sICAM-1) has been used as a marker of endothelial activation and severe malaria (Jakobsen, Morris-Jones et al. 1994; Turner, Morrison et al. 1994; Tchinda, Tadem et al. 2007). In addition to the molecular markers and regulators of endothelial quiescence and activation, IP-10, an interferon-gamma inducible chemokine involved in recruitment of activated Th1 cells, has been reported as a biomarker of fatal CM in studies from India and Ghana (Armah, Wilson et al. 2007; Jain, Armah et al. 2008).

Reliable diagnostic and prognostic biomarkers for CM and other forms of severe malaria may improve clinical management, resource allocation and outcome of serious childhood illness. The aim of this study was to evaluate the ability of endothelial biomarkers to discriminate between different clinical disease states in malaria and other conditions associated with fever and altered consciousness in Malawian children. We show that endothelium-based proteins are informative biomarkers of disease severity and clinical response and that a panel of biomarkers can discriminate between retinopathy positive CM and uncomplicated malaria or other CNS infections with a high degree of accuracy. Further, we demonstrate that a distinctive set of endothelium-based proteins is associated with retinopathy in a group of children with coma and parasitaemia.

5.3 Methods

5.3.1 Ethics Statement

Ethical approval for this study was granted from The College of Medicine Research Committee in Blantyre, Malawi (COMREC) and all parents or guardians gave written informed consent for children to enter the study.
5.3.2 Study Population

Children between 6 months and 14 years of age presenting with fever to the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi were prospectively enrolled between 1997 and 2009. Admission lithium heparin plasma samples were obtained from children after their parents or guardians had given their informed consent. The samples tested represented a subset of samples collected from larger prospective case-control studies of the pathogenesis and management of CM and central nervous system infections (Taylor, Fu et al. 2004; Beare, Taylor et al. 2006; Lewallen, Bronzan et al. 2008). Clinical and demographic data were collected from cases and controls at the time of blood collection, and all subsequent analyses were carried out blind to these details. All participants received standard treatment, including antimalarial and/or antibacterial therapy as indicated, according to Malawian National guidelines.

5.3.3 Ophthalmological Examination

After admission of a child with altered consciousness, the patient’s pupils were dilated by application of drops (tropicamide and phenylephrine) and the fundi were examined by direct and indirect ophthalmoscopy. The findings of an ophthalmologist or experienced clinician were recorded on standardized forms. Retinopathy was defined by the presence of any one or more of the following retinal findings: white-centred haemorrhages, retinal whitening, or vessel changes, with or without papilloedema, as previously described (Beare, Taylor et al. 2006; Lewallen, Bronzan et al. 2008). Papilloedema alone did not constitute retinopathy.

5.3.4 Definitions of clinical syndromes

_Cerebral Malaria (CM)_

Children meeting the case definition for CM (World Health Organization 2000; Taylor, Fu et al. 2004) including _P. falciparum_ asexual parasitaemia, a Blantyre coma score \( \leq 2 \) with no improvement following correction of hypoglycemia, and no evidence of an alternative cause for coma including meningitis on examination of cerebrospinal fluid, were eligible for enrolment. Children’s fundi were examined and they were classified as retinopathy positive (CM-R) or retinopathy negative (CM-N). CM-R children were considered to be confirmed CM and were
used for all analyses comparing clinical groups. Paired admission and 28 day convalescence plasma samples were collected for each surviving child available for follow-up.

*Uncomplicated Malaria (UM)*

Children were included in the UM group if they presented to the outpatient clinic at Queen Elizabeth Central Hospital, Blantyre, Malawi with febrile illness and a blood film positive for asexual *P. falciparum* without another explanation for fever, and with no malarial complications.

*CNS controls*

Children (aged 1 month to 10 years) with non-malarial fever and altered consciousness were included in the study as a further comparator group. Samples were taken from a study looking at suspected central nervous system (CNS) infections (unpublished data). Children with fever or history of fever, a negative malarial smear and at least one of the following: reduced level of consciousness, Blantyre coma score (BCS) ≤4, neck stiffness, photophobia, Kernig’s sign, tense fontanelle, focal neurological signs, convulsions, or unexplained irritability in infants, were eligible for enrolment.

### 5.3.5 Quantification of Biomarkers

Plasma concentrations of biomarkers Ang-1, Ang-2, sTie-2, VEGF-A, IP-10 and sICAM-1 (DuoSets, R&D Systems, Minneapolis, MN), von Willebrand factor (VWF [capture REF P0226, detection REF A0082]: DAKO, Denmark A/S) and von Willebrand factor propeptide (VWFpp [capture CLB-Pro35, detection CLB-Pro 14.3 HRP conjugated]: Sanquin, Netherlands) were measured by ELISA as follows. According to the manufacturer’s instructions, capture antibodies were diluted in PBS (Gibco) overnight at 4°C and were washed with PBS 0.05% Tween 20 (Sigma) five times and blocked for a minimum of 2 hours in PBS 1% BSA (reagent diluent). The samples were then diluted in reagent diluent and standard curves were generated using recombinant proteins (R&D Systems). Normal plasma from a pool of 40 adult donors served as a standard for VWFpp and VWF. The plasma pool contained 5.5 nM of VWFpp and 49 nM of VWF. Samples were incubated overnight at 4°C, washed five times and detection antibodies were added according to manufacturer recommended dilutions for 2 hours at room temperature (RT). For Ang-1 and Ang-2, the detection antibodies were resuspended one hour prior to use.
with 2% heat inactivated goat or mouse serum respectively. Following wash steps (7x), VWF and VWFpp were developed using TMB (eBioscience) and the reaction was stopped using 2N H$_2$SO$_4$. The plate was read at 450nM (Dynex Technologies Opsys MR plate reader) and concentrations were extrapolated from the standard curve (4-PL) using revelation Quicklink software (v4.04). The ELISA assays from R&D systems were washed (7x) and Extravidin-Alkaline phosphatase (AP) (Sigma) was added 1:1000 to each well for 1 hour at RT. The plates were then washed a final time (7x in PBS 0.05% Tween 20 and 2x in deionized water) before adding the substrate p-nitrophenyl phosphate (pNPP) (Sigma). The plates were read at 405nM and concentrations were extrapolated as above.

5.3.6 Statistics

Data were analyzed in GraphPad Prism v5.0 and SPSS v16.0. All analyses were non-parametric with Spearman’s correlation for two-way correlations between biomarkers, Mann-Whitney U tests to compare biomarkers between groups with Holms correction for multiple testing, and receiver operator characteristic (ROC) curves to assess the diagnostic accuracy of the tests. Optimal test thresholds were derived mathematically from the ROC curves using the point on the ROC curve with the lowest value for the formula: (1- sensitivity)$^2$ + (1-specificity)$^2$. Wilcoxon matched pairs test was used to compare biomarker levels measured at admission and convalescence.

5.4 Results

5.4.1 Patient Characteristics

A total of 123 febrile children with either UM (n=32), suspected CNS infections (n=24), or CM (n=67) were included in the study. Of the children that met study criteria for CM, 38 were retinopathy positive and were classified as retinopathy-validated CM (CM-R); whereas the other 29 had normal ocular fundi (CM-N) (Lewallen, Bronzan et al. 2008). Demographic and clinical data for these children are shown in Table 5.1.
Endothelial biomarkers differentiate retinopathy positive CM cases from those without retinopathy

Since retinopathy has been established as a discriminant tool in the diagnosis of CM, we examined biomarker levels in children with CM and malaria retinopathy (CM-R) and compared them to children with clinical CM without retinopathy (CM-N). Individually, Ang-2, Ang-2:Ang-1, sTie-2, VWFpp and sICAM-1 were significantly associated with retinopathy (Figure 5.1, Table 5.2). For each analyte tested, a receiver operator characteristic (ROC) curve was generated to assess the diagnostic accuracy of the biomarker to discriminate between CM-N and CM-R. The area under the ROC (AUROC) curve was computed and the sensitivity, specificity and positive and negative likelihood ratios were calculated at the optimal biomarker cut-off. sTie-2 and Ang-2 were the best individual predictors of retinopathy with AUROCs of 0.83 (95% CI: 0.73-0.93) and 0.77 (95% CI: 0.65-0.89) respectively.
Figure 5.1. Endothelial biomarkers are associated with retinopathy (CM-R) in children with clinically defined cerebral malaria.

Graphs showing the median and scatter of plasma biomarkers (A) Ang-2 (ng/mL), (B) Ang-1 (ng/mL), (C) Ang-2:Ang-1, (D) VWF propeptide (VWFpp, mmol/L), (E) VWF (mmol/L), (F) sTie-2 (ng/mL), (G) sICAM-1 (ng/mL), (H) VEGF (ng/mL) and (I) IP-10 (ng/mL) levels in retinopathy negative cerebral malaria (CM-N) or retinopathy positive cerebral malaria (CM-R) as measured by ELISA (Mann-Whitney, *p<0.05 after Holms correction for multiple comparisons (9 pair-wise comparisons). The limit of detection is represented by a dotted line (VEGF).
Table 5.2. Endothelial biomarkers are associated with retinopathy in a cohort of children with cerebral malaria (CM).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>AUROC CM-R vs CM-N</th>
<th>p-value</th>
<th>Cutoff</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>Positive likelihood ratio (95% CI)</th>
<th>Negative likelihood ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1</td>
<td>0.64 (0.51–0.78)</td>
<td>0.056</td>
<td>3.2</td>
<td>68 (53–81)</td>
<td>44 (28–62)</td>
<td>1.2 (0.84–1.8)</td>
<td>0.70 (0.38–1.3)</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.77 (0.66–0.88)</td>
<td>&lt;0.0001*</td>
<td>6.2</td>
<td>71 (55–83)</td>
<td>72 (54–85)</td>
<td>2.6 (1.4–4.8)</td>
<td>0.40 (0.23–0.69)</td>
</tr>
<tr>
<td>Ang-2:Ang-1</td>
<td>0.74 (0.66–0.87)</td>
<td>0.001*</td>
<td>2.0</td>
<td>74 (56–85)</td>
<td>66 (47–80)</td>
<td>2.1 (1.3–3.7)</td>
<td>0.40 (0.22–0.73)</td>
</tr>
<tr>
<td>sTie-2</td>
<td>0.83 (0.73–0.93)</td>
<td>&lt;0.0001*</td>
<td>56</td>
<td>74 (58–85)</td>
<td>72 (54–85)</td>
<td>2.7 (1.4–5.0)</td>
<td>0.35 (0.20–0.65)</td>
</tr>
<tr>
<td>VWF propeptide</td>
<td>0.71 (0.58–0.83)</td>
<td>0.005</td>
<td>51</td>
<td>76 (61–87)</td>
<td>62 (44–77)</td>
<td>2.0 (1.3–3.2)</td>
<td>0.38 (0.20–0.73)</td>
</tr>
<tr>
<td>VWF</td>
<td>0.58 (0.42–0.73)</td>
<td>0.305</td>
<td>310</td>
<td>68 (53–81)</td>
<td>58 (41–74)</td>
<td>1.7 (1.0–2.7)</td>
<td>0.54 (0.31–0.94)</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>0.73 (0.59–0.86)</td>
<td>0.008</td>
<td>100</td>
<td>71 (55–83)</td>
<td>69 (51–83)</td>
<td>2.3 (1.3–4.1)</td>
<td>0.42 (0.24–0.73)</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.65 (0.52–0.79)</td>
<td>0.040</td>
<td>0.25</td>
<td>74 (58–85)</td>
<td>58 (39–74)</td>
<td>1.7 (1.1–2.8)</td>
<td>0.46 (0.24–0.85)</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.58 (0.43–0.73)</td>
<td>0.312</td>
<td>0.71</td>
<td>66 (50–79)</td>
<td>54 (36–70)</td>
<td>1.4 (0.90–2.2)</td>
<td>0.64 (0.37–1.1)</td>
</tr>
</tbody>
</table>

Median (range). Mann-Whitney U test. *p<0.05 after Holm correction for 9 pair-wise comparisons.
CM-R: Cerebral malaria, retinopathy positive.
CM-N: Cerebral malaria, retinopathy negative.
AUROC: Area under the receiver operating characteristic curve.

5.4.3 Endothelial biomarkers differ between CM and UM

We compared admission levels of plasma biomarkers in children with CM-R (n=38) to children with UM (n=32). The median concentration of Ang-1 was significantly lower, and median levels of Ang-2:Ang-1, sTie-2, VWFpp, VWF, sICAM, VEGF were significantly higher in patients with CM-R than in patients with UM (Figure 5.2, Table 5.3).
Figure 5.2. Endothelial biomarkers differentiate retinopathy validated cerebral malaria (CM-R) from uncomplicated malaria (UM) and children with fever and decreased consciousness due to other causes (CNS).

Graphs showing the median and scatter of plasma biomarkers (A) Ang-2 (ng/mL), (B) Ang-1 (ng/mL), (C) Ang-2:Ang-1, (D) VWF propeptide (VWFpp, mmol/L), (E) VWF (mmol/L), (F) sTie-2 (ng/mL), (G) sICAM-1 (ng/mL), (H) VEGF (ng/mL) and (I) IP-10 (ng/mL) levels in UM, non-malarial febrile illness with decreased consciousness (CNS) or retinopathy validated cerebral malaria (CM-R) as measured by ELISA (Mann-Whitney, *p<0.05 after Holms correction for multiple comparisons (9 pair-wise comparisons). The limit of detection is represented by a dotted line (VEGF).
Table 5.3. Biomarker levels in Malawian children with uncomplicated malaria (UM), retinopathy confirmed cerebral malaria (CM-R) or non-malarial fever with altered consciousness (CNS).

<table>
<thead>
<tr>
<th></th>
<th>UM (n = 32)</th>
<th>CM-R (n = 38)</th>
<th>CNS (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1, ng/mL</td>
<td>14 (4.2–132)**</td>
<td>3.1 (0.94–9.2)</td>
<td>10 (3.1–69)**</td>
</tr>
<tr>
<td>Ang-2, ng/mL</td>
<td>5.2 (2.0–41)</td>
<td>7.6 (3.1–34)</td>
<td>5.08 (1.67–14.29)*</td>
</tr>
<tr>
<td>Ang-2:Ang-1</td>
<td>0.39 (0.04–2.6)**</td>
<td>2.3 (0.75–6.6)</td>
<td>0.34 (0.03–2.5)**</td>
</tr>
<tr>
<td>sTie-2, ng/mL</td>
<td>48 (32–84)**</td>
<td>66 (42–95)</td>
<td>67 (43–124)</td>
</tr>
<tr>
<td>VWFpp, nmol/L</td>
<td>32 (10–53)**</td>
<td>70 (13–1127)</td>
<td>23 (8.1–106)**</td>
</tr>
<tr>
<td>VWF, nmol/L</td>
<td>144 (23–301)**</td>
<td>357 (157–654)</td>
<td>169 (73–524)**</td>
</tr>
<tr>
<td>sICAM-1, ng/mL</td>
<td>660 (447–1761)**</td>
<td>1154 (718–2073)</td>
<td>1016 (675–4358)</td>
</tr>
<tr>
<td>VEGF, ng/mL</td>
<td>0.17 (0.02–9.1)*</td>
<td>0.55 (0.02–6.4)</td>
<td>0.46 (0.02–2.9)</td>
</tr>
<tr>
<td>IP-10, ng/mL</td>
<td>0.60 (0.05–8.3)</td>
<td>1.0 (0.13–8.1)</td>
<td>0.69 (0.02–2.6)</td>
</tr>
</tbody>
</table>

Median (range);
*p<0.05;
**p<0.01 for difference compared to CM-R (corrected for multiple testing at 18 pair-wise comparisons using Holm’s test).

ROC curves were generated to assess the diagnostic accuracy of the biomarkers to discriminate between UM and CM-R. The AUROC curve was computed and the sensitivity, specificity and positive and negative likelihood ratios were calculated at the optimal biomarker cut-off (Table 5.4). Ang-1, sTie-2, VWFpp, VWF, ICAM, VEGF were each able to differentiate between UM and CM-R whereas IP-10 was not. Although Ang-2 on its own was no longer significant after correcting for multiple comparisons, the Ang-2:Ang-1 ratio had an AUROC as good as Ang-1 and resulted in an improved positive likelihood ratio versus Ang-1 alone [Ang-1 LR(+) = 7.1 compared to Ang-2:Ang-1 LR(+) = 18].
5.4.4 Distinct biomarker profiles in CM differ between retinopathy validated CM and other causes of fever and altered mental status

Distinguishing CM-R from other causes of fever and altered level of consciousness is clinically challenging yet critical for instituting timely, specific, and potentially life-saving treatment. We hypothesized that CM-R is associated with a characteristic pattern of endothelial biomarker abnormalities, which may be clinically informative in distinguishing CM-R from other causes of fever and altered consciousness. Comparing children with CM-R (n=38) to a control group of children admitted with non-malarial febrile illness and altered level of consciousness (CNS) (n=24), median Ang-1 levels were lower, and median Ang-2, Ang-2:Ang-1, VWFpp and VWF higher in children with CM-R compared to CNS controls (Figure 5.2, Table 5.3).

Table 5.4. Receiver operating characteristic curves of endothelial biomarkers in children with uncomplicated malaria (UM) or cerebral malaria with retinopathy (CM-R)

<table>
<thead>
<tr>
<th></th>
<th>AUROC CM vs. CM-R (95% CI)</th>
<th>p-value</th>
<th>Cutoff (95%CI)</th>
<th>Sensitivity (95%CI)</th>
<th>Specificity (95%CI)</th>
<th>Positive likelihood ratio (95%CI)</th>
<th>Negative likelihood ratio (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1</td>
<td>0.96 (0.93–1.0)</td>
<td>&lt;0.0001*</td>
<td>5.3</td>
<td>54 (0.0–98)</td>
<td>87 (73–94)</td>
<td>7.1 (3.1–15)</td>
<td>0.272 (0.039–2.28)</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.65 (0.51–0.79)</td>
<td>0.0059</td>
<td>6.1</td>
<td>63 (45–77)</td>
<td>71 (55–83)</td>
<td>2.2 (1.2–3.8)</td>
<td>0.53 (0.32–0.96)</td>
</tr>
<tr>
<td>Ang-2: Ang-1</td>
<td>0.96 (0.93–1.0)</td>
<td>&lt;0.0001*</td>
<td>0.95</td>
<td>94 (60–98)</td>
<td>95 (83–99)</td>
<td>18 (6.6–69)</td>
<td>0.096 (0.017–0.25)</td>
</tr>
<tr>
<td>sTie-2</td>
<td>0.82 (0.72–0.92)</td>
<td>&lt;0.0001*</td>
<td>56</td>
<td>78 (51–89)</td>
<td>74 (59–85)</td>
<td>3.0 (1.7–5.2)</td>
<td>0.30 (0.15–0.59)</td>
</tr>
<tr>
<td>VWF propeptide</td>
<td>0.93 (0.87–0.99)</td>
<td>&lt;0.0001*</td>
<td>44</td>
<td>91 (75–97)</td>
<td>84 (70–93)</td>
<td>5.7 (2.7–12)</td>
<td>0.11 (0.036–0.33)</td>
</tr>
<tr>
<td>VWF</td>
<td>0.93 (0.88–0.99)</td>
<td>&lt;0.0001*</td>
<td>220</td>
<td>81 (65–91)</td>
<td>84 (70–93)</td>
<td>5.1 (2.4–11)</td>
<td>0.22 (0.11–0.46)</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>0.94 (0.87–1.0)</td>
<td>&lt;0.0001*</td>
<td>879</td>
<td>91 (76–97)</td>
<td>92 (79–97)</td>
<td>11 (3.9–34)</td>
<td>0.10 (0.035–0.30)</td>
</tr>
<tr>
<td>VSGF</td>
<td>0.71 (0.58–0.84)</td>
<td>0.0025*</td>
<td>0.25</td>
<td>72 (55–84)</td>
<td>74 (59–85)</td>
<td>2.7 (1.5–4.9)</td>
<td>0.38 (0.21–0.69)</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.64 (0.50–0.77)</td>
<td>0.0004</td>
<td>0.72</td>
<td>63 (51–82)</td>
<td>65 (49–79)</td>
<td>2.0 (1.2–3.3)</td>
<td>0.48 (0.27–0.83)</td>
</tr>
</tbody>
</table>

Median (range), Mann-Whitney U test;
*p<0.05 after Holm correction for 9 pair-wise comparisons;
CM-R: Cerebral malaria, retinopathy positive
UM: Uncomplicated malaria, AUROC: Area under the receiver operating characteristic curve.
Figure 5.3. Biomarker levels at admission and follow up.
Plasma levels of biomarkers were measured at admission and 28 days post-treatment in a cohort of retinopathy positive children with cerebral malaria. Wilcoxon signed rank test with Holms correction (9 pair-wise comparisons) was used to compare levels of (A) Ang-2 (ng/mL); sum of signed ranks (W), (W, p-value: 746, p<0.0009); (B) Ang-1 (ng/mL), (W, p-value: -741, p<0.0009); (C) Ang-2:Ang-1, (W, p-value: 741, p<0.0009); (D) VWF propeptide (nM), (W, p-value: 768, p<0.0009); (E) VWF (nM), (W, p-value: 740, p<0.0009); (F) sTie-2 (ng/mL) (W, p-value: 754, p<0.0009); (G) sICAM-1 (ng/mL), (W, p-value: 732, p<0.0009); (H) VEGF (ng/mL), (W, p-value: -388, p=0.001); and (I) IP-10 (ng/mL), (W, p-value: 607, p<0.0009).
Using ROC curve analysis, three biomarkers, Ang-1, VWFpp, and VWF, discriminated children with CM-R from children with other suspected CNS infections (Table 5.5). Median Ang-2 was elevated in CM-R compared to the CNS controls, and the Ang-2:Ang-1 ratio had an AUROC equal to that of Ang-1 alone but with a two-fold increase in the positive likelihood ratio (Table 5.5). sICAM-1, sTie-2, and VEGF, while useful biomarkers for UM vs. CM-R, did not discriminate between CM-R vs. CNS (Table 5.5).

Table 5.5. Receiver operating characteristic curves of endothelial biomarkers in children with fever and altered consciousness (CNS) or cerebral malaria with retinopathy (CM-R)

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>AUROC CNS vs. CM-R</th>
<th>p-value</th>
<th>Cutoff</th>
<th>Sensitivity (95%CI)</th>
<th>Specificity (95%CI)</th>
<th>Positive likelihood ratio (95%CI)</th>
<th>Negative likelihood ratio (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1</td>
<td>0.93 (0.88-0.99)</td>
<td>&lt;0.0001*</td>
<td>5.3</td>
<td>88 (69-96)</td>
<td>87 (73-94)</td>
<td>6.7 (2.9-13)</td>
<td>0.14 (0.05-0.42)</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.71 (0.55-0.84)</td>
<td>0.006*</td>
<td>6.3</td>
<td>67 (47-82)</td>
<td>71 (55-83)</td>
<td>2.3 (1.3-4.1)</td>
<td>0.47 (0.26-0.90)</td>
</tr>
<tr>
<td>Ang-2:Ang-1</td>
<td>0.93 (0.87-0.99)</td>
<td>&lt;0.0001*</td>
<td>71</td>
<td>51 (1-85)</td>
<td>95 (83-99)</td>
<td>13 (3.4-53)</td>
<td>0.31 (0.16-0.58)</td>
</tr>
<tr>
<td>sTie-2</td>
<td>0.62 (0.49-0.78)</td>
<td>0.108*</td>
<td>66</td>
<td>8.3 (2.3-26)</td>
<td>74 (58-85)</td>
<td>1.2 (0.99-1.6)</td>
<td>0.32 (0.076-1.3)</td>
</tr>
<tr>
<td>VWF peptide</td>
<td>0.89 (0.80-0.98)</td>
<td>&lt;0.0001*</td>
<td>38</td>
<td>79 (60-91)</td>
<td>84 (70-93)</td>
<td>5.0 (2.3-11)</td>
<td>0.25 (0.11-0.55)</td>
</tr>
<tr>
<td>VWF</td>
<td>0.80 (0.66-0.92)</td>
<td>&lt;0.0001*</td>
<td>310</td>
<td>63 (43-79)</td>
<td>84 (70-93)</td>
<td>4.0 (1.8-8.8)</td>
<td>0.45 (0.26-0.76)</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>0.99 (0.81-0.97)</td>
<td>0.254</td>
<td>1000</td>
<td>21 (21-57)</td>
<td>92 (79-97)</td>
<td>4.8 (1.4-16)</td>
<td>0.68 (0.49-0.94)</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.54 (0.39-0.69)</td>
<td>0.587</td>
<td>0.53</td>
<td>39 (22-59)</td>
<td>74 (58-85)</td>
<td>1.5 (0.71-3.1)</td>
<td>0.83 (0.37-1.2)</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.64 (0.59-0.78)</td>
<td>0.0073</td>
<td>0.91</td>
<td>52 (33-71)</td>
<td>66 (50-70)</td>
<td>1.5 (0.85-2.8)</td>
<td>0.73 (0.45-1.2)</td>
</tr>
</tbody>
</table>

Median (range), Mann-Whitney U test;  
*p<0.05 after Holm correction for 9 pair-wise comparisons; 
CM-R: Cerebral malaria, retinopathy positive, 
CNS: children with non-malarial fever with decreased consciousness, AUROC: Area under the receiver operating characteristic curve.

5.4.5 Correlation between endothelial biomarkers

Complex interactions have been reported between molecular regulators of endothelial function (Fiedler and Augustin 2006). Therefore we postulated that significant correlations would exist between endothelial biomarkers. After applying two-way rank correlations we found significant correlations between Ang-2 and sTie-2, VWFpp, VWF, sICAM-1, and IP-10 and inverse correlations between Ang-1, VWF, and sICAM-1 (Table 5.6).
Table 5.6. Two-way rank correlations between biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Ang-2</th>
<th>Tie-2</th>
<th>VWFpp</th>
<th>VWF</th>
<th>sICAM-1</th>
<th>VEGF</th>
<th>IP-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1</td>
<td>-0.21*</td>
<td>-0.10</td>
<td>-0.55*</td>
<td>-0.63*</td>
<td>-0.51*</td>
<td>0.034</td>
<td>-0.18*</td>
</tr>
<tr>
<td>Ang-2</td>
<td></td>
<td></td>
<td>0.33*</td>
<td>0.55*</td>
<td>0.42*</td>
<td>0.36*</td>
<td>0.12</td>
</tr>
<tr>
<td>sTie-2</td>
<td></td>
<td></td>
<td>0.251*</td>
<td>0.25*</td>
<td>0.44*</td>
<td>0.17</td>
<td>0.13</td>
</tr>
<tr>
<td>VWFpp</td>
<td></td>
<td></td>
<td></td>
<td>0.80*</td>
<td>0.58*</td>
<td>0.055</td>
<td>0.57*</td>
</tr>
<tr>
<td>VWF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.60*</td>
<td>0.14</td>
<td>0.37*</td>
</tr>
<tr>
<td>sICAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.16</td>
<td>0.43*</td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25*</td>
<td></td>
</tr>
</tbody>
</table>

Spearman’s rho;  
*p<0.05;  
**p<0.01;  
n = 123.

5.4.6  Endothelial biomarker levels are associated with clinical recovery

We hypothesized that sequential measurements of endothelial biomarker levels could provide objective and quantitative evidence of clinical recovery and disease resolution. To test this hypothesis, we obtained paired measurements of endothelial biomarkers at admission and at day 28, following treatment and recovery, from 38 survivors of CM-R. Levels of Ang-1 displayed a uniform and consistent increase in all participants, whereas levels of Ang-2, Tie-2, VWFpp, VWF, sICAM-1, and IP-10 decreased with convalescence (Figure 5.3). Overall, VEGF showed a significant increase in levels at convalescence (supplementary Table 1). Notably, the Ang-2:Ang-1 ratio showed the most dynamic range between levels at admission and follow-up and there was a universal and consistent decrease in Ang-2:Ang-1 levels associated with clinical recovery.

5.5  Discussion

The diagnosis of cerebral malaria in children is clinically challenging since the syndrome may be confused with other causes of fever and altered consciousness. Diagnostic tools that could accurately identify children with “true” CM would enable improved triage and management of these life-threatening infections. Currently retinopathy is the best tool to predict which febrile,
comatose children have true CM (Taylor, Fu et al. 2004), but it has operational constraints. Alternative methods of discriminating between these groups have not been described. Identifying retinopathy in a comatose child greatly increases the confidence with which the clinical syndrome can be attributed to malaria. Direct ophthalmoscopy through dilated pupils can be used to observe retinal changes, but a more accurate picture is provided by indirect ophthalmoscopy, a procedure that is usually not available where resources are limited. In this study, we tested the hypothesis that plasma biomarkers may function as surrogate markers for malarial retinopathy. We show that several biomarkers (Ang-2, sTie-2, VWFpp and sICAM-1) were significantly associated with retinopathy (Figure 5.1, Table 5.2). We report that a panel of endothelial and angiogenic biomarkers is able to discriminate, with a high degree of accuracy, children with retinopathy-confirmed CM (CM-R) from those with uncomplicated infection (UM) or with non-malarial febrile illness and altered consciousness (CNS controls) (Figure 5.2, Table 5.3, Table 5.4). This is the first study to demonstrate the specificity of Ang-1 for CM in children, and we confirm and extend previous observations of elevated levels of VWFpp and VWF in children with severe malaria compared to non-malarial febrile illness (Hollestelle, Donkor et al. 2006). Finally, we show a marked and uniform decrease in Ang-2:Ang-1 at follow-up (Figure 5.3), suggesting that the ratio between these two proteins may offer an approach to monitor clinical response.

The endothelium is a dynamic organ system representing the interface between the vascular space and vital organs. The regulation of the endothelial integrity is of critical importance, particularly in the face of infection-related injury. Endothelial adhesion of parasitized red cells and endothelial activation are prominent features in the pathology of fatal malaria. Parasitized erythrocytes bind to the endothelium directly through endothelial receptors and may indirectly bind through VWF and platelet complexes (Bridges, Bunn et al. 2009). There is evidence to suggest that blood-brain-barrier dysfunction and breakdown occurs in paediatric CM (Brown, Hien et al. 1999; Brown, Rogerson et al. 2001; van der Heyde, Nolan et al. 2006). However, the pathophysiology of CM is poorly understood. A detailed understanding of endothelial activation and regulation during infection may provide new insights into the molecular basis of severe and fatal malaria.
Angiopoietins are critical regulators of endothelial activation and integrity. Elevated Ang-2 levels have previously been associated with severe malaria in paediatric and adult populations and strategies to block Ang-2 have been suggested as novel interventions for severe malaria (Yeo, Lampah et al. 2008; Conroy, Lafferty et al. 2009; Lovegrove, Tangpukdee et al. 2009). In this study, Ang-2 was elevated in CM-R compared to UM and CNS controls but became of borderline significance after correcting for multiple comparisons. However, inclusion of Ang-2 as a component of the Ang2:Ang-1 ratio markedly improved the specificity and positive likelihood ratio compared to Ang-1 or Ang-2 alone. Levels of Ang-2 observed in this study were lower than those reported in Ugandan children with CM (Lovegrove, Tangpukdee et al. 2009). This difference is likely attributable to the present study design that excluded fatal cases, in which Ang-2 levels are highest. Ang-2 has been associated with increased disease severity (Yeo, Lampah et al. 2008; Conroy, Lafferty et al. 2009) and increased risk of death (Yeo, Lampah et al. 2008) in Asian adults with severe malaria. Together, these data suggest that changes in Ang-2 are reflective of disease severity and mortality and may be a good surrogate endpoint for trials investigating mortality or evaluating adjunctive therapies.

In the context of endothelial biology, the balance between Ang-1 and Ang-2 regulates the functional responsiveness of the endothelium. Ang-1 is synthesized by periendothelial cells to promote vascular quiescence under normal physiologic conditions; however, the release of Ang-2 from WP bodies can inhibit Ang-1 signalling in a dose-dependent manner, resulting in local destabilization of the endothelium (Yuan, Khankin et al. 2009). In this study, there were markedly lower Ang-1 levels at presentation in children with CM-R compared to those with UM or the CNS controls. The observed decreases in Ang-1 levels combined with increases in Ang-2 may contribute to the endothelial dysfunction observed in CM.

The functions of Ang-1 and Ang-2 are also modulated by interactions with VEGF. Under normal physiological conditions of high Ang-1 and low Ang-2, VEGF can stabilize the endothelium in an anti-apoptotic state and be neuroprotective (Carmeliet and Storkebaum 2002; Nicoletti, Shah et al. 2008). However, when Ang-1 levels are low, VEGF can act on the endothelium unopposed, resulting in the upregulation of Ang-2 mRNA, exocytosis of WP bodies, increased permeability of endothelial cells and upregulation of tissue factor and ICAM-1 (Oh, Takagi et al. 1999; Kim, Moon et al. 2001; Kim, Oh et al. 2002; Satchell, Anderson et al. 2004; Matsushita, Yamakuchi et al. 2005). This may be particularly important in the context of paediatric CM, where
coagulopathy and increased tissue factor expression may occur and Ang-1 levels are low (Francischetti, Seydel et al. 2007; Moxon, Heyderman et al. 2009). In this study, VEGF was elevated in children with retinopathy confirmed CM compared to children with uncomplicated malaria. These findings are in contrast to reports examining VEGF in severe malaria in adults from Southeast Asia, which have reported a decrease in VEGF in fatal CM cases (Jain, Armah et al. 2008) and decreased VEGF associated with increased disease severity (Yeo, Lampah et al. 2008). Other reports from children have shown no difference in CSF levels of VEGF between deaths due to malaria vs. those attributed to other causes (Armah, Wilson et al. 2007). However, a study of Kenyan children with CM showed plasma VEGF levels were positively correlated with TNF and inversely correlated with a neuroprotective agent, erythropoietin, and high levels of plasma VEGF were associated with an increased risk of seizures, raised intracranial pressure, and papilloedema (Casals-Pascual, Idro et al. 2008). These differences may be due to geographic location (Africa vs. Asia) or patient population (pediatric vs. adult).

Other studies in pediatric populations also suggest that VEGF is associated with disease severity. A study looking at VEGF levels in children with sepsis and meningococcemia reported the highest VEGF levels in patients with septic shock (Pickkers, Sprong et al. 2005) and elevated VEGF was a useful prognostic indicator in Kawasaki disease, an acute febrile vasculitis in children (Kentaro, Yuichi et al.). In our study, despite being elevated in CM-R, VEGF was not a particularly informative marker, as it was variable between admission and follow up in children with CM, and was non-specifically elevated in the CNS controls. However, VEGF may be playing an under-appreciated role in endothelial regulation based on the presence or absence of Ang-1 or Ang-2 in the local milieu. Consequently, it may be premature to rule out VEGF as a mediator of severe malaria, given its diverse functions. It is worth noting that VEGF is contained primarily within platelets, and plasma levels can be affected by differences in sample processing (anti-coagulant used, centrifugation time and speed, etc.).

ICAM-1 is upregulated in the cerebral endothelium during malaria infection and is associated with parasite sequestration within the cerebral vasculature, a pathological hallmark of CM (Turner, Morrison et al. 1994). sICAM-1 is released by activated endothelium during malaria and has been reported as a biomarker of disease severity (Turner, Ly et al. 1998; Tchinda, Tadem et al. 2007). Similarly, in our study, sICAM-1 was able to discriminate between UM and CM, but it
was also elevated in the CNS control group. Recent reports have described increased circulating concentrations of VWF and activation of the coagulation system in severe malaria, with possible implications for pathogenesis (Hollestelle, Donkor et al. 2006; Larkin, de Laat et al. 2009; Moxon, Heyderman et al. 2009). It has been postulated that an increase in ultra large VWF strings and a decrease in its cleavage protein ADAMTS13 can result in platelet accumulation and contribute to sequestration of parasitized erythrocytes (Bridges, Bunn et al. 2009). In the current study, both VWF and VWFpp were elevated in CM-R compared to UM or CNS controls, suggesting that these proteins are also good candidate biomarkers for CM.

The endothelial proteins measured in this study returned to a normal range in association with clinical recovery, suggesting that the alterations in biomarker levels at presentation were mediated by the infection status of the child rather than a natural host-mediated susceptibility as a result of genetic or epigenetic changes in the biomarkers examined. In previous studies, VWFpp was shown to return to baseline three days after the initiation of anti-malarial therapy (Hollestelle, Donkor et al. 2006), and Ang-2 and the RH-PAT index, a measure of peripheral endothelial dysfunction, returned to normal four days post treatment (Yeo, Lampah et al. 2008).

In summary, our study is the first to examine a panel of endothelial-based biomarkers in a well characterized patient population. Recent investigations have moved towards the inclusion of non-malaria febrile illness (NMFI) as a control group to investigate the specificity of markers for malaria infection. In this study, we included a NMFI group with decreased consciousness, which approximates the clinically ideal control group, i.e. children with incidental parasitemia and coma (bacterial meningitis, viral encephalopathy, etc.). CM is a clinical definition based on the presence of malaria parasitemia and coma with no other identifiable cause. However, in malaria endemic areas, there are often resource constraints that make exclusion of alternative causes of coma problematic. In order to better define biomarker specificity for severe malarial syndromes, it will be important that future studies include the most biologically and clinically informative control groups.

The results of this study are encouraging, and suggest that a limited panel of endothelial biomarkers may be useful in differentiating between retinopathy positive CM and non-malarial febrile illness with decreased consciousness. However, these results will need to be confirmed in
prospective studies and assessed alongside other infectious causes of coma in paediatric populations. Further validation of biomarkers needs to be performed to determine whether these markers: i) are informative in stratifying subjects in clinical trials or pathogenesis studies; ii) have clinical utility in the diagnosis of true CM, assessment of disease severity and response to therapy (allocation of resources), or to determine prognosis; and iii) represent novel therapeutic targets for adjunctive therapy.
Chapter 6
Combinations of Biomarkers of Inflammation and Endothelial Activation Predict Mortality among Ugandan Children with Severe Malaria: A Retrospective Case-Control Study

6.1 Abstract

**Background:** Severe malaria causes almost 1 million pediatric deaths annually. At presentation, it is difficult to predict which children with severe malaria are at greatest risk of death. Dysregulated host inflammatory responses and endothelial activation are thought to play pivotal roles in severe malaria pathogenesis. We hypothesized that biomarkers of these processes would accurately predict outcome among children with severe malaria.

**Methodology/Findings:** Plasma was obtained from children with uncomplicated malaria (n=53), cerebral malaria (n=44) and severe malarial anemia (n=59) at time of presentation to hospital in Kampala, Uganda. Levels of angiopoietin-2, von Willebrand Factor (vWF), vWF propeptide, soluble P-selectin, soluble intercellular adhesion molecule-1 (ICAM-1), soluble endoglin, soluble FMS-like tyrosine kinase-1 (Flt-1), soluble Tie-2, C-reactive protein, procalcitonin, 10 kDa interferon gamma-induced protein (IP-10), and soluble triggering receptor expressed on myeloid cells-1 (TREM-1) were determined by ELISA. Receiver operating characteristic (ROC) curve analysis was used to assess predictive accuracy of individual biomarkers. Six biomarkers (angiopoietin-2, soluble ICAM-1, soluble Flt-1, procalcitonin, IP-10, soluble TREM-1) discriminated well between children who survived severe malaria infection and those who subsequently died (area under ROC curve>0.7). Combinational approaches were applied in an attempt to improve accuracy. A biomarker score was developed based on dichotomization and summation of the six biomarkers, resulting in 95.7% sensitivity (95% CI: 78.1-99.9) and 88.8% specificity (79.7-94.7) for predicting death. Similar predictive accuracy was achieved with models comprised of 3 biomarkers. Classification tree analysis generated a 3-marker model with 100% sensitivity and 92.5% specificity (cross-validated misclassification rate: 15.4%, standard error 4.9%).

**Conclusions:** We identified novel host biomarkers of pediatric severe and fatal malaria (soluble TREM-1 and soluble Flt-1) and generated simple biomarker combinations that accurately
predicted death in our sample. While requiring validation in further studies, these results suggest the utility of combinatorial biomarker strategies as prognostic tests for severe malaria.
6.2 Introduction

*Plasmodium falciparum* malaria causes almost one million deaths annually, mostly among young children in sub-Saharan Africa (2009). The most common manifestations of pediatric severe malaria are severe malarial anemia (SMA) and cerebral malaria (CM). These syndromes can have case fatality rates as high as 20% (Murphy and Breman 2001). It is challenging at clinical presentation to accurately determine which children with severe malaria are at greatest risk of death. Simple and sensitive clinical scores have been developed to predict outcome, but they have low specificity and rely on subjective assessment of clinical signs (Marsh, Forster et al. 1995; Helbok, Kendjo et al. 2009). An accurate prognostic test would be useful for targeting limited health resources to high-risk children and for selecting patients to enroll in clinical trials of adjunctive therapies for severe malaria.

Investigations into malaria pathogenesis have implicated host pathways in disease progression. In particular, dysregulated inflammatory responses and endothelial activation are thought to be central processes in severe malaria pathogenesis (Clark, Alleva et al. 2004; van der Heyde, Nolan et al. 2006; Faille, El-Assaad et al. 2009). We hypothesized that plasma biomarkers of these pathways may have clinical utility as prognostic tools, particularly if used in combination. We selected biomarkers of these pathways and assessed their utility as indicators of disease severity and outcome in Ugandan children presenting to hospital with malaria.

Imbalanced inflammatory responses are observed in both CM and SMA (Grau, Taylor et al. 1989; Kurtzhals, Adabayeri et al. 1998; Othoro, Lal et al. 1999; Lyke, Burges et al. 2004). In this study, we measured plasma levels of acute-phase response components, C-reactive protein (CRP) and procalcitonin (PCT), which have been shown to increase during malaria infection (Chiwakata, Manegold et al. 2001; Gyan, Kurtzhals et al. 2002). We also measured 10 kDa interferon gamma-induced protein (IP-10), a chemokine reported to be elevated in fatal CM (Armah, Wilson et al. 2007). Moreover, we assessed levels of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), which is associated with inflammatory conditions (Ford and McVicar 2009) but has not been previously investigated in malaria.
Dysregulated inflammation is thought to promote CM in part through endothelial activation in the brain. Pro-inflammatory cytokines upregulate cell adhesion receptors (e.g., intercellular adhesion molecule-1 [ICAM-1]) that mediate sequestration of parasitized erythrocytes in brain microvasculature, leading to vessel occlusion (Beare, Harding et al. 2009) and blood-brain barrier dysfunction (Medana and Turner 2006). Upon endothelial activation, soluble endothelial cell receptors are released via ectodomain shedding or alternative splicing. We measured the soluble forms of ICAM-1 (sICAM-1) and the TGF-β receptor endoglin (s-endoglin), which have both been shown to be increased in severe malaria (Turner, Ly et al. 1998; Dietmann, Helbok et al. 2009), and soluble FMS-like tyrosine kinase-1 (sFlt-1), which has been implicated in placental malaria (Muehlenbachs, Fried et al. 2008). Endothelial activation also causes exocytosis of Weibel-Palade bodies (WPB), intracellular vesicles that contain a variety of effector molecules (Lowenstein, Morrell et al. 2005). We assayed WBP-associated factors angiopoietin-2 (Ang-2), von Willebrand factor (vWF), vWF propeptide, and soluble P-selectin (sP-selectin). Some of these molecules are elevated in CM (Hollestelle, Donkor et al. 2006; Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009) and have been suggested to contribute to pathology: Ang-2 may exacerbate vascular activation in malaria by antagonizing the quiescence-promoting interaction of the endothelial Tie-2 receptor with angiopoietin-1 (Ang-1) (Parikh, Mammoto et al. 2006), while vWF may help tether parasitized erythrocytes to endothelial cells via platelets (Bridges, Bunn et al.). In addition to CM patients, systemic endothelial activation has been shown to occur in adults with uncomplicated and non-CM severe malaria (Turner, Ly et al. 1998; Conroy, Lafferty et al. 2009); however, few studies have characterized the extent and significance of this process in pediatric SMA.

In this study, we examined plasma biomarkers of inflammation and endothelial activation in children presenting to hospital with malaria. We determined which markers were elevated in severe disease compared to uncomplicated malaria (UM), and which markers discriminated between children who survived severe malaria infection and those who subsequently died. Furthermore, we identified combinations of biomarkers from these two host pathways that predicted mortality among severe malaria patients with high accuracy.
6.3 Methods

6.3.1 Study site and participants

This retrospective case-control study was nested within a larger study conducted at Mulago Hospital in Kampala, Uganda between October 2007 and October 2009. Mulago Hospital is a national referral hospital that serves Kampala and surrounding districts. Malaria transmission in this region and the patient population at Mulago Hospital have been previously described (Opoka, Xia et al. 2008). Children presenting to hospital were eligible for enrollment if they were between 6 months and 12 years old and had microscopy-confirmed P. falciparum infection (asexual parasitemia with clinical signs or symptoms of malaria). Children were excluded if they had sickle cell trait/disease, HIV co-infection, or severe malnutrition. Clinical and demographic data were collected upon enrollment, and venous blood samples were collected for routine measurement of hemoglobin and platelet count, and for plasma banking. Thin blood smears were obtained at presentation for determination of parasitemia, which is reported as the arithmetic mean of two independent readings by expert microscopists. Treatment was in accordance with Ugandan national guidelines: artemether/lumefantrine was administered to children with uncomplicated malaria, and parenteral quinine was used in severe malaria cases (2006). All children with SMA received blood transfusions. Children were followed for recovery/survival or death.

For biomarker analysis, a sub-group (n=156) of UM outpatients, CM inpatients, and SMA inpatients in roughly equal numbers was selected from the larger study based on availability of an adequate volume of previously unthawed plasma. CM was defined as an unrousable coma not attributable to any other cause in a patient with P. falciparum malaria (i.e. Blantyre Coma Scale score <3, either before or >6 h after seizures or anticonvulsant medication (if applicable), or repeated (>3) seizures witnessed within a 24 h period, hypoglycaemia (<40 mg/dL or 2.2 mM) or any known alternative neurologic abnormalities). SMA was defined as hematocrit < 15% or hemoglobin < 5.0 g/dL in the presence of parasitemia.
6.3.2 Ethical approval and informed consent

Ethical approval for the study was obtained from the Mulago Hospital Research Ethics Committee, Makerere University Faculty of Medicine Research Ethics Committee, Uganda National Council for Science & Technology, and the University Health Network. The study was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent for participation in the study was obtained from parents/guardians before enrollment, and separate written consent was obtained for storage of a plasma sample for future analysis.

6.3.3 Biomarker assays

Plasma (sodium citrate anticoagulant) was stored at -20°C prior to testing. ELISAs were used to quantify plasma biomarker levels and were performed blinded to all associated clinical data. Ang-2, CRP, sTREM-1, s-endoglin, IP-10, sFlt-1, sICAM-1, sP-selectin, sTie-2 (R&D Systems), PCT (Ray BioTech), and vWF propeptide (Sanquin) were assayed according to the manufacturers’ instructions, with the following changes: assays were performed in a volume of 50 μL/well; plasma samples were incubated overnight at 4°C; and ELISAs were developed using Extravidin (Sigma, 1:1000 dilution, 45 min incubation) followed by addition of p-Nitrophenyl phosphate substrate (Sigma) and optical density readings at 405 nm. s-endoglin and sP-selectin were only measured in samples collected during the first year of the study (n=101). For vWF, plates were coated with anti-human vWF antibody (Dako, 1:600), incubated with samples and serial dilutions of recombinant vWF (American Diagnostica), then incubated with horseradish peroxidase-conjugated anti-human vWF (Dako, 1:8000). Assays were developed with tetramethylbenzidine, stopped with H₂SO₄, and read at 450 nm. Samples with concentrations below the limit of detection were designated as twice the background level.

6.3.4 Statistical analysis

GraphPad Prism v4, SPSS v18, and MedCalc software were used for analysis. For clinical and demographic variables, differences between groups were assessed using the Chi-square test (categorical variables) or the Kruskal-Wallis test with Dunn’s multiple comparison post-hoc tests (continuous variables). The Mann-Whitney U test was used to compare biomarker levels between groups, and p values were corrected for multiple comparisons using Holm’s correction. Receiver operating characteristic curves were generated using the non-parametric method of
Delong et al. (DeLong, DeLong et al. 1988). Cut-points were determined using the Youden index \( J = \max\{\text{sensitivity} + \text{specificity} - 1\} \). For logistic regression, linearity of an independent variable with the log odds of the dependent was assessed by including a Box-Tidwell transformation into the model and ensuring that this term was not significant. Bootstrapping (1000 sample draws) was used to generate variance estimates for \( b \). Model goodness-of-fit was assessed by the Hosmer-Lemeshow test and calibration slope analysis (Steyerberg, Eijkemans et al. 2001). Positive and negative predictive values were calculated using the estimated case fatality rate of 5.7% for microscopy-confirmed CM and SMA at Mulago Hospital (Opoka, Xia et al. 2008). Classification tree analysis was performed in SPSS with the following settings: minimum 10 cases for parent nodes and 5 for child nodes; customized prior probabilities based on the case fatality rate at Mulago Hospital; customized misclassification costs (as indicated); pruning to reduce overfitting; and cross-validation with 10 sample folds to generate an estimate of the misclassification rate and its standard error. There were no missing values from the dataset.

6.4 Results

6.4.1 Characteristics of study participants

Children presenting to Mulago Hospital in Kampala, Uganda with UM (n=53), CM (n=44), and SMA (n=59) were included in the study. There was some overlap between groups: six children with concurrent CM and SMA were categorized as “CM”, and five children with SMA exhibited decreased consciousness but did not strictly meet study criteria for CM. Table 6.1 presents the demographic and clinical characteristics of the three groups. Children with SMA were younger than children with UM and CM (p<0.001) and presented significantly later than the other groups (p<0.001, approximately one day later). Children with severe malaria had lower hemoglobin levels and platelet counts than children with UM.
Table 6.1. Demographic and clinical characteristics of study participants presenting with uncomplicated and severe malaria

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>UM (n = 53)</th>
<th>CM (n = 44)</th>
<th>SMA (n = 59)</th>
<th>Survivors (n = 80)</th>
<th>Fatalities (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (% female)</td>
<td>45.3</td>
<td>52.3</td>
<td>49.2</td>
<td>46.3</td>
<td>65.2 (p&lt;0.01)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>4.4 (2.1, 8.1)</td>
<td>3.0 (1.5, 4.3)</td>
<td>3.3 (2.0, 2.2) (***p&lt;0.001)</td>
<td>1.6 (1.0, 3.1)</td>
<td>1.9 (1.2, 3.3)</td>
</tr>
<tr>
<td>Days reported ill prior to presentation</td>
<td>3 (2, 4)</td>
<td>3 (2, 4)</td>
<td>4 (3, 6) (***p&lt;0.001)</td>
<td>3 (3, 4)</td>
<td>3 (2, 7)</td>
</tr>
<tr>
<td>Parachmeteria (parasites/μL)</td>
<td>3.8 (10^4) ((1.6 \times 10^6, 3.2 \times 10^7))</td>
<td>9.8 (10^4) ((1.5 \times 10^6, 2.7 \times 10^7))</td>
<td>2.6 (10^4) ((2.4 \times 10^5, 1.2 \times 10^6)) (***p&lt;0.001)</td>
<td>3.7 (10^4) ((2.5 \times 10^5, 1.5 \times 10^6))</td>
<td>1.6 (10^4) ((2.2 \times 10^5, 3.9 \times 10^6))</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>10.3 (9.4, 11.3)</td>
<td>6.5 (5.5, 8.4) (**p&lt;0.01)</td>
<td>8.8 (5.2, 4.4) (***p&lt;0.001)</td>
<td>4.3 (3.4, 5.9)</td>
<td>5.4 (6.2, 8.3) (p&lt;0.05)</td>
</tr>
<tr>
<td>Platelet count (×10^9/L)</td>
<td>166 (107, 219)</td>
<td>73 (47, 128) (***p&lt;0.001)</td>
<td>116 (71, 165) (p&lt;0.05)</td>
<td>103 (61, 162)</td>
<td>73 (41, 128)</td>
</tr>
<tr>
<td>Fetal cases</td>
<td>0</td>
<td>14</td>
<td>9</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>

*All variables except gender are presented as median (interquartile range). Groups were compared using the Mann Whitney U test or Kruskal-Wallis test with Dunn’s post-hoc tests (continuous variables) or Chi-square test (categorical variables).

CM, uncomplicated malaria; CM, cerebral malaria; SMA, severe malaria anemia.

6.4.2 Biomarker levels in uncomplicated vs. severe malaria patients

Samples obtained at presentation were assayed for biomarkers of endothelial activation and inflammation (Figure 6.1). sICAM-1, sTie-2, and sFlt-1 were increased in CM and SMA compared to UM (p<0.01), while s-endoglin, sP-selectin, and IP-10 did not differ between groups (p>0.05). WPB-associated proteins Ang-2, vWF, and vWF propeptide were elevated in children with severe malaria compared to UM, as were inflammatory biomarkers CRP, PCT, and sTREM-1 (p<0.01).
Figure 6.1. Plasma biomarker levels in Ugandan children with uncomplicated and severe malaria at time of presentation.
Biomarkers in the plasma of children with uncomplicated malaria (UM), cerebral malaria (CM), and severe malarial anemia (SMA) were measured by ELISA. Data are presented as dot plots with medians. A Mann Whitney U test was performed for each comparison, and p values were adjusted for multiple comparisons using Holm’s correction (n=24). ** p<0.01.
6.4.3 Biomarkers as predictors of mortality in children with severe malaria

To evaluate the prognostic utility of these biomarkers, we compared admission levels between children with severe malaria who survived infection and those who subsequently died. After correction for multiple comparisons, admission levels of Ang-2 were significantly increased in CM fatalities compared to survivors (Figure 6.2A; p<0.05), while Ang-2, sICAM-1, IP-10 (p<0.01), sTREM-1 and sFlt-1 (p<0.05) were elevated in SMA fatalities compared to survivors (Figure 6.2B).

The biomarkers that reached significance in the SMA group but not the CM group after correction for multiple comparisons (sICAM-1, IP-10, sTREM-1, sFlt-1) were significant or trending towards significance in the CM group before the correction was applied. This suggests that apparent differences between syndromes may have been due to low statistical power. Given these similarities, we combined all severe malaria patients for further analysis; this strategy also avoids the common problem of classifying mixed clinical phenotypes, as occurred in the present study population. Characteristics of survivors and fatalities were similar (Table 6.1), although among fatalities there was a greater proportion of females (p=0.007) and increased parasitemia (p=0.023). We found that Ang-2, sICAM-1, sFlt-1, IP-10, and sTREM-1 (p<0.01), as well as PCT (p<0.05), were elevated in fatal cases of severe malaria compared to survivors (Figure 6.2C).
Figure 6.2. Plasma biomarker levels in children with severe malaria who survived or subsequently died from infection. 
Presented are biomarkers that were significantly different for (A) CM patients only, (B) SMA patients only, and (C) all severe malaria patients combined. Biomarkers were measured by ELISA. Data are presented as dot plots with medians. A Mann Whitney U test was performed for each comparison, and p values were adjusted for multiple comparisons using Holm’s correction (n=12 for each group). * p<0.05 and ** p<0.01.
To assess how well these biomarkers discriminated between survivors and fatalities, we generated receiver operating characteristic (ROC) curves and determined area under the curve (AUC) (Figure 6.3). Ang-2, sICAM-1, and IP-10 had excellent predictive ability (AUC 0.8-0.9), and sTREM-1, sFlt-1 and PCT had acceptable predictive ability (AUC 0.7-0.8) (Hosmer 2000). The AUC for parasitemia, which is used in clinical practice as a prognostic factor, was 0.66.
Figure 6.3. Assessment of biomarker utility in predicting outcome in children with severe malaria
A receiver operating characteristic (ROC) curve was generated for each biomarker. The dashed reference line represents the ROC curve for a test with no discriminatory ability. Area under the ROC curve is displayed on each graph with 95% confidence intervals in parentheses. p values were adjusted for multiple comparisons using Holm’s correction (n=7). * p<0.05 and ** p <0.01.
We used the Youden index to obtain a cut-point for each biomarker, and evaluated clinical performance measures for these dichotomized biomarkers (Table 2). sTREM-1 achieved the highest sensitivity (95.7%) but had low specificity (43.8%), while IP-10 predicted death with the highest overall accuracy (82.6% sensitivity, 85% specificity).

Table 6.2. Clinical performance of biomarkers for predicting mortality among children with severe malaria.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cut-pointa</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PLRa</th>
<th>NLR</th>
<th>PPV (%)d</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-2</td>
<td>&gt;5.6 ng/mL</td>
<td>78.3 (56.3–92.5)</td>
<td>78.8 (68.2–87.1)</td>
<td>3.7</td>
<td>0.3</td>
<td>18.2 (6.8–38.7)</td>
<td>98.4 (92.4–99.9)</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>&gt;645.3 ng/mL</td>
<td>87.9 (66.4–97.2)</td>
<td>75.0 (64.1–84.0)</td>
<td>3.5</td>
<td>0.2</td>
<td>17.4 (5.9–35.9)</td>
<td>99.0 (93.2–100)</td>
</tr>
<tr>
<td>sFlt1</td>
<td>&gt;1056.5 pg/mL</td>
<td>82.6 (61.2–95.0)</td>
<td>57.5 (45.9–68.5)</td>
<td>1.9</td>
<td>0.3</td>
<td>10.5 (3.4–23.1)</td>
<td>98.2 (90.4–100)</td>
</tr>
<tr>
<td>PCT</td>
<td>&gt;43.1 ng/mL</td>
<td>56.5 (34.5–76.8)</td>
<td>82.5 (72.4–90.1)</td>
<td>3.2</td>
<td>0.5</td>
<td>16.3 (3.8–39.5)</td>
<td>96.9 (90.5–99.5)</td>
</tr>
<tr>
<td>IP-10</td>
<td>&gt;811.2 pg/mL</td>
<td>82.6 (61.2–95.0)</td>
<td>85.0 (75.3–92.0)</td>
<td>5.5</td>
<td>0.2</td>
<td>25.0 (8.3–49.0)</td>
<td>98.8 (93.4–100)</td>
</tr>
<tr>
<td>sTREM-1</td>
<td>&gt;289.9 pg/mL</td>
<td>95.7 (78.1–99.9)</td>
<td>43.0 (32.7–55.3)</td>
<td>1.7</td>
<td>0.1</td>
<td>9.3 (3.3–19.6)</td>
<td>99.4 (90.5–100)</td>
</tr>
</tbody>
</table>

aAll parameters are presented with 95% CIs in parentheses.
bCut-points were determined using the Youden Index ($D_{\text{max}}$=sensitivity+specificity–1).
cPLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.
dPPVs and NPVs were based on estimates that 5.7% of CM and SMA patients at Mulago hospital die of the malaria infection (28).

6.4.4 Predicting fatality using a “biomarker score”

We hypothesized that combining biomarkers would improve predictive accuracy. The modest number of deaths in the study precluded multivariable logistic regression analysis with more than 2-3 independent variables (Harrell, Lee et al. 1996). Therefore, as performed in other conditions (Vinueza, Chauhan et al. 2000; Morrow and Braunwald 2003), we combined the biomarkers into a score. For each marker, one point was assigned if the measured value was greater than the corresponding cut-point, and zero points were assigned if it was lower. A cumulative “biomarker score” was calculated for each patient by summing the points for all six markers. No two dichotomized biomarkers were highly correlated (Spearman’s rho <0.6; data not shown), suggesting that each biomarker would contribute unique information to the score.

Biomarker score was highly positively correlated with risk of death (Figure 6.4A; Spearman’s rho=0.96, p=0.003). Scores were elevated among fatalities compared to survivors (Figure 6.4B; median (interquartile range): 5 (4-6) and 1 (0-2.5), respectively). In a univariate logistic regression model, the biomarker score was a significant predictor of death with an odds ratio of
7.9 (95% CI 4.6-54.4) (Table 6.3, Model 1). After adjustment for parasitemia and age, which have been associated with malaria mortality, the score remained significant with an adjusted odds ratio of 7.8 (4.7-134) (Table 6.3, Model 2).
The biomarker score is significantly associated with risk of fatality among children with severe malaria.

The biomarker score for each patient was calculated as detailed in the text. (A) Biomarker scores were plotted against observed probability of death. The two variables were significantly related (Spearman’s rho=0.96, p=0.003). (B) Biomarker score distributions were plotted for severe malaria survivors and fatalities. (C) A receiver operating characteristic (ROC) curve was generated for the biomarker score. The dashed reference line represents the ROC curve for a test with no discriminatory ability. Area under the ROC curve is displayed on each graph with 95% confidence intervals in parentheses. *** p<0.001.
The association of biomarker score with outcome among children with severe malaria: logistic regression.

Table 6.3 Association of biomarker score with outcome among children with severe malaria: logistic regression.

<table>
<thead>
<tr>
<th>Variable</th>
<th>b [95% CI]</th>
<th>SE</th>
<th>Wald</th>
<th>df</th>
<th>p value</th>
<th>OR [95% CI]</th>
<th>Chi square</th>
<th>df</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biomarker score</td>
<td>2.1 (1.5-4.0)</td>
<td>2.3</td>
<td>18.6</td>
<td>1</td>
<td>0.001</td>
<td>7.9 (4.6-14.4)</td>
<td>3.3</td>
<td>5</td>
<td>0.06</td>
</tr>
<tr>
<td>Model 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biomarker score&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.1 (1.6-4.6)</td>
<td>2.15</td>
<td>18.2</td>
<td>1</td>
<td>0.001</td>
<td>7.8 (4.7-13.4)</td>
<td>1.1</td>
<td>8</td>
<td>1.0</td>
</tr>
<tr>
<td>Log parasitemia&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.030 (1.1-1.3)</td>
<td>2.8</td>
<td>0.01</td>
<td>1</td>
<td>0.01</td>
<td>1.1 (0.35-3.6)</td>
<td>0.053 (1.06-2.1)</td>
<td>3.5</td>
<td>0.052</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The reference category was “survival.”
<sup>b</sup>Pseudo-R² (Cox & Snell 0.473 and calibration slope 0.98).
<sup>c</sup>Pseudo-R² (Cox & Snell) 0.474 and calibration slope 1.0.
<sup>d</sup>Biomarker score and log parasitemia had a significant but low correlation (Spearman’s rho 0.292, p<0.01).
<sup>e</sup>Parasitemia was loop-transformed in order to achieve linearity with the log-odds of the dependent variable. SE, standard error; OR, odds ratio.

ROC curve analysis and cut-point determination were performed as above for the biomarker score. The AUC was 0.96 (0.90-0.99) (Fig. 4C), and we found that a score ≥4 was 95.7% sensitive and 88.8% specific for predicting death in our sample (Table 4, row 1). While the positive predictive value was low (33.9%) given a fatality rate of 5.7%, the negative predictive value (NPV) was 99.7%, indicating that a child with a score ≤3 will likely respond well to standard treatment protocols.

A score involving fewer biomarkers might be expected to improve practicality and facilitate potential translation to a clinical application. Using the same scoring scheme, 2-marker combinations performed poorly (data not shown). However, specific 3-marker combinations yielded sensitivity > 90% and specificity > 80% (Table 4).

Table 6.4 Clinical performance of biomarker combinations for predicting mortality among children with severe malaria.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Cut-point&lt;sup&gt;h&lt;/sup&gt;</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PLR&lt;sup&gt;i&lt;/sup&gt;</th>
<th>NLR&lt;sup&gt;i&lt;/sup&gt;</th>
<th>PPV (%)&lt;sup&gt;g&lt;/sup&gt;</th>
<th>NPV (%)&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomarker score (6 markers)</td>
<td>≥4</td>
<td>95.7 (78.1-99.9)</td>
<td>88.8 (79.7-94.7)</td>
<td>8.5 (7.6-9.6)</td>
<td>0.05 (0.007-0.4)</td>
<td>32.0 (12.8-61.3)</td>
<td>99.7 (95.2-100)</td>
</tr>
<tr>
<td>Ang-2, PCT, sICAM-1</td>
<td>≥2</td>
<td>91.3 (72.9-99.3)</td>
<td>86.6 (79.7-94.7)</td>
<td>8.1 (7.0-9.0)</td>
<td>0.1 (0.02-0.4)</td>
<td>22.9 (12.1-60.3)</td>
<td>99.4 (94.7-100)</td>
</tr>
<tr>
<td>Ang-2, IP-10, PCT</td>
<td>≥2</td>
<td>91.3 (72.9-99.3)</td>
<td>86.3 (76.7-92.9)</td>
<td>6.6 (5.7-7.7)</td>
<td>0.1 (0.02-0.4)</td>
<td>22.9 (12.1-60.3)</td>
<td>99.4 (94.6-100)</td>
</tr>
<tr>
<td>PCT, IP-10, sTRIM-1</td>
<td>≥2</td>
<td>91.3 (72.9-99.3)</td>
<td>81.3 (71.0-91.0)</td>
<td>4.9 (4.1-5.7)</td>
<td>0.1 (0.03-0.4)</td>
<td>22.7 (8.1-44.8)</td>
<td>99.4 (94.2-100)</td>
</tr>
</tbody>
</table>

<sup>h</sup>All parameters are presented with 95% CIs in parentheses.
<sup>i</sup>Cutpoints were determined using the Youden Index (Youden Index = sensitivity + specificity - 1).
<sup>j</sup>PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.
<sup>k</sup>PPVs and NPVs were based on estimates that 5.7% of CM and SMA patients at Mulaoo hospital died of the malaria infection [28].
6.4.5 Predicting fatality using classification tree analysis

To explore another combinatorial strategy, we used classification tree analysis, which selects and organizes independent variables into a decision tree that optimally predicts the dependent measure. Initially, a model based on IP-10 and sTREM-1 was generated with 43.5% sensitivity and 100% specificity for predicting mortality (data not shown). Since high sensitivity would be a crucial feature of a prognostic test for severe malaria, we repeated the analysis assigning the cost of misclassifying a death as a survivor as 10 times greater than the cost of misclassifying a survivor as a death. A model based on IP-10, Ang-2, and sICAM-1 was generated (Figure 6.5), with 100% sensitivity and 92.5% specificity for predicting outcome (cross-validated misclassification rate 15.4%, standard error 4.9%). In summary, combining dichotomized biomarkers using a scoring system or a classification tree predicted severe malaria mortality in our sample with high accuracy.
Figure 6.5. Classification tree analysis to predict outcome of severe malaria infection with host biomarkers.

All six biomarkers that discriminated survivors from fatalities were entered into the classification tree analysis. Prior probabilities of survival and death were specified (94.3% and 5.7%, respectively). The cost of misclassifying a true death was designated as 10 times the cost of misclassifying a true survivor. The cut-points selected by the analysis are indicated between parent and child nodes. Below each terminal node (i.e. no further branching), the predicted categorization of all patients in that node is indicated. This model yielded 100% sensitivity, 92.5% specificity, and a cross-validated misclassification rate of 15.4% (standard error 4.9%).
6.5 Discussion

Combinations of prognostic biomarkers, particularly if drawn from distinct pathobiological pathways, have been found to improve predictive accuracy (Morrow and Braunwald 2003). In this study, we demonstrated that simple schemes combining as few as 3 host biomarkers of inflammation and endothelial activation predicted mortality with high accuracy among a group of Ugandan children with severe malaria. These data provide support for the development of prognostic tests for severe malaria based on host biomarker combinations. Moreover, we further characterized WPB exocytosis in malaria infection and identified sFlt-1 and sTREM-1 as novel biomarkers of severe malaria in children, leading to new hypotheses regarding severe malaria pathogenesis.

sFlt-1 is generated by alternative splicing of VEGF receptor-1 mRNA and antagonizes the pro-inflammatory and pro-angiogenic effects of VEGF. Our observation of increased sFlt-1 in severe malaria parallels findings in sepsis patients (Shapiro, Yano et al. 2008). Data from murine models of sepsis suggest that sFLT-1 may have a protective role in this disease, as sFlt-1 administration reduced VEGF-mediated vascular permeability and mortality (Yano, Liaw et al. 2006). In pediatric CM, VEGF levels positively correlated with neurological complications (Casals-Pascual, Idro et al. 2008). Thus, elevated sFlt-1 in severe malaria may represent a host response to counter the pathological effects of excess VEGF.

We also identified sTREM-1 as a novel biomarker of severe and fatal malaria. The TREM-1 receptor is expressed on the cell membrane of monocytes and neutrophils and mediates pro-inflammatory responses (Bouchon, Dietrich et al. 2000). sTREM-1 is generated by cleavage of membrane TREM-1 upon myeloid cell activation (Gomez-Pina, Soares-Schanoski et al. 2007). Both membrane and soluble TREM-1 are increased in inflammatory pathologies in humans (Ford and McVicar 2009), and inhibition of TREM-1 improves outcome in murine sepsis models (Bouchon, Facchetti et al. 2001). Together, these findings raise the possibility that TREM-1 may contribute to the excessive inflammation characteristic of severe malaria. Interestingly, a recent report demonstrated increased levels of monocyte TREM-1 in uncomplicated malaria cases compared to uninfected individuals (Chimma, Roussilhon et al. 2009). Further studies are required to delineate the roles of sTREM-1 and sFlt-1 in severe malaria.
There were some discrepancies between our data and previous studies of biomarkers in pediatric severe malaria. s-endoglin was found to be increased in Gabonese children with severe malaria compared to UM (Dietmann, Helbok et al. 2009), but we did not replicate these results. We observed similar levels of IP-10 in CM and SMA fatalities, in contrast to a report that serum IP-10 was specifically elevated in Ghanaian children who died from CM (Armah, Wilson et al. 2007). However, these studies may not be comparable since blood was obtained post-mortem in the Ghanaian study rather than at admission.

As previously described (Holestelle, Donkor et al. 2006; Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009), we observed increased plasma levels of WPB components Ang-2, vWF, and vWF propeptide in CM vs. UM. We also demonstrated for the first time that these factors are specifically elevated among children with SMA, suggesting that extensive WPB exocytosis occurs not only in CM but also in SMA. Few studies have directly addressed endothelial activation in SMA (Tchinda, Tadem et al. 2007). WPB exocytosis can be induced by factors generated during malaria infection (e.g., cytokines, histamine, reactive oxygen species) that may be more elevated in SMA than UM (Othoro, Lal et al. 1999; Greve, Kremsner et al. 2000). It is biologically plausible that increased circulating levels of WPB contents could directly contribute to the pathogenesis of SMA. Ang-2 sensitization of endothelial cells to TNF (Fiedler, Reiss et al. 2006) may amplify secretion of endothelial cytokines, such as IL-6, that can promote anemia (Raj 2009). Interestingly, Ang-2 can impair maintenance of long-term hematopoietic stem cells (LT-HSCs) in bone marrow by inhibiting the Tie-2/Ang-1 interaction (Gomei, Nakamura et al. 2010). While the role of LT-HSCs in SMA requires clarification, it is interesting to speculate that dysregulated Ang-2 levels may contribute to anemia via LT-HSC depletion.

Regardless of whether these biomarkers mediate or simply reflect pathology, combinations of biomarkers accurately predicted mortality among children with severe malaria in our sample. Notably, some biomarker combinations showed excellent sensitivity, ensuring that the majority of children at high risk of death would be identified. While an effective adjunctive therapy for severe malaria remains elusive, prognostication could allow triage of patients for closer monitoring or intensive care resources, as available. Such a test may also assist in risk stratification and patient selection for clinical trials of adjunctive therapies, which are ongoing (Yeo, Lampah et al. 2007; Boggild, Krudsood et al. 2009).
Previous studies have developed clinical scores to prognosticate outcome in pediatric severe malaria (Marsh, Forster et al. 1995; Helbok, Kendjo et al. 2009). These scores incorporate clinical features such as prostration, coma, and respiratory distress. The simplicity and low costs of these tests are attractive features. However, a prognostic assay would ideally predict mortality with both high sensitivity and specificity based on a single criterion to avoid the uncertainty associated with non-extreme scores. The biomarker combinatorial strategies presented here appear to possess this attribute, although further studies are required to confirm our findings. Another advantage of a biomarker-based prognostic test over clinical assessment is its objective quality that is unaffected by between-clinician variability. Furthermore, advances in point-of-care platforms (Lee, Kim et al. 2009) may enable development of affordable tests that integrate malaria diagnostics with prognostic biomarkers.

The limitations to our study include a small sample size and the use of non-consecutive samples, which may have introduced a selection bias and inflated biomarker performance characteristics. Biomarker combinations that accurately predicted mortality require validation in larger prospective studies with adjustment for potential demographic and clinical confounders and head-to-head comparison with prognostic clinical scores. Furthermore, these combinations require validation across different ethnicities and malaria endemicities, as well as in children with non-CM/non-SMA severe malaria syndromes. Nevertheless, this study identified novel biomarkers in African children, who are at the greatest risk of malaria mortality, and specifically in SMA, for which few informative biomarkers have been described. We provide proof-of-concept that combining as few as 3 biomarkers using simple schemes may be able to accurately predict outcome in severe malaria infection.
Chapter 7
Angiopoietin-2 predicts mortality in a case-control study of Malawian children with cerebral malaria

7.1 Abstract

Background

The mortality due to cerebral malaria (CM) remains high despite the availability of effective antimalarial drugs. The ability to identify critical pathways of pathogenesis in CM is limited by the non-specific clinical diagnosis and high rate of incidental parasitaemia in malaria endemic areas. Malaria retinopathy has enabled better classification of children and enhanced our understanding of malaria pathogenesis.

Methods and Findings

This study represents a retrospective case-control design (n=155) examining how biomarkers of endothelial activation relate to retinopathy and mortality in children with clinical cerebral malaria. Levels of Angiopoietin (Ang)-1, Ang-2 and a soluble version of their receptor, sTie-2, were measured in plasma taken at time of admission. We find decreased Ang-1 and increased Ang-2 and sTie-2 in children with retinopathy compared to those without (Ang-1, p=0.0182; Ang-2, p<0.0001; sTie-2, p<0.0001). Ang-2 and sTie-2 remained independent predictors of retinopathy following dichotomization and adjustment for covariates (adjusted Odds ratio (95% CI), p-value: Ang-2, 4.3 (1.3-14.6), p=0.019; sTie-2, 9.7 (2.1-45.8), p=0.004). We were able to correlate biomarker levels with severity scores of the retinal abnormalities (haemorrhage, whitening, vessel changes) and found significant relationships between Ang-2 and sTie-2 with retinal whitening and vessel whitening (p<0.01 for all). We also investigated the prognostic ability of our biomarkers. Ang-2 and sTie-2 were significantly elevated in children at presentation who subsequently went on to die, and had comparable areas under the receiver operator characteristic curve (AUC) to lactate- a known prognostic marker in severe malaria (AUC (95% CI), p-value: Ang-2, 0.71 (0.63-0.68), p<0.0001; sTie-2, 0.64 (0.55-0.73), p<0.0001; lactate, 0.67 (0.59-0.78)). After inclusion in a multivariable logistic regression model, low Ang-1 and high Ang-2 were significantly associated with increased odds of death (adjusted
Odds ratio (95% CI): Ang-1, 2.5 (1.0-5.9), p=0.045; Ang-2, 3.9 (1.2-12.7), p=0.024). We further explored the utility of biomarkers to predict death using Kaplan-Meier analysis and classification and regression trees (CRT) and found Ang-2 to be the best predictive biomarker (Cut-off: 3.85ng/mL; p<0.0001 log-rank test). Finally, Ang-2 was informative when combined with known clinical parameters to improve classification of patients using either logistic regression or CRT.

**Conclusions**

These results provide insight into the endothelial dysfunction in cerebral malaria and demonstrate that Ang-2 is a promising predictive biomarker in cerebral malaria.
7.2 Introduction

Recent estimates put the global burden of malaria at approximately 250 million infections and 1 million deaths annually, with over 90% of cases attributed to *Plasmodium falciparum* and 85% of the mortality burden affecting children under the age of five (WHO 2009). Pathogenicity of cerebral malaria (CM) is mediated, in part, by the cytoadherence of mature stages of the parasite to the host endothelium via *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) to various host receptors (such as ICAM-1) in the brain. Severe malaria is characterized by the sequestration of parasites in major organs; and a report of parasite burden from fatal CM cases demonstrated the highest density of sequestration of parasites in the brain, intestine, and skin respectively (Seydel, Milner et al. 2006).

During malaria infection, the mechanisms leading to coma are incompletely understood. However, the brain endothelium, which represents the interface between the brain parenchyma and the intravascular compartment (containing sequestered parasites), likely plays a significant role during infection. In 75% of fatal CM cases, sequestration was associated with intravascular and perivascular pathology (Taylor, Fu et al. 2004). Additional findings include diffuse oedema, petechial haemorrhages in the brain parenchyma surrounding ruptured vessels, and ring haemorrhages. Initial CT findings are supportive of the clinicopathological findings seen at autopsy (Newton, Peshu et al. 1994; Potchen, Birbeck et al. 2009). Data from children with CM further suggest that acute endothelial activation is central to the pathophysiology of CM, as markers of endothelial activation, including endothelial microparticles, vonWillebrand factor, soluble cell-adhesion molecules (sCAMs), and endothelial regulator angiotropin-2 (Ang-2) are increased in malaria and have been positively correlated with disease severity (Jakobsen, Morris-Jones et al. 1994; Turner, Ly et al. 1998; Combes, Taylor et al. 2004; Hollestelle, Donkor et al. 2006; Tchinda, Tadem et al. 2007; Yeo, Lampah et al. 2008; Conroy, Lafferty et al. 2009; Larkin, de Laat et al. 2009).

Differentiating CM from other conditions that result in altered consciousness is challenging due to the high prevalence of incidental parasitaemia in malaria endemic areas and the non-specific clinical presentation (fever, coma, convulsions) (Taylor, Fu et al. 2004). Post-mortem examination revealed that almost one-quarter of children meeting the WHO case definition for
CM had been misclassified and were found to have an alternative cause of death by post-mortem examination (Taylor, Fu et al. 2004). Malaria retinopathy: consisting of haemorrhages, retinal whitening, or vessel abnormalities, has been shown to have a sensitivity of 95% and a specificity of 90% for identifying “true” CM using post-mortem examination as the gold standard (Beare, Taylor et al. 2006). Prospective studies from Malawian children with cerebral malaria show a prevalence of retinopathy around 60% in children with severe malaria (Beare, Southern et al. 2004). Data from retinal angiography is able to provide insight into the pathogenesis of retinopathy and CM: retinal whitening corresponds to areas of non-perfusion; vessel abnormalities relate to non-perfusion of capillaries or vessels (white) or to areas of reduced flow (orange); and haemorrhaging corresponds to loss of blood-retinal-barrier integrity. While data from angiography, autopsy specimens, and cerebral spinal fluid composition are supportive of blood-tissue-barrier dysfunction in CM, fluorescein angiography demonstrates it to be focal, relatively minor, and not associated with haemorrhages (Brown, Rogerson et al. 2001; Beare, Harding et al. 2009).

The mortality rate in cerebral malaria remains high (>10%), despite the initiation of appropriate anti-malarial therapy. Prognostic biomarkers that could identify individuals at risk of poor outcomes would enable better allocation of resources and could select a high-risk group, in whom novel treatment modalities could be evaluated. Based on the central role of the endothelium in mediating disease severity in malaria infection, we hypothesized that endothelial regulatory proteins Ang-1, Ang-2, and their soluble receptor, sTie-2, would relate to retinopathy and mortality in CM. We show that decreases in Ang-1, and increases in Ang-2 and sTie-2 are associated with retinopathy, clinical markers of disease severity, and mortality. Finally, we show that Ang-2 is a useful prognostic biomarker on its own or in combination with clinical findings at admission.
7.3 Methods

7.3.1 Ethics

Ethical approval for this study was granted from The College of Medicine Research Committee in Blantyre, Malawi (COMREC) and all parents or guardians gave written informed consent for children to enter the study.

7.3.2 Study Population

Children between 6 months and 14 years of age presenting with fever to the Queen Elizabeth Central hospital (QECH) in Blantyre, Malawi were prospectively recruited between 1997 and 2009. Admission EDTA plasma samples were obtained from children after their parents or guardians had given their informed consent. Archived samples collected as part of ongoing prospective studies of the pathogenesis and management of CM were used to assess the relationship between endothelium regulatory proteins (Ang-1, Ang-2, sTie-2) and retinopathy and mortality. This study represents a retrospective case control design of children with clinically defined cerebral malaria with retinopathy and mortality as outcome measures. Samples were selected based on the outcomes measures, the date of admission and sample availability, without any knowledge of clinical details. All participants received standard treatment, including antimalarial and/or antibacterial therapy as indicated, according to Malawian National guidelines. The clinical definition of CM was *P. falciparum* asexual parasitaemia; a Blantyre coma score ≤2 with no improvement following correction of hypoglycaemia, within 30 minutes of cessation of seizure activity, or within 4 hours of admission; and no evidence of meningitis on examination of cerebrospinal fluid.

7.3.3 Ophthalmological Examination

After admission of a child with altered consciousness, the patient’s pupils were dilated by application of topical ophthalmoplegic agents (tropicamide and phenylephrine) and the fundi were examined by direct and indirect ophthalmoscopy. The findings of an ophthalmologist or experienced clinician were recorded on standardized forms. Retinopathy was defined by the presence of any one of the following retinal findings: haemorrhaging, whitening, or vessel changes with or without papilloedema, as previously described (Beare, Taylor et al. 2006;
Clinical grading of retinal changes were recorded as follows: 0-4 for haemorrhages (0=none, 1=mild, 2=moderate, 3=severe, 4=very severe), and 0-3 for papilloedema, retinal whitening, orange vessels, white vessels, and white capillaries (0=none, 1=mild, 2=moderate, 3=severe) (Harding, Lewallen et al. 2006). Papilloedema alone did not constitute retinopathy.

7.3.4 Quantification of Biomarkers

Plasma concentrations of biomarkers Ang-1, Ang-2, and sTie-2 (DuoSets, R&D Systems, Minneapolis, MN) were measured by ELISA as follows. According to the manufacturer’s instructions, capture antibodies were diluted in PBS (Gibco) overnight at 4°C and were washed with PBS 0.05% Tween 20 (Sigma) five times and blocked for a minimum of 2 hours in PBS 1% BSA (reagent diluent). The samples were then diluted in reagent diluent and standard curves were generated using recombinant proteins (R&D Systems). Samples were incubated overnight at 4°C, washed five times and detection antibodies were added according to manufacturer recommended dilutions for 2 hours at room temperature (RT). For Ang-1 and Ang-2, the detection antibodies were resuspended one hour prior to use with 2% heat inactivated goat or mouse serum respectively. The plates were washed (7x) and Extravidin-Alkaline phosphatase (AP) (Sigma) was added 1:1000 to each well for 1 hour at RT. The plates were then washed a final time (7x in PBS 0.05% Tween 20 and 2x in deionized water) before adding the substrate p-nitrophenyl phosphate (pNPP) (Sigma). The plates were read at 405nM using a Dynex Technologies Opsys MR plate reader and concentrations were extrapolated from the standard curve (4-PL) using revelation Quicklink software (v4.04).

7.3.5 Statistics

Tests for comparison of continuous variables were based on the Mann-Whitney U test and Spearman rank correlation coefficient. Comparisons of proportions were based on Pearson chi-square test, linear-by-linear association, or Fisher’s exact test, as appropriate. Odds ratios (OR) were calculated using Pearson Chi-square or logistic regression models to adjust for confounders (p<0.05 by univariable analysis and changed the OR of any biomarker by ≥ 5%). Variables were excluded from multivariate models if >5% of the data was missing (to limit bias and a reduction
of power); the exception was thrombocytopenia, as platelets are a known source of Ang-1. We used Classification and regression tree (CRT) analysis to identify the cut-offs for dichotomizing biomarkers. The prognostic accuracy of biomarkers and individual laboratory findings were assessed using receiver operator characteristic (ROC) curves. The area under the ROC curves (AUC) or c-index was compared using the Delong-Delong Clarke Pearson method (DeLong, DeLong et al. 1988).

Multivariable logistic regression and CRT were used to generate predictive models of mortality. All variables except age were dichotomized prior to inclusion in the logistic regression models to circumvent issues of non-linearity and multicollinearity. Linearity of age with the log odds of the dependent (retinopathy and mortality) was confirmed by including a Box-Tidwell transformation into the model and ensuring that this term was not significant. Models were validated by ensuring the Hosmer-Lemeshow goodness-of-fit test was not significant (p>0.10).

CRT was performed including all significant univariable predictors with the following settings: 50 cases for parent nodes and 25 for child nodes; a prior probability of death of 23.8% and a misclassification cost of 10; and cross-validation with 10 sample folds to generate an estimate of the misclassification rate (when available). Surrogates were used to classify cases for variables with missing values. Analysis was performed with GraphPad Prism v5.0, SPSS v16.0 and MedCalc.

7.4 Results

7.4.1 Patient Characteristics

155 children between the ages of 8 months and 14 years were included in the study. All the children met the WHO case definition for cerebral malaria. There were 50 subjects that were retinopathy negative, 103 that were retinopathy positive, and 2 who died before fundoscopic exams could be performed. Overall, 59 (38.1%) of the children included in the study died compared to a mortality rate of 23.8% for all children admitted with CM to the research ward over the duration of the study (1997-2009). All subjects had plasma samples and clinical characteristics collected at admission. The demographic and clinical characteristics and laboratory findings at admission are summarized in Table 7.1.
Table 7.1. Demographic and clinical characteristics of population at admission

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All (n= 155)</th>
<th>Retinopathy Negative (n= 50)</th>
<th>Retinopathy Positive (n= 103)</th>
<th>P value</th>
<th>Survivors (n=96)</th>
<th>Deaths (n= 59)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, median</td>
<td>34 (27-51)</td>
<td>43 (31- 79)</td>
<td>32 (25- 44)</td>
<td>0.003 †</td>
<td>37 (29-51)</td>
<td>30 (22-51)</td>
<td>0.032 †</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>55.6%</td>
<td>58.0%</td>
<td>53.4%</td>
<td>0.727</td>
<td>55.2%</td>
<td>54.2%</td>
<td>0.996</td>
</tr>
<tr>
<td>Malnutrition, &lt;2SD</td>
<td>23.7%</td>
<td>32.0%</td>
<td>39.0%</td>
<td>0.402</td>
<td>37.9%</td>
<td>33.3%</td>
<td>0.571</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypotension</td>
<td>4.1%</td>
<td>4.1%</td>
<td>4.2%</td>
<td>0.981</td>
<td>3.2%</td>
<td>5.7%</td>
<td>0.468</td>
</tr>
<tr>
<td>Respiratory Distress</td>
<td>45.2%</td>
<td>28.0%</td>
<td>52.4%</td>
<td>0.004 †</td>
<td>34.4%</td>
<td>62.7%</td>
<td>0.001 †</td>
</tr>
<tr>
<td>History of Convulsions</td>
<td>80%</td>
<td>10.0%</td>
<td>33.8%</td>
<td>0.032</td>
<td>15.6%</td>
<td>27.1%</td>
<td>0.082</td>
</tr>
<tr>
<td>Blantyre Coma Score</td>
<td>0</td>
<td>12.0%</td>
<td>12.6%</td>
<td>6.2%</td>
<td>23.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperparasitaemia</td>
<td>12.3%</td>
<td>4.0%</td>
<td>15.5%</td>
<td>0.115</td>
<td>12.5%</td>
<td>11.9%</td>
<td>0.732</td>
</tr>
<tr>
<td>Severe anaemia</td>
<td>21.9%</td>
<td>4.0%</td>
<td>31.1%</td>
<td>&lt;0.0001 †</td>
<td>16.7%</td>
<td>30.5%</td>
<td>0.043 †</td>
</tr>
<tr>
<td>Leucocytosis</td>
<td>36.8%</td>
<td>27.7%</td>
<td>40.0%</td>
<td>0.149</td>
<td>28.0%</td>
<td>52.9%</td>
<td>0.003</td>
</tr>
<tr>
<td>Thrombocytopaenia</td>
<td>77.3%</td>
<td>52.3%</td>
<td>89.7%</td>
<td>&lt;0.0001 †</td>
<td>75.6%</td>
<td>81.0%</td>
<td>0.491</td>
</tr>
<tr>
<td>Hypoglycaemia,</td>
<td>5.8%</td>
<td>2.0%</td>
<td>7.8%</td>
<td>0.155</td>
<td>4.2%</td>
<td>8.5%</td>
<td>0.265</td>
</tr>
<tr>
<td>Hyperlactataemia</td>
<td>62.6%</td>
<td>49.0%</td>
<td>70.0%</td>
<td>0.017</td>
<td>54.7%</td>
<td>83.3%</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* n=147, † n=154, ‡ n=144, † n=132, ‡ n=131
†,‡ included in multivariable logistic regression models as covariates

Variables were dichotomized based on the following: hypotension (pediatric advanced lifesupport guidelines, (reference)); weight for age z-scores (WHO 2007 reference values); hyperparasitaemia (≥500,000 parasites/µL); severe anaemia (<15 % hematocrit); leucocytosis (>12,000 leukocytes/ µL); thrombocytopaenia (<150,000 x 10^3 platelets/ µL); hypoglycaemia (<2.2mmol/L); hyperlactataemia, >5mmol/L; and retinopathy (any one of: hemorrhage, whitening, vessel changes).

7.4.2 Biomarkers of endothelial activation are associated with malaria retinopathy

Based on the hypothesis that the endothelium is central to the pathophysiology of retinopathy, markers of endothelial activation were measured in children with retinopathy compared to those without. Ang-1 was decreased (p=0.018), and Ang-2 and sTie-2 were increased (p<0.0001) in subjects with retinopathy compared to those without retinopathy (Figure 7.1). Venous lactate was also elevated in children with malarial retinopathy (p=0.0035). The association of demographic, clinical and laboratory findings with retinopathy were evaluated: children with malaria retinopathy were significantly younger, and were more likely to have respiratory distress, severe anaemia and thrombocytopaenia compared to children without retinopathy (Table 7.1).
Figure 7.1. Endothelial biomarkers and lactate are associated with retinopathy in children with cerebral malaria.

Bar graphs showing the median and scatter of endothelial biomarkers and venous lactate. (A) Ang-1 (ng/mL), (B) Ang-2 (ng/mL), (C) sTie-2 (ng/mL), and (D) venous lactate (mmol/L) were measured in parasitemic children by elisa with (n= 103) or without (n= 50) malarial retinopathy as defined by the presence of haemorrhage, retinal whitening or vessel changes. Analysis by Mann-Whitney (U statistic, p-value), *significant after Holms correction for 5 pair-wise comparisons: (A) Ang-1 (1969, p=0.0184)*, (B) Ang-2 (1288, p<0.0001)*, (C) sTie-2 (1258, p<0.0001)*, and (D) venous lactate (1358, p=0.0035)*.
The relationship between endothelial biomarkers and retinopathy was further explored following dichotomization of biomarkers. Positive biomarker tests (Ang-1 ≤ 6.76ng/mL, Ang-2 > 3.85ng/mL, sTie-2 >67.8ng/mL) were associated with increased odds of having retinopathy (Table 7.2: Odds ratio (OR) (95% CI), p-value: Ang-1, 5.9 (2.7-12.8), p<0.001; Ang-2, 10.6 (4.6-24.6), p<0.001; sTie-2, 11.7 (3.9-35.0), p<0.001). Ang-2 and sTie-2 remained independent predictors of retinopathy after adjusting for covariates (age, respiratory distress, severe anaemia, thrombocytopenia).

### Table 7.2. Odds ratios (OR) for positive biomarker tests in retinopathy and mortality

<table>
<thead>
<tr>
<th>Retinopathy</th>
<th>Mortality ¶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Decreased Ang-1</td>
<td>5.9 (2.7-12.8)</td>
</tr>
<tr>
<td>Increased Ang-2</td>
<td>10.6 (4.6-24.6)</td>
</tr>
<tr>
<td>Increased Tie-2</td>
<td>11.7 (3.9-35.0)</td>
</tr>
</tbody>
</table>

Endothelial biomarkers dichotomized by CRT: Ang-1 (Cut-point: <=6.76 ng/mL †, <=5.89 ‡), Ang-2 (Cut-point: >3.85 ng/mL), sTie-2 (Cut-point: >67.80 ng/mL).

* Adjusted P values: multivariable logistic regression model adjusting for covariates; † age, respiratory distress, severe anaemia, thrombocytopenia; ‡ age, respiratory distress, Blantyre coma score, severe anaemia. See supplementary Table 1 and 2 for model validation.

### 7.4.3 Relationship between specific retinal changes and endothelial activation

The breakdown of retinal changes for this population is described in Figure 7.2. Whitening (94.5%) was the most common retinal change followed by vessel changes (76.9%) and haemorrhages (70.3%). Less than 10% of children had a single retinal change recorded, while 51.6% of children had all three retinal changes. Papilloedema alone did not constitute retinopathy. Only one child had papilloedema without other retinal changes; whereas 33% (n=30) of retinopathy positive children had papilloedema, and 53.3% (n=16) of the children with retinopathy and papilloedema had all three changes (haemorrhages, whitening and vessel changes).
Figure 7.2. Venn-diagram depicting the distribution of retinal changes in the study population

A Venn diagram depicting the distribution of retinal changes according to the number of children with haemorrhages (H), retinal whitening (W), or vessel abnormalities (V: orange vessels, white vessels, white capillaries). Data are presented as percentages followed by the number. Detailed classification of retinal changes was available for 91 of the 103 retinopathy positive children. A total of 70.3% children had haemorrhages (n=64), 94.5% had retinal whitening (n=86) and 76.9% had vessel abnormalities (n=70). There was significant overlap between the various components of retinopathy with 51.6% of the children having all three retinal changes.
We assessed the severity of retinal changes using a clinical score (0- none, 1- mild, 2- moderate, 3- severe and 4- very severe). Ang-2 and sTie-2 were positively associated with the number of haemorrhages, the severity of retinal whitening, and the number and extent of white vessels and capillaries (Table 7.3; p<0.01). Venous lactate was associated with retinal whitening (p<0.01) and orange vessels (p<0.05), and Ang-1 was negatively correlated with orange vessels (p<0.05). Ang-2 was negatively associated, albeit weakly, with papilloedema (p<0.05).

Table 7.3. Angiopietins and lactate correlate with the severity score of retinal abnormalities

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Haemorrhage</th>
<th>Whitening</th>
<th>Orange vessels</th>
<th>White vessels</th>
<th>White capillaries</th>
<th>Papilloedema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1</td>
<td>-0.082</td>
<td>-0.161</td>
<td>-0.188*</td>
<td>0.015</td>
<td>-0.051</td>
<td>-0.038</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.328**</td>
<td>0.448**</td>
<td>0.081</td>
<td>0.254**</td>
<td>0.296**</td>
<td>-0.212*</td>
</tr>
<tr>
<td>sTie-2</td>
<td>0.309**</td>
<td>0.431**</td>
<td>0.111</td>
<td>0.222**</td>
<td>0.284**</td>
<td>-0.107</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.135</td>
<td>0.270**</td>
<td>0.180*</td>
<td>0.131</td>
<td>0.138</td>
<td>-0.117</td>
</tr>
</tbody>
</table>

Spearman’s rho correlating biomarker levels with clinical grading score of retinal components. Haemorrhage (0=none, 1= mild, 2=moderate, 3=severe, 4=very severe); Whitening, orange vessels, white vessels, white capillaries, and papilloedema (0=none, 1=mild, 2=moderate, 3=severe). *p<0.05, **p<0.001

7.4.4 The angiopietin-Tie-2 system is associated with disease severity

The relationship between the endothelial biomarkers and disease severity were investigated using two-way correlations between the biomarkers (Ang-1, Ang-2 and sTie-2) and clinical and laboratory characteristics taken at admission.
Table 7.4). Ang-1 was positively correlated with haematocrit and platelet count (p<0.01) and negatively associated with the Blantyre Coma Score (p<0.05). Increased sTie-2 was associated with an increase in respiratory rate and white blood cell count (p<0.05) and with a decrease in hematocrit (p<0.001); increased sTie-2 was uniquely associated with a decrease in blood glucose (p<0.05). Increased Ang-2 was associated with a younger age (p<0.05), and decreased weight-for-age Z score (p<0.05), Blantyre coma score (p<0.05), haematocrit (p<0.001) and platelet count (p<0.001). Finally, increases in Ang-2 were positively associated with pulse (p<0.01), respiratory rate (p<0.001), parasitaemia (p<0.001), white blood cell count (p<0.01) and venous lactate (p<0.001).
Table 7.4. Correlation of the angiopoietin-Tie-2 system with clinical characteristics at admission

<table>
<thead>
<tr>
<th></th>
<th>Ang-1</th>
<th>Ang-2</th>
<th>sTie-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (months)</td>
<td>0.032</td>
<td>-0.162*</td>
<td>-0.111</td>
</tr>
<tr>
<td>Weight-for-age z score</td>
<td>-0.096</td>
<td>-0.164*</td>
<td>-0.102</td>
</tr>
<tr>
<td>Temperature (C)</td>
<td>0.072</td>
<td>-0.038</td>
<td>-0.018</td>
</tr>
<tr>
<td>Pulse</td>
<td>-0.068</td>
<td>0.207**</td>
<td>0.098</td>
</tr>
<tr>
<td>Systolic Blood Pressure</td>
<td>0.113</td>
<td>-0.088</td>
<td>-0.154</td>
</tr>
<tr>
<td>Respiratory Rate</td>
<td>-0.015</td>
<td>0.309***</td>
<td>0.166*</td>
</tr>
<tr>
<td>Blantyre Coma Score</td>
<td>-0.196*</td>
<td>-0.189*</td>
<td>-0.092</td>
</tr>
<tr>
<td>Parasitaemia</td>
<td>0.092</td>
<td>0.432***</td>
<td>0.133</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.223**</td>
<td>-0.289***</td>
<td>-0.293***</td>
</tr>
<tr>
<td>White Blood Cell Count</td>
<td>0.101</td>
<td>0.351***</td>
<td>0.180*</td>
</tr>
<tr>
<td>Platelet Count</td>
<td>0.308***</td>
<td>-0.351***</td>
<td>-0.154</td>
</tr>
<tr>
<td>Blood Glucose</td>
<td>-0.027</td>
<td>-0.115</td>
<td>-0.182*</td>
</tr>
<tr>
<td>Venous Lactate</td>
<td>-0.088</td>
<td>0.387***</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Spearman’s rho correlating biomarkers with clinical characteristics at time of admission. * p<0.05, **p<0.01, ***p<0.001

7.4.5 Biomarkers of endothelial activation are associated with mortality

We next investigated the hypothesis that markers of endothelial activation would be associated with poor prognosis. Increases in median levels of Ang-2 and sTie-2, but not Ang-1, were associated with a fatal outcome (Figure 7.3, top panel: p<0.0001). Venous lactate was also elevated in children who died (p=0.002). We generated receiver operator characteristic (ROC) curves to assess the prognostic accuracy of the biomarkers. Ang-2, sTie-2 had comparable areas under the ROC (AUC) (p>0.05) with: Ang-2 (AUC (95% CI)), 0.71 (0.63-0.78); sTie-2, 0.64 (0.55-0.73); and venous lactate, 0.67 (0.59-0.78) (Figure 7.3, middle panel). Finally, decision plots were constructed to visualize the trade-off between sensitivity and specificity of the biomarkers at the various cut-offs along the ROC curve (Figure 7.3, bottom panel). The cut-points for each marker, as determined by CRT, are represented in the decision plots (Ang-1 ≤5.89ng/mL, Ang-2 > 3.85ng/mL, sTie-2 >67.8ng/mL).
Endothelial markers and venous lactate predict mortality. (A-D) Graphs showing the median and scatter of endothelial biomarkers and venous lactate, with the associated (E-F) receiver operator characteristic (ROC) curves and (I-L) decision plots of sensitivity and specificity generated from the ROC curves. Prognostic accuracy was assessed using receiver operator characteristic (ROC) curves and comparing the area under the ROC curve (AUC: 95% CI). Levels of Ang-2 (B, F, J), sTie-2 (C, G, K) and venous lactate (D, H, L) were significantly elevated in children who died compared to those who survived (AUC (95% CI), p-value: Ang-2, 0.71 (0.63-0.78), p<0.0001; sTie-2, 0.64 (0.55-0.73), p=0.003; venous lactate, 0.67 (0.59-0.78), p=0.002). There was no difference in Ang-1 levels (A, E, I) between children who survived and children who died (AUC (95% CI), p-value: 0.56 (0.47-0.65), p=0.20). The AUC were compared between lactate, sTie-2, and Ang-2 and there were no significant differences (method of Delong et al.).
We also investigated the relationship between demographic, clinical and laboratory findings and mortality, and children who died were significantly younger, had lower Blantyre Coma Scores, and were more likely to have respiratory distress, severe anemia, leukocytosis, and hyperlactatemia (Table 7.1). The relationship between endothelial biomarkers and mortality were further explored following dichotomization; and positive biomarker tests were associated with increased odds of death (Table 7.2; Odds ratio (OR) (95% CI), p-value: Ang-1, 2.4 (1.2-5.1), p=0.017; Ang-2, 7.9 (2.6-23.6), p<0.001; sTie-2, 3.2 (1.6-6.3), p=0.001). Ang-2 and Ang-1 remained independent predictors of mortality after adjusting for covariates (age, respiratory distress, Blantyre Coma Score, severe anaemia).

Finally, using Kaplan-Meier curves, we examined the time to death for children stratified by biomarker test results. As shown in Figure 7.4, high Ang-2 (>3.85 ng/mL) is associated with a lower 28 day survival rate (52.6%) compared to those with low Ang-2 levels (≤3.85 ng/mL, 89.7% survival) (Log rank (Mantel Cox): Chi-square, 14.7; p<0.0001).
Children were stratified based on whether they had high Ang-2 levels (>3.85 ng/mL) or low Ang-2 levels (≤3.85 ng/mL) at admission. Children with high Ang-2 levels had lower rates of cumulative survival (52.6%) compared to those with low Ang-2 levels (89.7%) (p<0.0001: Log-rank test).
7.4.6 Incorporating endothelial biomarkers into prognostic clinical models

We used logistic regression and classification and regression tree (CRT) analysis to generate prognostic models using routine clinical parameters and our plasma biomarkers. A clinically predictive model of mortality was generated using parameters that were associated with poor outcome and are readily available to clinicians (age, Blantyre coma score, respiratory distress, severe anemia). We used the predicted probabilities from this clinical model to generate a c-index (equivalent to the AUC) of 0.73 (95% CI: 0.65-0.79). Then, using the clinical model as a foundation, we added in the biomarker tests to determine whether they would significantly improve the predictive ability. The clinical model including all three biomarker had a c-index of 0.79 (0.72-0.84), which is significantly better than the clinical model alone (p=0.03). In order to minimize cost and maximize practicality, we assessed whether a more parsimonious model could be achieved using a single biomarker. Inclusion of Ang-2 alone (but not Ang-1 or sTie-2) into the clinical model is significantly better than the clinical model alone (c-index (95%CI), p-value: 0.78 (0.68-0.82), p=0.02).

Finally, we used CRT as an alternative method to develop a prognostic model for its intuitive approach and easy to interpret decision tree. CRT works by splitting data into two mutually exclusive groups in order to maximize the homogeneity within each “node”. When all variables significantly associated with death are included in the analysis, the program generates a decision tree using Ang-2 at a cut-off of 3.85ng/mL to discriminate between survivors and non-survivors. In this model, Ang-2 levels above 3.85ng/mL correspond to sensitivity to predict death of 93.2% and a misclassification rate of 23.1% (with 10-fold cross-validation) (Figure 7.5A). Next, we explored models where we enter a clinical sign (BCS or respiratory distress) as the first variable to maximize clinical application, since a predictive model should adhere to clinical sensibilities. In both clinically informed models, Ang-2 was used to identify non-survivors in children considered lower risk (BCS 0 or 1; Figure 7.5B, or children without respiratory distress; Figure 7.5C). The model with BCS has comparable sensitivities and specificities to those of Ang-2 alone with a sensitivity of 94.9%, a specificity of 34.4%, and a misclassification rate of 19.8%.
Classification and regression tree models for the prediction of mortality in children with cerebral malaria

Classification and regression tree analysis (CRT) was performed for mortality as the outcome measure including all significant univariable predictors of mortality with following conditions: misclassification cost, 10; cross-validation, 10-fold (if appropriate); prior probability of death, 23.8%; minimum number per child node, 25; and impurity measure, gini criterion. (A) Model generated from all significant independent variables (Ang-2 (cut-point >3.85ng/mL): sensitivity-93.2%, specificity-36.5%, cross-validated misclassification rate (standard error)- 0.231 (0.032); Clinically informed models: (B) Blantyre Coma Score plus Ang-2 (sensitivity-94.9%, specificity-34.4%, misclassification rate (standard error)- 0.198 (0.038); (C) Respiratory distress plus Ang-2 (sensitivity-93.2%, specificity-27.1%, misclassification rate (standard error)- 0.228 (0.035).
7.5 Discussion

In this study we had two main objectives: the first, to examine the role of the angiopoietin-Tie-2 system in the pathophysiology of cerebral malaria; and the second to investigate whether markers of endothelial activation are potential prognostic biomarkers in severe malaria. To this aim, we examined how changes in levels of Ang-1, Ang-2 and sTie-2 related to retinopathy, clinical markers of disease severity and outcome. We demonstrate that perturbations in the angiopoietin-Tie-2 system are independently associated with retinopathy; and levels of Ang-2 and sTie-2 are related to the severity of haemorrhages, retinal whitening and vessels changes in the retina. By comparing biomarker levels with clinical characteristics at admission, we show that Ang-2 is consistently associated with a number of markers of disease severity (including parasitaemia, WBC count, low platelet count, and venous lactate). The prognostic ability of the angiopoietins was also evaluated, and we found elevations in Ang-2 and sTie-2, and decreases in Ang-1 were associated with increased odds of death. Further, Ang-2 on its own was a good predictor of mortality based on Kaplan-Meier and CRT analysis and was informative when used in combination with important clinical markers. Together, these data demonstrate an association between the angiopoietins and the microvascular disturbances involved in cerebral malaria pathogenesis.

Angiopoietins engage the Tie family of receptor tyrosine kinases on the vascular endothelium to exert their effects. The most well characterised members of this family are Tie-2 ligands Ang-1 and Ang-2. Ang-1 is released from mural cells (vascular smooth muscle cells and pericytes) and activated platelets and helps promote endothelial cell survival and helps maintain the integrity of endothelial tight junctions through the phosphorylation of Tie-2 (Milner, Hansen et al. 2009) (Davis, Aldrich et al. 1996). Ang-2 exists pre-stored in endothelial Weibel-Palade (WP) bodies, and is rapidly mobilized and released following activation of the endothelium (Fiedler, Scharpfenecker et al. 2004); Ang-2 is thought to function primarily to oppose Ang-1 function, but can exert partial agonist activity in the absence of Ang-1 (Yuan, Khankin et al. 2009). A soluble form of Tie-2 has been identified in supernatants from cultured endothelial cells, as well as in normal human sera and plasma (Reusch, Barleon et al. 2001). Soluble Tie-2 is shed from endothelial cells as a result of ectodomain cleavage and is stimulated by a variety of physiological factors, including basic fibroblast growth factor and VEGF (Reusch, Barleon et al. 2001).
Recent data suggest that Tie-2 shedding is dependent on matrix metalloproteinase-14 (Onimaru, Yonemitsu et al. 2010). sTie-2 has been identified as a biomarker in a number of conditions: including cancer (Homer, Greenman et al. 2002; Chin, Greenman et al. 2003; Figueroa Vega, Diaz et al. 2010), sepsis (van der Heijden, van Nieuw Amerongen et al. 2010), and ischemia (Findley, Mitchell et al. 2008). To date, the function of sTie-2 is incompletely understood, as Tie-2 can bind several ligands with both activating (Ang-1, Ang-4) and inhibitory (Ang-2) functions; however, its association with cancer metastasis suggests a role for sTie-2 in regulating angiogenesis (Figueroa-Vega, Alfonso-Perez et al. 2009).

As the retina and the brain are formed from the same embryonic tissue and have analogous blood-tissue-barriers, investigations into the pathophysiology of retinopathy can provide critical insights into microvascular and tissue disturbances in the brain; as illustrated by the correlation between the number hemorrhages in the retina to those seen in brain (White, Lewallen et al. 2001). The accessibility of the retina enables clinicians and researchers to use indirect ophthalmoscopy to visualize subtle vascular and tissue changes in the eye, which cannot be appreciated in the brain using standard immunohistochemistry. In addition, fluorescein angiography has been used to visualize the extravasation of fluorescein corresponding to vascular leak (Beare, Harding et al. 2009); the intravascular filling defects with mottling in the blood column associated with parasite sequestration in orange vessels; areas of non-perfusion and hypoxia in white vessels and in retinal whitening; and non-perfusion in hemorrhaging. Retinopathy is often (Lewallen, Bakker et al. 1996) (Olumese, Adeyemo et al. 1997; Beare, Southern et al. 2004), but not always (Olumese, Adeyemo et al. 1997), associated with worse outcome in cerebral malaria.

In this study, retinopathy was associated with a younger median age, respiratory distress, history of convulsions, severe anemia, thrombocytopenia and increased lactate (Figure 7.1, Table 7.2). By univariable analysis, Ang-1, Ang-2 and sTie-2 were associated with retinopathy; however, the relationship between Ang-1 and retinopathy was lost following adjustment for thrombocytopenia. We saw decreased platelet counts in retinopathy positive children, which is consistent with other reports of retinopathy in cerebral malaria (Chimalizeni, Kawaza et al.; Lewallen, Bronzan et al. 2008). Together, these data suggest that decreased Ang-1 levels in
retinopathy may be a reflection of reduced platelet-derived Ang-1. We postulate that activated platelets bound to the damaged endothelium may release Ang-1 to limit endothelial damage, and thus a decrease in Ang-1 levels in severe disease may reflect both a decrease in platelet number and in platelet Ang-1 content. However, this will need to be confirmed in future studies.

sTie-2 and Ang-2 were associated with a 9.7-fold and 4.3-fold increased odds of retinopathy following adjustment for covariates (Table 2); and were positively associated with the number of haemorrhages, the degree of retinal whitening, and the number of white vessels and capillaries. We hypothesize that Ang-2 may be involved in mediating the pathophysiological processes by: sensitizing the endothelium to sub-threshold levels of TNF (Fiedler, Reiss et al. 2006); promoting increased endothelial cell permeability (Fiedler and Augustin 2006); and promoting the upregulation of cellular adhesion molecules like ICAM-1 (Fiedler and Augustin 2006), which bind to parasitized erythrocytes. In this way, Ang-2 may be promoting a positive feedback loop of endothelial activation, inflammation, and parasite sequestration. It is plausible that sTie-2 levels could then be released in response to Ang-2 as an attempt to normalize the Ang-2: Ang-1 ratio and promote endothelial quiescence. However, we acknowledge that Ang-2 may simply be a reflection of endothelial activation and hypoxia (Abdulmalek, Ashur et al. 2001; Oh, Takagi et al. 1999). Together, these data implicate the angiopoietin-Tie-2 system in the vascular dysfunction seen in cerebral malaria, but further studies will be required to investigate a possible causal role for the angiopoietin-Tie-2 system in the pathology of cerebral malaria.

The second aim of this study was to investigate the relationship between our endothelial biomarkers and prognosis in cerebral malaria. We saw a significant increase in median levels of sTie-2 and Ang-2 in fatal cases, and these biomarkers had comparable AUC to venous lactate (Table 7.3). Following standardized dichotomization of the biomarkers, Ang-1 and Ang-2 remained significant independent predictors of mortality after controlling for covariates (Age, BCS, respiratory distress, severe anemia: Table 7.2). These data support a role for the angiopoietins in the pathogenesis of severe malaria and further suggest that these proteins may be good biomarkers. As Ang-2 was the best predictor, we assessed the ability of Ang-2 levels to discriminate between survivors and non-survivors using a Kaplan-meier curve (Figure 7.4). High Ang-2 levels clearly discriminate between children who live and those who die. These results are
in agreement with those of Yeo et al. and Erdman et al., which show a significant difference between Ang-2 levels and outcome.

The most striking results in this study arise from the prognostic role of Ang-2. By univariable analysis, Ang-2 was as good as lactate (arguably the best biochemical marker for severe malaria in children (Waller, Krishna et al. 1995; Newton, Valim et al. 2005)) at predicting mortality (Figure 7.3). Further, we demonstrate that Ang-2 adds value to a clinical model to predict mortality using logistic regression; and Ang-2 alone, or in combination with clinical parameters, is informative in a classification-based approach to predicting outcome (Figure 7.5). Previously, lactate was evaluated to see whether it would add value to clinical models; however, a multicentre trial determined it did not add value to clinical predictors. Likewise, if we use logistic regression with our clinical model (on the subset of children with lactate measurements available), lactate does not add value to the clinical model, but Ang-2 does. These results suggest that Ang-2 may be an informative biomarkers when used alongside clinical parameters. A number of other studies have found elevated Ang-2 in severe (Matsuyama, Hashiguchi et al. 2000; Yeo, Lampah et al. 2008; Conroy, Lafferty et al. 2009; Lovegrove, Tangpukdee et al. 2009) and fatal malaria (Yeo, Lampah et al. 2008; Erdman, Dhabangi et al.).

Our data, together with previous reports, suggest that Ang-2 is a robust, quantitative and objective marker of malaria severity that may have utility in clinical practice and/or trials of novel therapeutic agents. Ang-2 is elevated in severe compared to uncomplicated malaria and healthy controls across a range of ethnicities and ages (Yeo, Lampah et al. 2008) (Lovegrove, Tangpukdee et al. 2009) (Conroy, Lafferty et al. 2009), mirrors clinical progression or deterioration when measured longitudinally over the course of illness (Yeo, Lampah et al. 2008), and predicts mortality independently of clinical signs. Given its recognized role as a mediator of endothelial quiescence and activation, Ang-2 may be useful as a surrogate endpoint in clinical trials of adjunctive therapies for malaria that target the host endothelium (Yeo, Lampah et al. 2008), as well as a promising molecular target for development of “theranostic” tools.

The results of this study, while promising, must be interpreted with caution. The retrospective study design and use of convenience samples may result in the inclusion of bias. Further, this study only includes children with well-defined cerebral malaria. While this homogeneity in study
participants enables us to better investigate the role of the biomarkers in disease pathogenesis, it reduces the generalisability of the data. These results will need to be confirmed prospectively in larger- preferably multicentre- trials. Nonetheless, the data suggest that: i) the angiopoietin-Tie-2 system is involved in CM pathogenesis, and may be a target for adjunctive therapy; and ii) Ang-2 is a promising prognostic biomarker in severe malaria.
Chapter 8
Nitric oxide for the adjunctive therapy of severe malaria: hypothesis and rationale

8.1 Abstract

We hypothesize that supplemental inhaled nitric oxide (iNO) will improve outcomes in children with severe malaria receiving standard antimalarial therapy. The rationale for the hypothesized efficacy of iNO rests on: (1) biological plausibility, based on known actions of NO in modulating endothelial activation; (2) pre-clinical efficacy data from animal models of experimental cerebral malaria; and (3) a human trial of the NO precursor L-arginine, which improved endothelial function in adults with severe malaria. iNO is an attractive new candidate for the adjunctive treatment of severe malaria, given its proven therapeutic efficacy in animal studies, track record of safety in clinical practice and numerous clinical trials, inexpensive manufacturing costs, and ease of administration in settings with limited healthcare infrastructure. We plan to test this hypothesis in a randomized controlled trial (ClinicalTrials.gov Identifier: NCT01255215).
8.2 Introduction

Malaria causes approximately 800,000 deaths annually, mostly among children in sub-Saharan Africa (Kamijo, Le et al. 1993). Although the use of artemisinin-based antimalarial therapy has improved outcomes in severe malaria, the mortality rates remain high (Dondorp, Nosten et al. 2005). Adjunctive therapies that target the underlying pathophysiology of severe malaria may further reduce morbidity and mortality in severe malaria (Serghides, Patel et al. 2009). Nitric oxide (NO) is an attractive, as yet untested, potential adjunctive treatment for severe malaria because it modulates endothelial activation, a critical pathway in the pathogenesis of severe malaria. Based on promising pre-clinical data from animal models (Gramaglia, Sobolewski et al. 2006) and a human trial using the NO precursor L-arginine (Yeo, Lampah et al. 2007), together with its established record of safety in clinical practice, a clinical trial evaluating nitric oxide for the adjunctive treatment of severe malaria is warranted.

The most common life-threatening clinical syndromes associated with *P. falciparum* infection in children are cerebral malaria (CM), respiratory distress with metabolic acidosis, and severe malarial anemia (WHO, 2000). Critical pathogenic mechanisms in these severe malaria syndromes represent potential targets for adjunctive therapies, including nitric oxide. CM presents as a diffuse symmetrical encephalopathy with altered level of consciousness and/or repeated seizures (WHO, 2000). The pathogenesis of CM is incompletely understood but may be due to one or more of the following mechanisms: sequestration of parasitized erythrocytes within the cerebral microvasculature (MacPherson, Warrell et al. 1985; Aikawa, Iseki et al. 1990; Turner 1997), reduction in microvascular flow (Idro, Jenkins et al. 2005), metabolic alterations including hypoglycemia and hypoxia, host inflammatory response (Kwiatkowski, Hill et al. 1990; Brown, Turner et al. 1999; Akanmori, Kurtzhals et al. 2000; Lyke, Burges et al. 2004), blood-brain barrier dysfunction (Brown, Chau et al. 2000; Brown, Rogerson et al. 2001), and cerebral edema (Newton, Peshu et al. 1994; Looareesuwan, Wilairatana et al. 1995). In African children with severe malaria, lactic acidosis secondary to impaired tissue perfusion appears to explain most cases of respiratory distress, characterized by deep (Kussmaul) respirations. Respiratory distress therefore represents a manifestation of decompensated shock, frequently associated with multi-system organ failure and widespread endothelial dysfunction. Severe malarial anemia is caused by both increased destruction of parasitized and non-parasitized
erythrocytes, as well as impaired hematopoiesis (McDevitt, Xie et al. 2004). Our hypothesis focuses on two of the three common manifestations of severe pediatric malaria: cerebral malaria and respiratory distress with metabolic acidosis. We postulate that nitric oxide may modulate the deleterious host responses that characterize these syndromes, including systemic inflammation and endothelial dysfunction.

8.3 The Hypothesis

We hypothesize that Ugandan children with severe malaria will benefit from adjunctive iNO in addition to standard anti-malarial therapy. We will test this hypothesis by comparing the change of angiopoietin-2 (Ang-2), an objective and quantitative biomarker of malaria severity, measured longitudinally over the hospital admission, between two parallel groups randomized to receive iNO or placebo (primary outcome). We will also compare relevant clinical outcomes including therapeutic efficacy, safety and neurocognitive outcome (secondary outcomes). The secondary hypotheses are that adjunctive iNO will reduce mortality, accelerate recovery times, and shorten length of hospital stay in severe malaria. Furthermore, we hypothesize that iNO will ameliorate biomarkers of host response to severe malaria including whole blood lactate without affecting parasite clearance. We hypothesize that iNO will reduce the rate of adverse neurocognitive sequelae following severe malaria. Finally, we hypothesize that adjunctive iNO will be safe and well tolerated in children treated for severe malaria. We plan to test these hypotheses in a resource-constrained field setting in Uganda.

8.4 Rationale

8.4.1 Endogenous NO synthesis and regulation

NO is a gaseous free radical that is endogenously produced by the conversion of L-arginine and molecular oxygen to L-citrulline by members of the NOS family of enzymes. Three isoforms have been described to date: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Tissue expression of nNOS is primarily in neurons of the brain and peripheral nervous system and eNOS is expressed mainly in epithelial cells, although these isoforms also operate in the immune system. Active NOS is a tetramer of two NOS proteins and two calmodulin molecules. Cofactors for the enzyme include (6R)-tetrahydrobiopterin (BH₄), FAD, FMN and iron protoporphyrin IX (haem).
Both nNOS and eNOS are constitutively expressed, but remain inactive in resting cells, regulated by intracellular calcium flux. Increased free intracellular calcium stabilizes the interaction of calmodulin to nNOS or eNOS and stimulates the production of NO. This form of regulation leads to transient and short-lasting production of NO, which functions in neuronal signalling and vasodilation. In contrast, iNOS is found in most resting cells, has calcium-independent activity due to tight binding to calmodulin even at low levels of intracellular calcium, and produces high levels of NO for prolonged periods of time. Although all three NOS isoforms have similar NO production rates (~1 µM min⁻¹ mg⁻¹), iNOS is responsible for high-level production of NO by phagocytes because it is highly expressed after activation (Bogdan 2001). Expression of iNOS in inflammatory and tissue cells is upregulated by exposure to microbial products such as lipopolysaccharide (LPS) and dsDNA or cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and interferon-γ (IFN-γ) (Bogdan 2001; Korhonen, Lahti et al. 2005). Enzyme levels are regulated at the level of transcription as well as mRNA stability (Bogdan 2001).

**8.4.2 Physiologic actions of NO**

NO is a highly reactive molecule due to its unpaired electron, mediating a range of effects through three main molecular mechanisms. First, NO reacts readily with transition metals such as iron, copper and zinc, thereby modifying the function of numerous enzymes. Second, NO reacts with thiol groups (e.g., cysteine residues) abundantly present on many proteins to produce S-nitrosothiols, and alters the function of several proteins including p21ras, coxsackievirus protease A2, as well as transcription factors, kinases involved in signaling cascades, caspases, ion channels, and metabolic proteins (Korhonen, Lahti et al. 2005). Third, nitric oxide reacts rapidly with superoxide anion (O₂⁻) to produce peroxynitrite which is a powerful oxidant, capable of modifying proteins, lipids and nucleic acids. Peroxynitrite plays an important microbicidal role; however, excessive peroxynitrite formation may lead to cytotoxicity through nitration of proteins and inhibition of mitochondrial respiration.

NO has a half-life of several seconds. It can readily diffuse across cell membranes into neighbouring cells, acting as an intercellular messenger. In addition, NO may produce effects distant from its site of production transported by vehicles such as low-molecular weight S-nitrosothiols, S-nitrosylated proteins including haemoglobin and albumin, and nitrosyl-metal complexes which liberate NO spontaneously or after cleavage by ectoenzymes (Bogdan 2001).
The activity of NO \textit{in vivo} can be monitored indirectly by measurement of the stable byproducts of NO oxidation, nitrite (NO$_2^-$) and nitrate (NO$_3^-$), collectively termed NO$_x$. Dietary factors, exercise and renal insufficiency affect levels of NO$_x$ in body fluids such as plasma and urine, and need to be controlled in comparative studies. Other measures of NO activity are functional (e.g., the NO-mediated reactive hyperaemia peripheral arterial tonometry (RH-PAT) index), or biochemical (e.g., levels of cGMP, a downstream signalling molecule) (Baylis and Vallance 1998).

One important molecular target of NO is soluble guanylate cyclase (sGC), an enzyme containing a heme moiety with ferrous iron. Formation of a ferrous-nitrosyl-heme complex alters the porphyrin ring structure and leads to activation of sGC with a 400-500 fold increase in the rate of cGMP synthesis (Korhonen, Lahti et al. 2005). Intracellular signalling by cGMP is mediated to a large extent by cGMP dependent protein kinase (PKG) which promotes smooth muscle relaxation, as well as platelet and neutrophil activation.

Through its action on numerous target molecules, NO is involved in a broad range of physiologic processes. NO was identified originally as the endothelium-derived relaxation factor that mediates vasodilation (Palmer, Ferrige et al. 1987). Subsequent studies have demonstrated a role for NO in platelet aggregation (Moncada, Palmer et al. 1991), endothelial-cell activation (Matsushita, Morrell et al. 2003), apoptosis, inflammation, chemotaxis, neurotransmission and antimicrobial defense.

### 8.5 NO in infectious disease

NO plays a complex and versatile role in the pathogenesis and control of infectious diseases. Protective and toxic effects of NO are frequently seen in parallel in the setting of infection because of its variety of molecular targets, widespread production by diverse cell types, and broad capacity for intra- and intercellular signalling (Bogdan 2001).

NO has direct microbicidal activity against numerous viral, bacterial and parasitic agents. The mechanism of action may involve mutation of DNA; inhibition of DNA repair and synthesis; inhibition of protein synthesis; alteration of proteins by S-nitrosylation, ADP-ribosylation or tyrosine nitration; inactivation of iron, copper or zinc-dependent enzymes; and peroxidation of
lipid membranes (Bogdan 2001). Peroxynitrite (ONOO\(^{-}\)), a reaction product of NO and superoxide anion (O\(_2\)^{-}), may mediate these effects. Highlighting the central role of ONOO\(^{-}\) in host defense, successful human pathogens, including \textit{M. tuberculosis} and \textit{S. typhimurium}, possess counteracting peroxiredoxins that detoxify ONOO\(^{-}\) to nitrite (Bryk, Griffin et al. 2000).

NO attenuates neutrophil respiratory burst and neutrophil-derived oxidative stress (Gessler, Nebe et al. 1996), and reduces neutrophil rolling and adhesion in microvascular endothelial cells (Kubes, Suzuki et al. 1991). In adaptive immune responses, NO inhibits T-cell and B-cell proliferation (van der Veen 2001). NO alters cytokine responses, down-regulating pro-inflammatory IL-1, IL-2, TNF and IFN-\(\gamma\) and increasing the production of IL-4, IL-13, and transforming growth factor-\(\beta\) (Bogdan 2001).

8.6 NO in malaria

NO plays a role at multiple stages of malaria infection, beginning with the innate defences of the \textit{Anopheles} mosquito vector, where NO antagonizes the \textit{Plasmodium} parasite (Luckhart, Vodovotz et al. 1998). On the other hand, by reversibly binding to salivary proteins (nitrophorins), NO facilitates the mosquito blood meal by enhancing vasodilation and antagonizing hemostasis (Ribeiro, Hazzard et al. 1993).

Despite its potent microbicidal properties against a number of human pathogens (Bogdan 2001), NO does not appear to have a direct effect against \textit{Plasmodium} species. Exogenous NO did not inhibit the growth of \textit{P. falciparum in vitro}, even at saturating concentrations (2 mM) (Rockett, Awburn et al. 1991). Byproducts of NO metabolism, including NO\(_2\), NO\(_3\)^{-} and the nitrosothiol derivatives of cysteine and glutathione, exhibit anti-parasitic activity in vitro (Rockett, Awburn et al. 1991), albeit at concentrations 2 to 3 orders of magnitude higher than those in human plasma during malaria infection (Sobolewski, Gramaglia et al. 2005). In experimental murine infection in vivo, some studies have found no effect of iNOS deficiency or pharmacologic inhibition of NOS on \textit{P. berghei} (Kremsner, Nussler et al. 1993; Favre, Ryffel et al. 1999), \textit{P. chabaudi} (Jacobs, Radzioch et al. 1995; Favre, Ryffel et al. 1999; van der Heyde, Gu et al. 2000; Gillman, Batchelder et al. 2004) and \textit{P. yoelii} (Amante and Good 1997) parasitemia, suggesting a minimal direct anti-parasitic role of endogenous NO, despite important modifying effects on host disease severity (Jacobs, Radzioch et al. 1995; Amante and Good 1997). Likewise, in human
observational studies, NOx levels are not consistently associated with parasitemia, although they are inversely correlated with disease severity (Hobbs, Udhayakumar et al. 2002; Cramer, Nussler et al. 2005).

8.6.1 NO dampens endothelial activation

The vascular endothelium plays a critical role in the pathogenesis of cerebral malaria. Parasitized erythrocytes (PEs) adhere to the microvascular endothelium resulting in sequestration and vascular obstruction, impaired perfusion and tissue hypoxia (Day, Phu et al. 2000). Autopsy studies in fatal cerebral malaria reveal sequestration of PEs in the capillaries and post-capillary venules of multiple organs (MacPherson, Warrell et al. 1985). Cytoadherence is mediated through constitutive and cytokine-inducible receptors on the endothelial cells, including intercellular cell adhesion molecule-1 (ICAM-1) (Turner, Morrison et al. 1994). NO decreases endothelial cell adhesion molecule expression (De Caterina, Libby et al. 1995), and has been shown to reduce the adherence of PEs to endothelial cells in vitro under flow conditions (Serirom, Raharjo et al. 2003).

Severe malaria is characterized by marked activation of the microvascular endothelium. One prominent feature of endothelial activation is the exocytosis of intracellular Weibel-Palade bodies (WPB), causing the release of von Willebrand factor (vWF) (Hollestelle, Donkor et al. 2006; de Mast, Groot et al. 2007) and angiopoietin-2 (Ang-2) (Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009) into the circulation. Ang-2 functions as an autocrine regulator by sensitizing the endothelium to the effects of TNF, resulting in increased adhesion receptor expression (Fiedler, Reiss et al. 2006). NO inhibits the exocytosis of WPB contents through S-nitrosylation of critical regulatory enzymes (Matsushita, Morrell et al. 2003). Thus, Ang-2 serves as critical regulator of endothelial activation.

Elevated Ang-2 levels are associated with poor clinical outcome in severe malaria (Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009) and Ang-2 has been used to follow disease progression and recovery in previous studies of malaria (Yeo, Lampah et al. 2008). Among survivors of severe malaria, Ang-2 levels have been shown to decrease linearly during recovery at a mean rate of 2700 pg/mL per 24h (Yeo, Lampah et al. 2008). Thus, Ang-2 is an objective, quantitative marker of disease severity, validated for longitudinal follow-up of patients with
malaria. As a result, measurement of Ang-2 levels will allow us to test our hypothesis with precision, and we have chosen Ang-2 as the primary outcome of our clinical trial and have

8.6.2 NO reduces inflammatory injury in the pulmonary vascular bed

Pulmonary involvement in adults with severe malaria is associated with high mortality and represents another manifestation of severe malaria for which iNO may be beneficial (Deaton 1970; Taylor, Canon et al. 2006). Mechanisms of lung injury in severe malaria in adults share common features with acute lung injury/adult respiratory distress syndrome (ALI/ARDS) in sepsis. Increased permeability of the capillary-alveolar barrier is considered to be the principal functional abnormality underlying ALI/ARDS due to malaria and other causes. Other key events include erythrocyte sequestration and host inflammatory response to parasite products released into the circulation (Mohan, Sharma et al. 2008). Lung histopathologic and ultrastructural studies from adults with fatal *P. falciparum* infection have found septal and interstitial edema, monocytes and parasitized erythrocytes (PE) adherent to the capillary microvasculature, and endothelial cell cytoplasmic swelling (Duarte, Corbett et al. 1985; MacPherson, Warrell et al. 1985). Similarly, in a murine model of experimental malaria-induced lung injury, disruption of the capillary-alveolar membrane barrier and septal inflammation was observed (Lovegrove, Gharib et al. 2008). Thus, the pulmonary vascular endothelium plays a central role in ALI during malaria infection, as the site of PE and leukocyte adhesion, and the target of parasite-induced inflammation. Endogenous NO inhibits inflammatory injury in murine ALI models, as evidenced by increased vascular leak and pathology in iNOS−/− mice (Speyer, Neff et al. 2003; Zeidler, Millecchia et al. 2004). Some studies have documented that iNO decreases pulmonary capillary pressure through selective vasodilatory effects on post-capillary venules (Benzing and Geiger 1994), and reduces pulmonary edema in patients with ALI (Benzing, Brautigam et al. 1995). Furthermore, in experimental models of ALI, mice lacking inducible nitric oxide synthase had fewer neutrophils sequestered in the pulmonary vasculature (Razavi, Wang le et al. 2004), and inhaled NO reduced the accumulation of neutrophils in the pulmonary vasculature and air space (Sato, Walley et al. 1999). Similar effects of inhaled nitric oxide on leukocyte kinetics are observed outside the lung in rodent models of severe sepsis (Neviere, Mordon et al. 2000). iNO may therefore be a useful adjunct for the treatment of adults with ALI secondary to severe malaria.
On the other hand, ALI appears to be rare in children with *P. falciparum* infection. Respiratory distress in children with severe malaria is more often associated with metabolic acidosis, and represents respiratory compensation for primary lactic acidosis related to impaired tissue perfusion. In children hospitalized with malaria in sub-Saharan Africa, metabolic acidosis and hypovolemia are common presenting signs (Maitland, Pamba et al. 2003; Pamba and Maitland 2004), fluid replacement rather than fluid restriction restores cardiopulmonary homeostasis (Pamba and Maitland 2004), and echocardiography frequently reveals tachycardia, low stroke volume index, and high inferior vena cava collapsibility index, which improve with fluid replacement therapy (Yacoub, Lang et al. 2010). Of note, lactic acidosis is a prognostic marker for mortality in children with severe malaria (Newton, Valim et al. 2005), and its association with respiratory distress represents a final common pathway of decompensated shock, cardiopulmonary insufficiency and impending death. Respiratory distress with deep (Kussmaul) breathing in children with severe malaria carries a mortality rate higher than cerebral malaria (Marsh, Forster et al. 1995).

Despite these important differences in the pathophysiology underlying respiratory distress in adults and children with severe malaria, we hypothesize that iNO may also benefit African children with respiratory distress/metabolic acidosis. We speculate that the local action of iNO on the endothelium of the pulmonary vascular bed and/or more distal effects on the endothelium systemically, may modulate the cascade of inflammation and reduce the associated systemic inflammatory response syndrome.

### 8.6.3 Action of iNO beyond the pulmonary vasculature

While iNO has been primarily used for the treatment of pulmonary disease, it produces pharmacologic activity outside the pulmonary vasculature through well elucidated mechanisms of blood NO transport. Gaseous nitric oxide in inspired air readily diffuses across the alveolar-capillary membrane to reach the pulmonary vasculature. Although NO is short-lived in biological fluids by virtue of its unpaired electron, reaction products of NO with blood act to conserve its biological activity and transduce an endocrine function (Wang, Tanus-Santos et al. 2004). Numerous species subserve this activity, including S-nitroso-albumin, nitrite, iron-nitrosylated hemoglobin, S-nitroso-hemoglobin, plasma haptoglobin-hemoglobin complexes, nitrated lipids, N-nitrosamine, and other iron-nitrosyl complexes (Jia, Bonaventura et al. 1996;
Cosby, Partovi et al. 2003; Wang, Tanus-Santos et al. 2004). A physiologic example is the regulation of systemic vasomotor tone through blood NO transport to smooth muscle targets distal from its site of production. In clinical trials, NO administered via the inhalational route produces systemic effects in the treatment of vaso-occlusive crises in patients with sickle cell disease (Head, Swerdlow et al.; Weiner, Hibberd et al. 2003). Likewise, inhaled NO exerts distant effects in the cerebral vasculature and brain parenchyma. iNO is neuroprotective in animal models of brain injury (Olivier, Loron et al.; Pansiot, Loron et al.). Furthermore, in humans, iNO has been shown to decrease the risk of severe intraventricular hemorrhage or periventricular leukomalacia (Schreiber, Gin-Mestan et al. 2003), and improve long-term neurocognitive outcomes in premature neonates (Mestan, Marks et al. 2005). These precedents suggest that iNO may also produce systemic and cerebral end-organ effects in the setting of severe malaria.

8.6.4 Severe malaria syndromes are characterized by low nitric oxide bioavailability

In an animal model, reduced NO bioavailability contributed to the pathogenesis of ECM (Gramaglia, Sobolewski et al. 2006). NO supplementation with either a NO donor (dipropylenetriamine NONOate) or NO gas provided marked protection against severe disease (Gramaglia, Sobolewski et al. 2006). Our laboratory has generated evidence that inhaled NO decreases inflammation, rescues blood-brain barrier dysfunction and reduces parasite sequestration in the brain (Serghides, Kim, et al., unpublished data). Likewise, African children with severe malaria have impaired production of NO (Anstey, Weinberg et al. 1996), low levels of mononuclear cell iNOS expression (Anstey, Weinberg et al. 1996), and low plasma levels of the NOS substrate arginine (Lopansri, Anstey et al. 2003).

Natural variation in the genes encoding the NOS enzymes in human populations lends additional evidence for a protective role of endogenous NO against severe malaria syndromes. Polymorphisms in the promoter region if the iNOS gene at positions -954 (G-->C) and -1173 (T-->C), located at a putative gene repressor binding site (Kun, Mordmuller et al. 2001), are associated with higher NOS enzymatic activity (Kun, Mordmuller et al. 2001) and higher plasma and urine levels of NOx (Hobbs, Udhayakumar et al. 2002). These polymorphisms are common in African but not Asian populations(Kun, Mordmuller et al. 2001; Ohashi, Naka et al. 2002;
Boutlis, Hobbs et al. 2003; Dhangadamajhi, Mohapatra et al. 2009) and are associated with protection from severe malaria in several reports (Kun, Mordmuller et al. 1998; Kun, Mordmuller et al. 2001; Hobbs, Udhayakumar et al. 2002). Another iNOS promoter polymorphism consisting of CCTTT\(_n\) pentanucleotide microsatellite repeats 2.5kb upstream from the iNOS transcription start site has been associated with susceptibility to malaria, although findings from published reports are inconsistent, with long forms of the allele associated with severe disease in some studies (Ohashi, Naka et al. 2002; Cramer, Mockenhaupt et al. 2004; Dhangadamajhi, Mohapatra et al. 2009) but not others (Burgner, Xu et al. 1998). Genetic variation in other NOS isoforms has also been shown to influence the risk of cerebral malaria. A single amino acid substitution at position 298 (Glu-→Asp) in eNOS was associated with increased plasma levels of NOx and protection from cerebral malaria in Indian adults (Dhangadamajhi, Mohapatra et al. 2009). A single nucleotide polymorphism at position -84 (G-→A) of the nNOS gene is responsible for decreased basal transcriptional level, and is associated with increased risk of cerebral malaria in Indian adults (Dhangadamajhi, Mohapatra et al. 2009). Taken together, these findings from human population genetic studies indicate that nitric oxide synthase gene polymorphisms affect susceptibility to malaria via alterations in NO production and lend support to a protective role for NO against severe malaria syndromes. Findings from the published reports are summarized in Table 1.
Table 8.1. Polymorphisms in nitric oxide synthase genes and their association with malaria disease severity

<table>
<thead>
<tr>
<th>NOS gene</th>
<th>Polymorphism</th>
<th>Population studied</th>
<th>Effect on nitric oxide levels</th>
<th>Susceptibility to malaria</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>1173 C--&gt;T</td>
<td>Tanzanian children</td>
<td>Increased NOx</td>
<td>Protection against cerebral malaria and severe malarial anaemia</td>
<td>(Hobbs, Udhayak umar et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>954 G--&gt;C</td>
<td>Children in Gabon</td>
<td>7-fold higher baseline NOS activity</td>
<td>Reduced number of malarial attacks</td>
<td>(Kun, Mordmull er et al. 1998; Kun, Mordmull er et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>CCTTT&lt;sub&gt;n&lt;/sub&gt; repeat</td>
<td>Indian and Thai adults, Ghanaian children</td>
<td>NR</td>
<td>Longer repeats associated with severe malaria</td>
<td>(Ohashi, Naka et al. 2002; Cramer, Mockenhaupt et al. 2004; Dhangad damajhi, Mohapatra et al. 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gambian children</td>
<td>NR</td>
<td>Shorter repeats associated with severe malaria</td>
<td>(Burgner, Xu et al. 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asymptomatic children and adults, Papua New Guinea</td>
<td>No association between CCTTT repeat number and plasma NOx levels</td>
<td>No association with severe malaria</td>
<td>(Hobbs, Udhayak umar et al. 2002; Boutlis, Hobbs et al. 2003)</td>
</tr>
<tr>
<td>eNOS</td>
<td>298 G--&gt;A</td>
<td>Indian adults</td>
<td>Increased NOx</td>
<td>Protection from cerebral malaria</td>
<td>(Dhangad damajhi, Mohapatra et al. 2009)</td>
</tr>
<tr>
<td>nNOS</td>
<td>84 G--&gt;A</td>
<td>Indian adults</td>
<td>Decreased basal transcripational activity</td>
<td>Increased risk of cerebral malaria</td>
<td>(Dhangad damajhi, Mohapatra et al. 2009)</td>
</tr>
</tbody>
</table>

NR; not reported

8.6.5 Inhaled nitric oxide in clinical practice and clinical trials

NO is approved for use by the US FDA for the treatment of neonates with hypoxic respiratory failure (Finer and Barrington 2006). NO has been used in numerous clinical trials involving older children and adults, reviewed in Table 2. One meta-analysis of 12 trials including 1237 patients with acute lung injury/acute respiratory distress syndrome demonstrated that iNO has an
excellent safety profile, although renal dysfunction may occur in critically ill adult ICU patients (Adhikari, Burns et al. 2007).

Unlike NO donors such as L-arginine, nitroglycerine and sildenafil, iNO has not been reported to cause systemic vasodilation, electrolyte disturbance or effects on blood glucose. Furthermore, unlike NO donors, inhaled NO does not require functional endothelial cell NO synthase. NOS may be compromised in ill patients and therefore there may be a deficit in their ability to generate NO from these donor molecules. It has been used in a wide variety of clinical settings in children and adults, including acute respiratory distress syndrome, pulmonary hypertension, and pregnancy-induced pulmonary hypertension (Sokol, Jacobs et al. 2003). Outside the current licensing context, NO may be cheaply manufactured, and may be easily administered by mask with minimal infrastructure. These pragmatic considerations make iNO an appealing candidate adjuvant, appropriate for resource-constrained malaria-endemic areas. Although iNO is not routinely used in clinical practice in most hospitals in sub-Saharan Africa and most clinicians in this setting will not be immediately familiar with its use, the ease of administration with cylinder and mask, in a manner similar to oxygen supplementation, may allow for rapid uptake and scale-up of iNO in the African context, should it prove effective.

8.7 Evaluation of the Hypothesis

To test our hypothesis, we plan to conduct a randomized controlled trial of adjunctive iNO among Ugandan children with severe malaria. Details of the experimental plan (trial protocol) are outlined in the US NIH trials registry, clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT01255215), and are summarized briefly below.

Study Design

The study will be a prospective, parallel arm, randomized, placebo-controlled, blinded clinical trial of adjunctive continuous inhaled nitric oxide at 80 ppm versus placebo (both arms in addition to standard anti-malarial therapy), among children aged 1-10 years of age with severe malaria.

Inclusion criteria

1. Age 1-10 years
2. Positive malaria rapid diagnostic test
3. Features of severe malaria (2000): repeated seizures, prostration, impaired consciousness, or respiratory distress
4. Willing and able to complete follow up schedules for the study – 14 day and 6 months after hospital discharge

Exclusion criteria
1. Baseline methemoglobinemia (>2%)
2. Known chronic illness: renal, cardiac, or hepatic disease, diabetes, epilepsy, cerebral palsy, or clinical AIDS
3. Severe malnutrition
4. Severe malarial anemia (Hb <50g/L) without other signs of severe malaria.

8.8 Study setting
The study will be conducted at a single pediatric hospital in Jinja, Uganda. Uganda is a low-income country with a severely resource-constrained healthcare system. Malaria transmission is moderate and seasonal in Jinja, which lies on the northern shore of Lake Victoria in the central area of Uganda near the capital, Kampala. Jinja Regional Referral hospital admits at least 175 children with severe malaria annually (excluding cases of severe malarial anemia), representing over 30% of all admissions. *P. falciparum* resistance to chloroquine and sulfadoxine-pyrimethamine is widespread in the country (34% to 67%) (Idro, Aloyo et al. 2006).

8.9 Treatment groups
Participants in the intervention group will receive iNO at a concentration of 80 ppm, in addition to Ugandan standard of care of severe malaria, which includes a potent antimalarial agent (either parenteral quinine or artesunate). iNO will be administered continuously via non-rebreather face mask for a maximum period of 72 hours, but may be discontinued earlier if a patient recovers and no longer tolerates the face mask. An air compressor will be used to deliver continuous flow
of vehicle air. Participants in the control group will receive a continuous flow of room air delivered using an air compressor (indistinguishable in odour and appearance from the mixture of 80 ppm iNO), in addition to Ugandan standard of care of severe malaria.

8.10 Randomization and blinding methods

Simple randomization will be employed, using a computer-generated randomization list. Treatment allocation will be recorded on paper and kept in sequentially numbered sealed opaque envelopes, which will be drawn for each randomized participant by an unblinded investigator who is not responsible for patient care, laboratory or data analysis.

Trial participants, their parents/guardians, and all study personnel involved in the clinical management, assessment of outcomes, and laboratory testing will be blinded to the treatment assignment. An unblinded investigator not involved in patient care will be responsible for the administration, monitoring and recording of iNO, NO₂, and methemoglobin levels. Cylinders containing NO will be attached to the ventilation circuit in all patients, but the flow of NO will be controlled according to treatment arm assignment in a blinded manner. Laboratory analysis for Ang-2 levels (primary outcome) and all other parameter will occur in a manner blinded to treatment allocation.

8.11 Outcome measures

The longitudinal change in serum Ang-2 concentration over the first 72 hours of hospital admission will be the primary efficacy endpoint. Ang-2 is an objective, quantitative marker of disease severity, validated for longitudinal follow-up of patients with malaria (Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009). A mixed-effects linear model will be used to compare the change in Ang-2 over time between treatment arms.

Secondary outcomes of the trial will include relevant clinical, laboratory and neurocognitive endpoints. We will compare the following clinical endpoints, consistent with other therapeutic trials for malaria (Tran, Day et al. 1996; van Hensbroek, Onyiorah et al. 1996; Dondorp, Nosten et al. 2005): mortality at 48 hours and 14 days after admission; recovery times (time to fever resolution, time to sit unsupported); and length of hospital stay. Parasitological outcomes including time to parasite clearance and parasite recrudescence/re-infection at 14 day follow-up.
will also be compared between treatment groups. Biomarkers of disease severity (in addition to Ang-2, the primary study endpoint), including whole blood lactate, will also be followed. Lactate is produced by the anaerobic metabolism of glucose in the absence of adequate tissue oxygenation, and elevated lactate levels represent a final common pathway of tissue hypoxia and decompensated shock, the forerunner of cardiovascular collapse and death. We will measure lactate as an independent biomarker of disease severity during the clinical trial. Biomarkers of endothelial activation, inflammation, and coagulopathy will also be followed as they may provide additional insight into the pathways and processes altered in cerebral malaria and modulated by iNO delivery. Finally, neurocognitive outcomes in children with severe malaria will be followed in order to determine if adjunctive iNO may have a neuroprotective effect. The overall cognitive deficit at 6 months after discharge will be assessed by performing neuropsychological tests as previously described (John, Bangirana et al. 2008).

8.12 Consequences of the Hypothesis

Advances in malaria therapeutics have broad potential for global impact because of the large number of deaths attributable to severe malaria (Kamijo, Le et al. 1993). Residual mortality remains high despite potent antiparasitic treatment, underscoring the need for adjunctive therapies that target critical pathways in malaria pathogenesis (Dondorp, Nosten et al. 2005). Previously tested adjunctive treatment strategies for severe malaria include immunomodulation, iron chelation, reduction of oxidative stress, anti-coagulation, volume expansion, reduction of intracranial pressure, and prevention of seizure activity (John, Kutamba et al.). Only volume expansion with albumin has been associated with a mortality benefit (John, Kutamba et al.; Maitland, Nadel et al. 2005; Akech, Gwer et al. 2006). In this context, iNO, if demonstrated to be effective, would represent an important advance, filling a gap in the existing armamentarium against malaria. Given its low manufacturing cost and applicability in resource-constrained settings, NO could be rapidly scaled up to reach peripheral zones where severe malaria is most prevalent. Thus, our hypothesis that supplemental inhaled nitric oxide (iNO) will improve outcomes in children with severe malaria receiving standard antimalarial therapy, if borne out by experimental evidence from randomized controlled trials, has the potential to alter clinical practice and save lives.
Chapter 9
Inhaled Nitric Oxide for the Adjunctive Therapy of Severe Malaria: Protocol for a Randomized Controlled Trial

9.1 Abstract

**Background.** Severe malaria remains a major cause of global morbidity and mortality. Despite the use of potent anti-parasitic agents, the mortality rate in severe malaria remains high. Adjunctive therapies that target the underlying pathophysiology of severe malaria may further reduce morbidity and mortality. Endothelial activation plays a central role in the pathogenesis of severe malaria, of which angiopoietin-2 (Ang-2) has recently been shown to function as a key regulator. Nitric oxide (NO) is a major inhibitor of Ang-2 release from endothelium and has been shown to decrease endothelial inflammation and reduce the adhesion of parasitized erythrocytes. Low-flow inhaled nitric oxide (iNO) gas is a US FDA-approved treatment for hypoxic respiratory failure in neonates.

**Methods/Design.** This prospective, parallel arm, randomized, placebo-controlled, blinded clinical trial compares adjunctive continuous inhaled nitric oxide at 80 ppm to placebo (both arms receiving standard anti-malarial therapy), among Ugandan children aged 1-10 years of age with severe malaria. The primary endpoint is the longitudinal change in Ang-2, an objective and quantitative biomarker of malaria severity, which will be analysed using a mixed-effects linear model. Secondary endpoints include mortality, recovery time, parasite clearance and neurocognitive sequelae.

**Discussion.** Noteworthy aspects of this trial design include its efficient sample size supported by a computer simulation study to evaluate statistical power, meticulous attention to complex ethical issues in a cross-cultural setting, and innovative strategies for safety monitoring and blinding to treatment allocation in a resource-constrained setting in sub-Saharan Africa.

**Trial Registration.** ClinicalTrials.gov Identifier: NCT01255215
9.2 Background

Malaria is the leading parasitic cause of morbidity and mortality worldwide, causing an estimated 240 million clinical cases and 800,000 deaths annually (Kamijo, Le et al. 1993). Children in sub-Saharan Africa bear the greatest burden of disease, where one in every five childhood deaths is due to malaria and 25% of survivors of cerebral malaria develop long-term neurocognitive impairment (Kamijo, Le et al. 1993; John, Bangirana et al. 2008). Despite the use of highly effective anti-malarial medications, 10-30% patients with severe malaria will die (Dondorp, Fanello et al.; Newton and Krishna 1998; Dondorp, Nosten et al. 2005), underscoring the need for adjunctive therapies that can be applied in endemic areas. To date, effective adjunctive treatments have been elusive despite numerous clinical trials (John, Kutamba et al.). New therapies, appropriate for use in endemic areas, are therefore urgently needed to address the unacceptably high residual mortality associated with severe malaria in pediatric populations.

NO is a gaseous, lipid-soluble free radical that is produced in vivo by the enzymatic conversion of L-arginine and molecular oxygen to L-citrulline by members of the nitric oxide synthase (NOS) family of proteins. A free radical, NO is a highly labile molecule with a half-life of several seconds that reacts with transition metal or thiol groups of numerous target proteins (Korhonen, Lahti et al. 2005). NO readily diffuses across cell membranes into neighbouring cells, or may produce effects distant from its site of production transported by vehicles such as low-molecular weight S-nitrosatiols, S-nitrosylated proteins including haemoglobin and albumin, and nitrosyl-metal complexes which liberate NO spontaneously or after cleavage by ectoenzymes (Bogdan 2001). NO regulates numerous cellular processes including cytoplasmic granule exocytosis, platelet aggregation, endothelial-cell activation, apoptosis, inflammation, chemotaxis, neurotransmission and antimicrobial defense by modulating the activity of regulatory proteins (Matsushita, Morrell et al. 2003). A well-recognized example is the role of NO as the endothelium-derived relaxation factor that mediates vasodilation by activating smooth muscle soluble guanylate cyclase (sGC) (Palmer, Ferrige et al. 1987).

Evidence from a murine model suggests that reduced NO bioavailability contributes to the pathogenesis of experimental cerebral malaria (Gramaglia, Sobolewski et al. 2006). “Footprint” molecules of labile nitric oxide including cGMP and nitrite were markedly decreased over the
course of infection, and NO supplementation with either a NO donor (dipropylenetriamine NONOate, DPTA/NO) or NO gas provided marked protection against severe disease (Gramaglia, Sobolewski et al. 2006). Data from human studies support the hypothesis of reduced bioavailable NO in severe malaria: African children with severe malaria have impaired production of NO (Anstey, Weinberg et al. 1996), low levels of mononuclear cell iNOS expression (Anstey, Weinberg et al. 1996), low levels of the NOS substrate arginine (Lopansri, Anstey et al. 2003), and elevated levels of the NOS inhibitor, asymmetric dimethyl arginine (Yeo, Lampah et al.). Furthermore, genetic variation in NOS isoforms that affect plasma and urine levels of NO and its metabolites are common in African populations and have been shown to influence the risk of cerebral malaria (Kun, Mordmuller et al. 1998; Kun, Mordmuller et al. 2001; Ohashi, Naka et al. 2002; Boutlis, Hobbs et al. 2003; Dhangadamajhi, Mohapatra et al. 2009). Factors contributing to reduced bioavailable NO in malaria include scavenging of NO by free haemoglobin and superoxide anion, and reduced levels of nitrate, a NO precursor molecule (Lopansri, Anstey et al. 2003; Sobolewski, Gramaglia et al. 2005; Gramaglia, Sobolewski et al. 2006).

The mechanism of action by which NO might improve outcomes in malaria may involve the vascular endothelium, which plays a central role in the pathogenesis of cerebral malaria. Activation of endothelial cells is characterized by increased surface expression of cellular adhesion molecules, the exocytosis of Weibel-Palade bodies (WPB), and the breakdown of intracellular tight junctions with transudation of intravascular fluid producing end organ dysfunction. In malaria, parasitized erythrocytes (PEs) adhere to the microvascular endothelium, resulting in sequestration and vascular obstruction, impaired perfusion and tissue hypoxia (Day, Phu et al. 2000). Autopsy studies in fatal cerebral malaria reveal sequestration of PEs in the capillaries and post-capillary venules of multiple organs (MacPherson, Warrell et al. 1985). NO decreases endothelial cell adhesion molecule expression, including intercellular cell adhesion molecule-1 (ICAM-1) (Turner, Morrison et al. 1994; De Caterina, Libby et al. 1995) and has been shown to reduce the adherence of PEs to endothelial cells (Jakobsen, Morris-Jones et al. 1994). The release of intracellular WPB contents from endothelial cells liberates von Willebrand factor (vWF) (Hollestelle, Donkor et al. 2006; de Mast, Groot et al. 2007) and angiopoietin-2 (Ang-2) (Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009) into the circulation. Interactions of vWF with the coagulation cascade may contribute to vessel obstruction and may
help tether parasitized erythrocytes to endothelial cells via platelets (Bridges, Bunn et al.). Ang-2 acts in an autocrine and paracrine fashion to sensitize the endothelium to the effects of TNF, resulting in increased adhesion receptor expression (Fiedler, Reiss et al. 2006). In addition, Ang-2 antagonizes the interaction of the Tie-2 receptor with angiopoietin-1 (Ang-1), thereby promoting endothelial permeability and reducing vessel stability (Thurston, Suri et al. 1999; Parikh, Mammoto et al. 2006; Fukuhara, Sako et al. 2008; Saharinen, Eklund et al. 2008). NO inhibits the exocytosis of WPB contents through S-nitrosylation of critical regulatory factors (Matsushita, Morrell et al. 2003) and may therefore promote endothelial quiescence, reduce vascular fluid leak, and reduce end-organ damage. A clinical trial demonstrated that a strategy of NO supplementation using the NOS substrate L-arginine improved endothelial function, as measured by reactive-hyperemia-peripheral arterial tonometry, in Indonesian adults with moderately severe malaria (Yeo, Lampah et al. 2007).

Low-flow iNO at a concentration of 5-80 ppm is approved for use by the US FDA for the treatment of neonates with hypoxic respiratory failure, in whom it reduces requirements for extracorporeal membrane oxygenation (ECMO) and improves survival (Finer and Barrington 2006). After a decade of use in clinical practice and in numerous clinical trials of iNO in critically ill older children and adults, iNO has a well-established safety profile. Pragmatic considerations, including a theoretically cheap manufacturing cost and ease of administration by mask, make NO an attractive therapeutic option for unresponsive patients in resource-limited settings.

In summary, a clinical trial of adjunctive inhaled nitric oxide (iNO) in severe malaria is warranted on the basis of firm proof of concept from animal models (Gramaglia, Sobolewski et al. 2006) and a clinical trial using the NO donor L-arginine (Yeo, Lampah et al. 2007), together with evidence of safety from clinical experience and numerous clinical trials of iNO for other conditions (Adhikari, Burns et al. 2007).

9.3 Methods

9.3.1 Study Design

The study is a prospective, parallel arm, randomized, placebo-controlled, blinded clinical trial of adjunctive continuous inhaled nitric oxide at 80 ppm versus placebo (both arms in addition to
standard anti-malarial therapy), among children aged 1-10 years of age with severe malaria. Figure 9.1 shows a participant flow diagram for the trial, consistent with the Consolidated Standards of Reporting Trials (CONSORT) 2010 statement (Schulz, Altman et al.).
Figure 9.1. Participant flow diagram

The participant flow diagram illustrates randomization of 180 children with severe malaria to inhaled nitric oxide (iNO) or placebo, consistent with the Consolidated Standards of Reporting Trials (CONSORT) 2010 statement. RDT: rapid diagnostic test; metHb: methemoglobin; SMA: severe malarial anemia.
9.3.2 Study Objectives

The primary objective of this trial is to compare the longitudinal change in angiopoietin-2 (Ang-2), an objective and quantitative biochemical marker of malaria severity, among children who are randomized to receive inhaled nitric oxide in addition to standard antimalarial therapy compared to those randomized to placebo in addition to standard antimalarial therapy.

Secondary objectives are:

- To determine the efficacy of adjunctive iNO in severe malaria based on clinical parameters: mortality, recovery times, length of hospital stay.
- To assess the effect of adjunctive iNO on laboratory parameters: parasite clearance, whole blood lactate levels, and other biomarkers of malaria severity.
- To determine the efficacy of adjunctive nitric oxide in preventing neurocognitive sequelae after severe malaria.
- To assess the tolerability and safety of iNO in severe malaria.

9.3.3 Study Hypotheses

The working hypothesis is that young children hospitalized with malaria will benefit from adjunctive iNO, as determined by more rapid improvement in serum angiopoietin-2 (Ang-2) levels. We will test this hypothesis by comparing the change in Ang-2 over the hospital admission between the two groups randomized to receive iNO or placebo (room air) using a mixed-effects linear statistical model.

The secondary hypotheses are that adjunctive iNO will reduce mortality, accelerate recovery times, and shorten length of hospital stay in severe malaria. Furthermore, we hypothesize that iNO will accelerate improvements in biomarkers of host response to severe malaria, including whole blood lactate, but will not affect parasite clearance. We hypothesize that iNO will reduce the rate of adverse neurocognitive sequelae following severe malaria. Finally, we hypothesize that adjunctive iNO will be safe and well tolerated in children treated for severe malaria.

9.3.4 Eligibility criteria

Children will be eligible for the trial if they meet the following inclusion criteria:
1. Age 1-10 years

2. Positive malaria rapid diagnostic test (RDT)

3. One or more features of severe malaria: repeated seizures (two or more generalized seizures in 24 h); prostration (in children 1 year and older, the child is unable to sit unsupported or stand although was able to before the illness); impaired consciousness (Blantyre Coma Score <5 in children 1 to 4 years, GCS <14 for children ≥ 5 years); respiratory distress: age related tachypnea with sustained nasal flaring, deep breathing or substernal retractions

4. Willing and able to complete follow up schedules for the study – 14 day and 6 months after hospital discharge.

The inclusion criteria require timely parasitological confirmation of malaria infection prior to enrolment, which poses logistical challenges at our peripheral centre with limited laboratory resources. For diagnosis, we will use commercially available immunochromatographic RDTs, complemented where possible with microscopy of peripheral smears. Despite well-recognized variability in the test performance characteristics of RDTs under field conditions, one commercially available RDT (First Response Malaria Ag Combo (pLDH/HRP2, Premier Medical Corporation Ltd., India) is highly ranked by the World Health Organisation (WHO), with a 95% detection rate even at low parasitemia and a false positive rate of 0% (Organization 2008). This RDT includes detection bands for two *P. falciparum* antigens, histidine-rich protein-2 (HRP-2) and parasite lactate dehydrogenase (pLDH), and we will require positivity for both antigens for trial inclusion. Thus, our parasitologic criterion is expected to be highly specific, in order to include only patients who are truly parasitemic.

The exclusion criteria for this study are as follows:

1. Baseline methemoglobinemia (>2%)

2. Known chronic illness: renal, cardiac, or hepatic disease, diabetes, epilepsy, cerebral palsy, or clinical AIDS

3. Severe malnutrition, defined as weight-for length or height below -3 standard deviations based on WHO reference charts, or symmetrical edema involving at least the feet (Organization 1999).

4. Severe malarial anemia (SMA; Hb <50g/L) without other signs of severe malaria.
The latter exclusion criterion was chosen because of differences in the pathophysiology of SMA (increased clearance of infected and uninfected erythrocytes, and dysregulated hematopoiesis) compared to other malaria syndromes characterised by excessive inflammation and endothelial activation. Based on its postulated mechanism of action, it is less clear that iNO would benefit children with SMA.

9.3.5 Study setting

The study will be conducted at a single pediatric hospital in Jinja, Uganda. Uganda is a low-income country with a severely resource-constrained healthcare system. Malaria transmission is moderate and seasonal in Jinja, which lies on the northern shore of Lake Victoria in the central area of Uganda near the capital, Kampala. Jinja Regional Referral hospital admits at least 175 children with severe malaria annually (excluding cases of severe malarial anemia), representing over 30% of all admissions. *P. falciparum* resistance to chloroquine and sulfadoxine-pyrimethamine is widespread in the country (34% to 67%) (Idro, Aloyo et al. 2006).

9.3.6 Treatment groups

Participants in the intervention group will receive iNO at a concentration of 80 ppm, in addition to Ugandan standard of care of severe malaria, which includes a potent anti-parasitic agent. In light of the recent AQUAMAT trial that demonstrated a mortality benefit of parenteral artesunate over quinine for the treatment of African children with severe malaria (Dondorp, Fanello et al.), artemesunate will be used as the antimalarial of choice for this study. iNO will be administered continuously via non-rebreather face mask for a maximum period of 72 hours, but may be discontinued earlier if a patient recovers and no longer tolerates the face mask. The dose of iNO (80 ppm) was chosen as it is the highest approved dose by the US-FDA for use in neonates, with the greatest potential for a therapeutic effect. In pre-clinical animal studies, a dose of 80 ppm provided greater protection from experimental cerebral malaria than lower doses (Serghides, Kim, et al., unpublished data). At least five published clinical trials across different age groups and clinical conditions have safely used this dose (Head, Swerdlow et al.; 1997; Davidson, Barefield et al. 1998; Weiner, Hibberd et al. 2003; Long, Jones et al. 2005). With careful monitoring for dose-dependent adverse events and titration of iNO concentration accordingly, a dose of 80 ppm can safely be used in our trial.
Participants in the control group will receive room air (indistinguishable in odour and appearance from the mixture of 80 ppm iNO), in addition to Ugandan standard of care treatment for severe malaria, including parenteral artesunate. An air compressor will be used to deliver continuous flow of vehicle air in both groups.

9.3.7 Randomization and blinding methods

Simple randomization will be employed, using a computer-generated randomization list. Treatment allocation will be recorded on paper and kept in sequentially numbered sealed opaque envelopes, which will be drawn for each randomized participant by an unblinded investigator who is not responsible for patient care, laboratory or data analysis. We will retain all envelopes and records for quality monitoring purposes.

In previous clinical trials using iNO, one of the design and implementation challenges was establishing the blinding procedures while titrating and monitoring concentrations of iNO, as well as anticipated dose-related increases of methemoglobin and NO\textsubscript{2} concentrations (Adhikari, Burns et al. 2007). The establishment of two teams, one blinded team making all clinical assessments and therapeutic decisions, and another unblinded team monitoring the delivery of the treatment gas and assessing the development of potential toxicities, allowed iNO to be delivered safely while minimizing the possibility that direct knowledge of treatment allocation would influence the care delivered to the patient (Adhikari, Burns et al. 2007). Thus, an unblinded investigator not involved in patient care will be responsible for the administration, monitoring and recording of iNO, NO\textsubscript{2}, and methemoglobin levels. Cylinders containing NO will be attached to the ventilation circuit in all patients, but the flow of NO will be controlled according to treatment arm assignment in a manner blinded to patients, caregivers, and healthcare providers. Only the unblinded study site investigator will administer, monitor, and titrate the delivery of NO or placebo (room air), as well as monitor and record the safety parameters of methemoglobin and NO\textsubscript{2} levels. Laboratory analysis for Ang-2 levels (primary outcome) and all other parameter will occur in a manner blinded to treatment allocation.

9.3.8 Outcome measures

The longitudinal change in serum Ang-2 concentration over the first 72 hours of hospital admission will be the primary efficacy endpoint. Elevated Ang-2 levels are associated with poor
clinical outcome in severe malaria (Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009) and Ang-2 has been used to follow disease progression and recovery in previous studies of malaria (Yeo, Lampah et al. 2008). Among survivors of severe malaria, Ang-2 levels have been shown to decrease linearly during recovery at a mean rate of 2700 pg/mL per 24h (Yeo, Lampah et al. 2008). Thus, Ang-2 is an objective, quantitative marker of disease severity, validated for longitudinal follow-up of patients with malaria. Ang-2 levels will be measured longitudinally at admission (day 0), day 1, day 2 and day 3 of hospitalization. Angiopoietin-2 will be measured by enzyme-linked immunosorbent assay (ELISA) from plasma or serum samples, and is readily detectable in samples frozen for storage and later thawed (Conroy, Lafferty, et al. 2009). Commercially available ELISA kits will be used (DuoSets, R&D Systems, Minneapolis, MN). A mixed-effects linear model will be used to compare the change in Ang-2 over time between treatment arms.

Secondary outcomes of the trial will include relevant clinical, laboratory and neurocognitive endpoints. We will compare the following clinical endpoints, consistent with other therapeutic trials for malaria (Tran, Day et al. 1996; van Hensbroek, Onyiorah et al. 1996; Dondorp, Nosten et al. 2005): mortality at 48 hours and 14 days after admission; recovery times (time to fever resolution, time to sit unsupported); and length of hospital stay. Parasitological outcomes including time to parasite clearance and parasite recrudescence/re-infection at 14 day follow-up will also be compared between treatment groups. Biomarkers of disease severity (in addition to Ang-2, the primary study endpoint), including whole blood lactate, will also be followed. Lactate is produced by the anaerobic metabolism of glucose in the absence of adequate tissue oxygenation, and elevated lactate levels represent a final common pathway of tissue hypoxia and decompensated shock, the forerunner of cardiovascular collapse and death. We will measure lactate as an independent biomarker of disease severity during the clinical trial. Biomarkers of endothelial activation, inflammation, and coagulopathy will also be followed as they may provide additional insight into the pathways and processes altered in cerebral malaria and modulated by iNO delivery. Finally, neurocognitive outcomes in children with severe malaria will be followed in order to determine if adjunctive iNO may have a neuroprotective effect. The overall cognitive deficit at 6 months after discharge will be assessed by performing neuropsychological tests as previously described (John, Bangirana et al. 2008).
9.3.9 Duration of Study Participation

After admission to hospital for severe malaria, disease survivors will typically be discharged after a week or less. Trial participants will be administered iNO during the first 72 hours of admission (or less if a patient recovers and no longer tolerates the mask), which represents the period of highest mortality. During hospitalization, detailed data on the interim medical history will be collected, with attention to complications like coma, seizures and hypoglycemia that might affect neurocognitive outcome. After discharge, patients will return for a follow-up visit at 14 days to test for *P. falciparum* recrudescence, and at 6-months to undergo neurocognitive testing.

9.3.10 Safety

NO is approved by the US FDA for the treatment of neonates with hypoxic respiratory failure, where it has been shown to improve oxygenation, decrease pulmonary hypertension, reduce the requirement for extracorporeal membrane oxygenation, and improve survival (Finer and Barrington 2006). In addition, iNO is widely used in clinical practice across North America and Europe in older children and adults with respiratory failure, where it improves oxygenation but has not been shown to confer a survival benefit (Troncy, Collet et al. 1998). As adjunctive therapy, iNO is safe and well tolerated in these critically ill patient populations and a large number of randomized controlled trials have demonstrated a favourable safety profile of iNO. One meta-analysis of 12 trials including 1237 patients with acute lung injury or acute respiratory distress syndrome demonstrated that iNO is generally safe, but was associated with a statistically elevated risk of developing renal dysfunction in these critically ill adults (Adhikari, Burns et al. 2007). We anticipate that our pediatric target population may be less susceptible to renal injury, particularly after exclusion of patients with underlying chronic renal disease, but we will monitor renal function in all patients enrolled in our trial with daily creatinine and urine output measurement.

Methemoglobinemia and elevated nitrogen dioxide (NO₂) levels in the inspiratory ventilation circuit are well-recognized, dose-dependent, and reversible adverse effects of nitric oxide administration; however, at doses commonly used in clinical practice, these are not common or clinically important consequences (Adhikari, Burns et al. 2007). Methemoglobinemia results
from the reaction of NO with oxyhemoglobin, thereby reducing oxygen carrying capacity (Griffiths and Evans 2005). NO$_2$ is generated from the gas phase reaction of NO with molecular oxygen, and is a known pulmonary irritant (Elsayed 1994; Leavey, Dubin et al. 2004). In previous clinical trials of iNO, methemoglobin and NO$_2$ were routinely monitored and elevated levels constituted a criterion for NO dose reduction. Among neonates receiving iNO at 80ppm, 35% developed methemoglobinemia (>7%) and 19% had elevated NO$_2$ levels (>3%), requiring reduction of the iNO concentration in the ventilation circuit (Davidson, Barefield et al. 1998). Similarly, in our trial, methemoglobin and NO$_2$ will be monitored and the iNO dose will be titrated downward according to these defined thresholds. Furthermore, elevated baseline methemoglobinemia will be used as an exclusion criterion from study participation as a possible indicator of genetic susceptibility to methemoglobinemia.

Adverse events may occur commonly in a trial involving children with severe malaria, although the majority of events are likely due to the clinical course of the infection and not to study medications. For example, mortality among Ugandan children with severe malaria receiving standard care including potent antimalarial agents was as high as 16% among children with impaired consciousness and 21% among children with deep acidotic breathing as presenting clinical signs (Idro, Aloyo et al. 2006). In order to carefully and rationally monitor the frequency of deaths in our trial for deviations from the expected baseline level, we plan to use statistical control charts. The control chart is a commonly used tool to monitor output of processes in a variety of settings, including clinical trials (Svolba and Bauer 1999). This method continuously follows a process outcome (e.g., patient mortality), allowing early detection of deviations from a state of “statistical control,” thereby prompting a search for assignable causes.

Other adverse events will be monitored using pediatric toxicity tables modified from the US National Institute of Allergy and Infectious Diseases (NIAID, 2007). Using this comprehensive checklist of potential adverse events, investigators will grade the severity of the event and the likelihood that the event is causally associated with the study gas according to scales defined \textit{a priori} (NIAID, 2007; WHO, 2010).

In addition to the ethical oversight provided by both the Ugandan and North American institutions, an independent Data and Safety Monitoring Board (DSMB) has been convened to
supervise the trial. The DSMB is composed of medical and biostatistical experts with representation from Uganda and North America who will meet periodically and as necessary to review trial progress, safety data, and indices of study quality. Severe adverse events, including all deaths in the trial, will be reported in a timely fashion to the DSMB and to the ethics boards that approved the study.

9.3.11 Ethical considerations

Ethical approval has been obtained from the Makerere University Research and Ethics Committee (Kampala, Uganda), the Uganda National Council on Science and Technology, and the Research Ethics Board of the University Health Network, Toronto, Canada (REB# 10-0607-B). The research is being conducted in accordance with the Declaration of Helsinki.

Informed consent will be obtained from the parent/guardian of all children that participate in the study. Trained study team personnel will seek consent after a comprehensive discussion with the parent/guardian of a prospective participant in the local language (Lusoga) at an education-appropriate level. Assent will also be sought from those children who are alert and able to understand the trial (age 8 and above), and sustained dissent on the part of children will be honoured (Wendler 2006). Specific aspects included in the consent discussion include: the acuity and potential lethality of severe malaria, residual mortality despite antiparasitic treatment, the absence of proven effective adjunctive therapies, potential benefits and harms of iNO, the concept of randomization and potential allocation to placebo control (although all patients will receive standard care including potent antimalarials), blinding of treatment allocation, blood samples required for the trial above what is necessary for clinical care, and the distinction between the experimental intervention and clinical care.

International collaborative research may face complex community challenges (Newman 2006; Tindana, Singh et al. 2007). Community engagement (CE), a participatory process of collaboration and exchange between the various key stakeholders in the research process, may mitigate risks with respect to trial success, optimize participant retention, and minimize social disruption by providing a platform to seek input from and provide ongoing feedback to community members (Lavery, Tinadana et al.). There is currently no consensus on what CE activities are required in clinical trials, but a recently published model of CE in global health
research provides a useful framework of key CE activities and their ethical implications (Lavery, Tinadana et al.).

We will use CE to improve awareness of our trial and its findings in the catchment area of the Jinja Regional Referral Hospital. We also hope the CE activities will contribute to key ethical objectives for the trial, including respect for communities, fairness, transparency and accountability of the trial overall. We will focus on the following CE activities and their associated aims: (1) understanding the relevant community by consciously reaching out beyond the hospital to its catchment areas and listening to their issues and concerns; (2) providing information about the trial, including the pre-clinical evidence behind iNO, timeframe, procedures, and what will happen if the trial is successful; (3) building relationships and trust with local frontline healthcare workers; (4) specific educational/training activities, based on consultation with the nurses and/or frontline healthcare workers to ascertain what would be most relevant and beneficial for them; and (5) feedback of trial results, guided by the community itself as to how and what types of feedback activities would be most appropriate. Several levels of community will be targeted, including parents and primary caregivers of children, who comprise the group at highest risk of malaria, as well as healthcare professionals within the hospital catchment area. These activities do not constitute a mechanism for recruitment of participants to the trial, since only children with severe malaria will be eligible. Instead the community engagement process is intended to build trust and avoid misunderstandings through a dynamic exchange of information and ideas between trial scientists and community members.

9.3.12 Sample Size Calculation

We will enrol 180 patients (approximately 90 in each treatment arm). To arrive at this sample size estimate, we began with a preliminary calculation based on data from a recent clinical data in severe malaria, in which Ang-2 decreased by 2700pg/mL/day (95% CI 1800-3600pg/mL/day) (Yeo, Lampah et al. 2008). We assume that a 50% change in this parameter would represent a clinically significant therapeutic effect. By standard calculations for normally distributed data, 80 patients per group will provide 80% power to detect a difference between two treatment arms of 1350 pg/mL/day at p=0.05 (two-sided). To account for possible dropout, loss to follow-up, and/or non-evaluable data of 10% of patients, approximately 90 patients per study arm are required.
To validate this preliminary sample size estimate, consistent with our analytic plan (mixed-effects linear model), a simulation study was performed. A number of assumptions needed to be made, such as within-patient correlation and Ang-2 variability. Patient Ang-2 data were simulated using a multivariate normal distribution. A simple autoregressive correlation structure was used with correlations of 0.75, 0.5 and 0.25 for time lags of 1, 2 and 3 days, respectively. In previous studies, variability appears proportional to the mean (Yeo, Lampah et al. 2008). Simulations were run with three different relationships where the standard deviation was taken to be 40%, 50% and 80% of the Ang-2 mean at each time point. Both groups were assumed to start at Ang-2 levels of 15,000 pg/mL (Yeo, Lampah et al. 2008) and the average values at each of days 1, 2 and 3 were based on the hypothesized slopes of Ang-2. One thousand replications were performed at each standard deviation relationship and treatment effect. The mixed effects models were fit and the likelihood ratio test was used to test the hypothesis of no time-by-treatment interaction at the 5% level (two-tailed). Table 1 shows the results of the power simulations.

9.3.13 Interim Analysis

An interim analysis for efficacy, safety and trial quality is planned at the midpoint of patient enrolment (approximately 45 patients per group). There is no plan to stop the trial prematurely for efficacy or futility based on the primary endpoint (Ang-2), other biochemical parameters, clinical recovery times or parasitological outcomes, given the modest size of the trial and limited statistical power at the midpoint. Data at the time of the interim analysis will be presented to the DSMB members for review, who may advise that the trial continue without modification, continue with changes to the protocol, or be discontinued prematurely. With respect to the statistical interpretation of safety data, the DSMB may recommend termination or modification of the trial if mortality rates exceed statistical thresholds as described above. However, we do not propose that the DSMB be strictly bound by pre-specified criteria, because of the complexity of the trade-offs between safety, efficacy, and the possibility that new information will change considerations. Rather, consideration of stopping guidelines requires a reasoned judgment based on all information that is available at the time of data review.
9.3.14 Primary Analysis

The primary focus is whether or not the rate of reduction in Ang-2 differs between the treatment groups. In statistical terms, this is a \textit{time-by-treatment} interaction. Given that we will have repeated measurements, possibly incomplete, over time, a linear mixed-effects model will be used to estimate and test the magnitude of the \textit{time-by-treatment} interaction. A linear time trend will not be assumed by treating “day” as a categorical variable in the model. The primary analysis will be by \textit{intention-to-treat} (ITT). That is, patients will be analysed in the group to which they were randomized, regardless of deviations from study protocol. A secondary \textit{per-protocol} analysis may be considered if important deviations from the protocol compromise the validity of the ITT analysis.

9.3.15 Secondary outcomes

\textit{Mortality}. Analysis will follow standard methods in other clinical trials for malaria (Tran, Day et al. 1996; van Hensbroek, Onyiorah et al. 1996; Dondorp, Nosten et al. 2005). Mortality at 48 h and 14 days will be coded as a binary variable. Absolute and relative risk reduction will be reported with binomial 95% confidence intervals. Analysis will be by $\chi^2$ test or Fisher’s exact test. In addition, we will present Kaplan-Meier survival curves comparing patients treated with iNO and placebo. Time to death will be analysed using survival analysis (log-rank test for difference between treatment arms).

\textit{Time to recovery}. Among survivors (a subgroup of randomized participants), recovery times will be analysed by survival analysis (log-rank test). Time to sit unsupported, time to coma resolution (in the subset of patients with coma at study admission) and time to discharge will be documented by treating clinicians blinded to treatment allocation. Time to fever resolution, defined as the time required to achieve a temperature $<38^\circ C$ and the time to maintenance of temperature $<38^\circ C$, will be determined from frequent vital sign monitoring. The time required to achieve a reduction in parasite density of 50%, 90% and to undetectable levels will be determined from daily blood smears. Results will be expressed as the median time to each event, with 95% confidence intervals.
Additional biomarkers (continuous variables, repeated longitudinal measurements) will be analysed using mixed-effects linear models, with the raw value or log-transformed value of the biomarker level as the dependent variable, as appropriate.

Neurocognitive outcome. As in previous studies involving children in sub-Saharan Africa (John, Bangirana et al. 2008), standardized instruments will be used for neurocognitive testing: Kaufman Assessment Battery for Children (working memory), the visual form of the computerized Test of Variables of Attention (executive attention), and the Tactual Performance Test (tactile-based learning). Summary variables from each test, converted to age-specific standardized (z) scores, will provide quantitative measures of these three cognitive domains (working memory, attention and tactile learning). Details of these tests are described elsewhere (Boivin, Bangirana et al. 2007; John, Bangirana et al. 2008). Frequencies of overall cognitive and neurologic deficits in children treated with iNO and children receiving placebo will be compared by χ² test or Fisher’s exact test. Differences in cognitive areas affected in the two age groups (18 mo-4 years, 5-10 years) will be assessed by comparing frequency of individuals with deficits in each area by χ² test or Fisher’s exact test. This will also serve as the best surrogate of impairment in a particular area in one age group versus another, since the type of testing for each cognitive area will be different for the two age groups, so no direct comparison of level of impairment will be possible across age groups. For individual tests, age-adjusted z-scores (determined from normative data in previous studies among healthy community controls) will be analyzed by means of mixed-effects models to examine study group differences in relation to neurocognitive outcomes. The models will provide estimated mean differences between children treated with iNO to placebo controls.

9.3.16 Subgroup Analyses

Although our sample size is modest, we will explore subgroup effects by examining interaction terms in the mixed-effects linear model. Subgroup analyses related to important prognostic factors will be performed: age < 5 years or ≥5 years; HIV seropositivity; and bacterial co-infection.
9.3.17 Quality management

Quality management (QM), both quality control and assurance, is a continuous, ongoing process of evaluation of the quality of the conduct and documentation of studies. The first step in QM will be training/re-training of the research staff to ensure consistency in clinical management, sample processing and data collection. Standard operating procedures (SOPs) have been developed for all study related procedures and protocols, and study personnel will document any deviation from SOPs together with the reason for the deviation. The next step for QM will be monitoring of collected data on a prospective basis, with daily review of source documents for completeness, accuracy and consistency. Next, data entry will be verified periodically, and discrepancies will be reviewed with the nurses and medical officers to discover the reason for errors, take corrective measures and prevent future errors. Collection, storage and transport of clinical samples will also be monitored on a regular basis. The ethical conduct of the study will be monitored through initial training in research ethics for study staff, and documentation, mediation and resolution of any perceived violations of ethical standards by participants, their parents/guardians, or members of the community at large. Measures to minimize bias in the trial will be subjected to formal evaluation. Quality of randomization and allocation concealment will be evaluated by keeping sequentially numbered opaque envelopes containing the randomization code, which will be opened, signed and dated at the time of randomization. Quality of trial blinding will be evaluated by asking key trial persons (participants, parents/guardians, medical officers, and nurses) to guess patients’ treatments at the end of their trial participation, and compare the answers with the actual treatments, as previously described (Hrobjartsson, Forfang et al. 2007). External independent oversight of trial quality will be performed by the DSMB, who will review trial quality indices periodically, as well as an external trial auditor.

9.4 Discussion

Nitric oxide is an attractive candidate for an adjunctive therapeutic agent for severe malaria given pre-clinical data on its efficacy in animal models and an established track record of safety in clinical practice and previous trials. Unlike other NO donor molecules, iNO has not been reported to cause systemic vasodilation and hypotension (Adhikari, Burns et al. 2007). Furthermore, unlike the NO precursor L-arginine, iNO does not require functional endothelial cell NOS, which may be compromised in patients with severe disease. It is routinely used in
clinical practice as an approved agent for hypoxic respiratory failure in neonates, and has an established track record of safety in critically ill patient populations. NO has been used in a wide variety of clinical settings in older children and adults including acute respiratory distress syndrome, pulmonary hypertension, and pregnancy-induced hypertension (Sokol, Jacobs et al. 2003).

Outside the existing patent, iNO is relatively inexpensive (Hansen 2004), and can feasibly delivered by mask (Long, Jones et al. 2005) in areas with minimal health infrastructure. As currently marketed, INOmax from INO Therapeutics is cost-effective in high-income countries for the treatment of respiratory failure in neonates (Angus, Clermont et al. 2003), but may be prohibitively expensive in low- or middle-income countries. The real cost of iNO (not just the price from a single company) is much lower (medical grade iNO $1.99/h compared to $125/h for INOmax) (Pierce, Peters et al. 2002). The patent for INOmax expires in 2013, leaving open the possibility for cheap manufacturing and commercialization of iNO in low-income settings, should iNO prove to be beneficial in severe malaria.

Other adjunctive treatment strategies for severe malaria previously tested in randomized controlled trials include immunomodulation, iron chelation, reduction of oxidative stress, anti-coagulation, volume expansion, reduction of intracranial pressure, and prevention of seizure activity (John, Kutamba et al.). Only one agent (albumin) was associated with a mortality benefit (John, Kutamba et al.; Maitland, Nadel et al. 2005; Akech, Gwer et al. 2006), although poor methodologic quality in some of these trials may have limited their ability to detect meaningful treatment effects (Enwere 2005). The search for an effective adjuvant in severe malaria remains a worthwhile goal given the significant residual mortality with primary antiparasitic treatment (Dondorp, Fanello et al.; Dondorp, Nosten et al. 2005). In this context, iNO, if demonstrated to be effective, would represent an important advance in malaria therapeutics.

Our hypothesis that children with severe malaria will benefit from adjunctive iNO can be answered using a modest sample size (n=180). This parsimonious design was made possible by selecting a quantitative biomarker of malaria severity, Ang-2, as the primary endpoint and by using a powerful statistical technique (mixed-effects linear model). In contrast, a study using mortality (dichotomous variable) as the primary endpoint would require a total of over 1,000
patients to detect a 30% reduction from the baseline mortality of 20% with 80% power, likely necessitating multicentre recruitment over several years and monumental resources. Ang-2 is a surrogate but well validated measure of malaria severity, appropriate for an early efficacy trial in a human population. Repeated longitudinal measurement of Ang-2 allows for increased precision in the quantitative outcome variable, thereby reducing the necessary sample size. The analytic plan, a mixed-effects linear statistical model, includes random-effect terms, appropriate for representing clustered and therefore dependent data arising when data are gathered over time on the same individuals (Laird and Ware 1982). We performed a computer simulation study to validate our sample size estimate using assumptions based on previous studies of Ang-2 in severe malaria. In 9,000 simulated trial outcomes under different assumptions for the treatment effect size and baseline variability in Ang-2, the mixed-effects linear model detected a significant treatment effect with >80% power under most plausible scenarios. This a priori power calculation provides further refinement on a crude sample size calculation and provides additional evidence that the planned number of patients is adequate to test our hypothesis. This sample size validation is particularly important in light of a review highlighting that numerous previous trials of adjunctive treatments for cerebral malaria had insufficient statistical power to detect even large treatment effects (Enwere 2005).

Some unique aspects of our trial relate to its setting in a peripheral, resource-constrained pediatric hospital in sub-Saharan Africa. Standard clinical investigations including quality-controlled microscopy, biochemistry and microbiology, as well as equipment to monitor gas delivery (NO, NO₂ and methemoglobin monitoring) need to be introduced to the facility for the trial. A commercially available portable biochemistry instrument (i-STAT®, Abbott Point of Care Inc., Princeton, NJ) and a pulse CO-oximeter for non-invasive methemoglobin monitoring (Masimo Rad-57™, Masimo Corporation, Irvine, CA) will allow for onsite monitoring of critical investigations, with outsourcing of other clinical testing to reference laboratories in Kampala. Objective qualitative determination of parasitemia at presentation for the purposes of trial enrolment using commercially available lateral flow immunochromatographic tests for parasite antigen detection (rapid diagnostic tests) will be used to supplement local microscopy, which may be subject to error in the absence of rigorous laboratory quality control (Hawkes and Kain 2007). While upgrading hospital capacity for our trial requires the infusion of considerable resources, it is hoped that this will result sustained local capacity development in clinical
management, laboratory diagnostics, modern therapeutics, and innovation at the Jinja Regional Referral Hospital, consistent with the ethical objectives of our trial.

The cross-cultural setting of the trial poses certain ethical challenges, demanding a sensitive approach to the informed consent process. While some of these ethical considerations are of a universal nature, others may be more specific to the sub-Saharan African context. First, the trial involves a vulnerable pediatric population with surrogate decision-makers. In addition to parent/guardian consent in the local and education-appropriate language, we have built in a second assent process for competent participants, given that children may legitimately and autonomously participate in decisions related to their own healthcare (Henschel, Rothenberger et al.; Wendler 2006). Next, the acuity and lethality of the underlying infection demand that consent be obtained early after admission for maximal treatment benefit, yet must not interfere with the emergency management of critically ill participants and must give adequate time for stressed parents/guardians to give due consideration before consenting. The complexity of the scientific design (randomized, placebo-controlled adjunctive therapy, with blinding of treatment allocation) together with variable education level and familiarity with biomedical research of parents/guardians presents difficulties requiring careful explanation during the consent discussion (e.g., conveying the position of scientific equipoise in order to accept possible randomization to placebo). Additional ethical considerations which may be more specific to the African context include the “therapeutic misconception,” (participants may not clearly distinguish research from health interventions) (Lynoe, Hyder et al. 2001; Molyneux, Peshu et al. 2005). Furthermore, African parents frequently express concern about blood taking, including fears about the misuse of the blood, unauthorized testing for HIV, long-term storage, genetic testing, and harm to the child from excessive blood loss (Molyneux, Peshu et al. 2005). Our consent process explicitly addresses the distinction between study and clinical interventions, as well as detailed descriptions of volumes, frequency and subsequent handling of blood samples in the trial. Finally, we have incorporated a community engagement (CE) plan into our trial design to foster a trusting relationship with the surrounding catchment population.

Monitoring patient safety in a trial involving critically ill children in a resource-limited environment poses additional challenges. The administration of study gas will be tightly regulated and monitored with state-of-the-art technology, with particular attention to two dose-
dependent, reversible adverse effects: elevated inspired NO\textsubscript{2} and methemoglobinemia. Strict criteria for study gas discontinuation have been established. Standardized pediatric toxicity tables will be used to monitor for other adverse events in a blinded and objective manner. We will also monitor mortality using statistical control charts, in order to rationally detect deviations from the expected baseline mortality of 20\% (Idro, Aloyo et al. 2006; Kyu and Fernandez 2009). This approach involves striking a balance between the earliest detection of elevated mortality in order to institute corrective measures (“true alarm”), and the risk of halting the trial unnecessarily for variations in mortality due to chance alone (“false alarm”). An upper control limit of mortality that will prompt a safety review has thus been defined using statistical principles, together with the predicted performance of this surveillance strategy. Trial safety oversight will ultimately be provided by an independent DSMB who will meet regularly to review recruitment, interim evidence of efficacy, safety and trial quality.

Limitations of this trial design include its use of a surrogate marker, Ang-2, as the primary endpoint. This allows for an efficient design but may be less compelling to clinicians than would a demonstrable mortality benefit (of note, mortality has been included as a secondary trial endpoint). On the other hand, Ang-2 has been extensively validated as an objective and quantitative biomarker of malaria severity (Conroy, Phiri et al.; Yeo, Lampah et al. 2008; Conroy, Lafferty et al. 2009; Lovegrove, Tangpukdee et al. 2009). Thus, Ang-2 is an appropriate endpoint for an initial efficacy study of iNO in a human population, but a promising treatment effect would require validation in costly, large, multicentre trials. Blinding presents a challenge for a gaseous therapy requiring monitoring for dose-dependent toxicities, which we have addressed by separating study tasks between a blinded team (responsible for clinical care, assessment of endpoints and laboratory testing) and an unblinded team (responsible for administering treatment and monitoring safety parameters) to minimize potential bias. Risks and costs related to conducting the study in a resource-constrained setting are balanced with potential benefits in terms of local capacity building, the high incidence of severe malaria allowing timely completion of the study, and applicability of the therapy in other malaria-endemic areas.

In summary, based on compelling data supporting the efficacy of iNO in experimental cerebral malaria in animal models, coupled with the documented safety of iNO in clinical practice and trials for other diseases, we have outlined a protocol for a randomized clinical trial of iNO for the
adjunctive treatment of severe malaria in Ugandan children. If our study demonstrates a significant treatment effect, this would represent a major and important advance in the treatment of severe malaria with broad potential for global public health impact.
Chapter 10
Summary and Future Directions

10.1 Thesis overview

Together, malaria and tuberculosis account for more than 2 million deaths annually. Furthermore, the economic impact of each disease is considerable: both diseases disproportionately affect the most disadvantaged populations globally, and an annual global budget of $200 million and $3.3 billion is spent on control programs for malaria and TB, respectively (Lau, Chan et al. 1998; Winkelstein, Marino et al. 2000). The present thesis examines co-infection with malaria and tuberculosis and explores shared pathways in innate immunity to both pathogens. Chapter 2 examines the biological interaction between malaria and mycobacteria in a mammalian host, showing that malaria may exacerbate primary or re-activation mycobacterial disease. Chapter 3 explores the role of the macrophage scavenger receptor CD36, previously shown to contribute to parasite clearance in the context of malaria, in a murine model of tuberculosis. Chapters 4 to 7 show that biomarkers of host defense pathways common to both malaria and tuberculosis distinguish between clinical disease phenotypes and predict mortality in severe malaria. One of these biomarkers, Ang-2, is released from activated endothelial cells early in the course of severe malaria, and elevated levels of Ang-2 are prognostic of a poor outcome including mortality. One strategy to inhibit Ang-2 release, inhaled nitric oxide (Matsushita, Morrell et al. 2003), is explored in Chapters 8 and 9. These chapters describe a hypothesis and protocol to test whether adjunctive therapy with inhaled nitric oxide, previously tested in clinical trials of pulmonary tuberculosis (Long, Jones et al. 2005), may also improve outcomes in African children with severe malaria.

10.2 A comparison of selected aspects of host response to malaria and tuberculosis

It is interesting to note, given obvious differences between the prokaryotic bacteria *M. tuberculosis* and the apicomplexan parasite *P. falciparum*, that host responses to both pathogens share some similarities. Clinical and pathobiological aspects of malaria and tuberculosis are compared and contrasted in Table 10.1.
Chapter 1 provided an overview of selected clinical aspects of each disease, both of which may produce disease across a spectrum of severity: fatalities are common in severe and cerebral malaria as well as miliary TB and TB meningitis, and neurocognitive deficits commonly follow CNS involvement in both diseases (Table 10.1).

Chapter 3 addressed the role of the class B scavenger receptor CD36 in experimental mycobacterial infection. CD36 also has a recognized role in malaria pathogenesis, contributing to non-opsonic phagocytic uptake of parasitized erythrocytes, sequestration in the peripheral vasculature, and immune evasion through interactions with dendritic cells (Serghides, Smith et al. 2003) (Table 10.1).

Likewise, Chapters 4-7 described host biomarkers of disease severity in malaria, which collectively pointed to alterations in circulating levels of key mediators of inflammation, coagulation and endothelial activation. Several of these pathways have also been implicated in immunity to *M. tuberculosis* (Table 10.1), and are discussed below.

Chapter 6 described the utility of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) as a biomarker of malaria severity. TREM-1 mediates pro-inflammatory responses in experimental models of sepsis (Bouchon, Dietrich et al. 2000; Bouchon, Facchetti et al. 2001) and the membrane density of TREM-1 on peripheral monocytes has been previously shown to be increased in patients with uncomplicated malaria (Chimma, Roussilhon et al. 2009). With respect to its role in tuberculosis, experimental systems demonstrate that TREM-1 activation using an agonist monoclonal antibody did not reduce intracellular replication of *M. tuberculosis* in cultured macrophages *in vitro* (Bleharski, Kiessler et al. 2003). However, soluble-TREM-1 levels are elevated in the sputum of patients with pulmonary tuberculosis and correlate with clinical severity score (Tintinger, van der Merwe et al.). Although TREM-1 has not yet been demonstrated to participate directly in intracellular antimycobacterial defense programs, it appears to be a marker of disease severity in patients with pulmonary TB as in malaria (Chapter 6).

Circulating levels of the leukocyte-endothelial adhesion molecule sICAM-1 were shown to have predictive value as a biomarker of disease severity in Chapter 6. In an analogous manner, elevated levels of soluble ICAM-1 have been detected in the sera of patients with TB compared
with normal controls, and sICAM-1 concentrations correlate well with indicators of disease severity including the mycobacterial count, radiologic severity score, presence of cavitary disease, and the inflammatory markers ESR, CRP and peripheral neutrophil count (Lai, Wong et al. 1993; Shijubo, Imai et al. 1993). Thus, ICAM-1 appears to be a clinically informative surrogate measure of disease severity in both malaria and active pulmonary TB (Mukae, Ashitani et al. 2003). In contrast, levels of sP-selectin, another leukocyte-endothelial adhesion molecule, are significantly higher in the serum of active TB patients compared to healthy controls, and correlate with bacillary burden and other markers of inflammation (Mukae, Ashitani et al. 2003) but are no different in severe compared to uncomplicated malaria (Chapter 6).

The angiogenic regulatory factor sFLT-1 was described in Chapter 6 as a biomarker of malaria severity. Other components of the VEGF signaling pathway have also been implicated in cerebral malaria pathogenesis (Deininger, Winkler et al. 2003). In the context of tuberculosis, intense angiogenesis has been found in pathologic specimens obtained from patients with active pulmonary tuberculosis (Ridley, Heather et al. 1983). Activated macrophages appear to be the main source of the pro-angiogenic factor VEGF in tuberculosis lesions (Matsuyama, Hashiguchi et al. 2000). VEGF concentrations are increased in the serum of patients with active pulmonary tuberculosis and in the CSF of patients with active tuberculous meningitis (Matsuyama, Hashiguchi et al. 2000; Husain, Awasthi et al. 2008). VEGF levels decline with treatment, suggesting that they represent a measure of disease activity and possibly an indicator of response to therapy (Husain, Awasthi et al. 2008). The associated soluble VEGF receptor, sFlt-1, is present at elevated concentrations in exudative pleural effusions of patients with pleural or pulmonary TB (Tomimoto, Yano et al. 2007). Thus, sFLT-1 and possibly other components of the VEGF signaling pathway may be important molecular determinants of clinical outcome in both malaria and tuberculosis.

Peripheral levels of angiopoietins were examined as biomarkers of malaria severity in Chapters 4-7. Limited studies have also suggested a possible role for angiopoietins in the pathobiology of tuberculosis, by modulating the process of granuloma neovascularization. A central core of caseous necrosis, characteristic of tuberculous lesions in humans may become fibrotic or mineralized over time, or may liquefy and erode into a bronchus, resulting in intrapulmonary dissemination of mycobacteria and transmission of tuberculosis in the population. CD4+ T cells
and their cytokine product, interferon-gamma (IFN-γ), are major determinants of granuloma necrosis (Ehlers, Benini et al. 2001). Downstream of IFN-γ, experimental models have demonstrated that an imbalance between angiostatic and angiogenic mediators, including the angiopoietins, and a concomitant reduction in granuloma vascularization results in hypoxia and caseation at the granuloma core. Although the implications for human disease have yet to be fully explored, angiopoietins may be important molecular determinants of tuberculosis progression, as in malaria (Chapters 4-7).

Chapters 8 and 9 describe experiments to test the role of nitric oxide in severe malaria in children. Nitric oxide also has an established role in host defense against tuberculosis. Inducible nitric oxide synthase (iNOS)-mediated production of toxic reactive nitrogen intermediates (RNI) by activated macrophages is cidal against M. tuberculosis both in vitro (Chan, Xing et al. 1992) (Denis 1991) and in vivo (Flynn, Chan et al. 1993; Chan, Tanaka et al. 1995; Flynn, Goldstein et al. 1995). In a murine model, inhibition of endogenous NO generation by chemical inhibitors of iNOS during acute M. tuberculosis infection leads to rapidly fatal disease progression with heavy bacterial burden in end-organs (Chan, Tanaka et al. 1995; Flynn, Scanga et al. 1998).

In summary, shared host defense pathways in tuberculosis and malaria provide an illustration that conserved cellular and molecular pathways act to contain and eradicate a broad array of pathogens (Table 10.1).
Table 10.1. Comparison of selected clinical and pathobiological aspects of malaria and tuberculosis

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Malaria</th>
<th>Tuberculosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Etiology</strong></td>
<td><em>P. falciparum</em> Eukaryotic apicomplexan parasite of RBCs</td>
<td><em>M. tuberculosis</em> Prokaryotic mycobacterium</td>
</tr>
<tr>
<td><strong>Life-threatening clinical syndromes</strong></td>
<td>Cerebral malaria Respiratory distress with acidosis Severe malarial anemia</td>
<td>Miliary tuberculosis Tuberculous meningitis</td>
</tr>
<tr>
<td><strong>Diagnosis</strong></td>
<td>Light microscopy Immunochromatographic rapid diagnostic tests</td>
<td>Active disease: Culture LTBI: tuberculin skin test, IGRA</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>Uncomplicated: Artemisinin combination therapy Severe malaria: parenteral artesunate</td>
<td>1st line agents: Rifampin, isoniazid, ethambutol, pyrazinamide</td>
</tr>
<tr>
<td><strong>Prognosis</strong></td>
<td>Mortality in severe disease 20% despite potent antimalarials</td>
<td>14-21% (miliary tuberculosis)</td>
</tr>
<tr>
<td></td>
<td>Neuro-cognitive sequellae 25% of survivors of CM</td>
<td>80% of meningitis survivors followed long-term</td>
</tr>
<tr>
<td><strong>Molecular pathobiology</strong></td>
<td>CD36 Receptor for non-opsonic phagocytic uptake of PEs Mediates sequestration of pRBCs in peripheral vasculature May contribute to immune evasion by inhibition of dendritic cell function</td>
<td>CD36 deficiency attenuates experimental mycobacterial infection. CD36 may participate in cellular processes (apoptosis, macrophage migration, phagocytosis) involved in mycobacterial expansion (Chapter 3).</td>
</tr>
<tr>
<td></td>
<td>sTREM-1 Elevated plasma levels are associated with severe disease and mortality in severe malaria in children (Chapter 6)</td>
<td>Sputum levels of sTREM-1 reflect clinical severity</td>
</tr>
<tr>
<td></td>
<td>sICAM-1 Elevated plasma levels are associated with severe disease and mortality (Chapter 6)</td>
<td>Elevated serum levels are associated with severe disease</td>
</tr>
<tr>
<td></td>
<td>sFLT-1 Elevated plasma levels are associated with severe disease and mortality (Chapter 6)</td>
<td>Elevated in tuberculous pleural effusions</td>
</tr>
<tr>
<td></td>
<td>Angiopoietins Low Ang-1, elevated Ang-2, and elevated sTie-2 are associated with disease severity and mortality (Chapters 4-7)</td>
<td>May contribute to granuloma neovascularization</td>
</tr>
<tr>
<td></td>
<td>Role of nitric oxide Inhibits WP body release and promotes endothelial quiescence</td>
<td>Reactive nitrogen intermediates, including NO, mediate intracellular killing of mycobacteria by activated macrophages</td>
</tr>
</tbody>
</table>
10.3 Interactions between malaria, tuberculosis and HIV

Among the three leading infectious diseases globally, we have discussed malaria and tuberculosis, as well as their interaction in an experimental model. Human immunodeficiency virus, the causative agent of acquired immunodeficiency syndrome (HIV/AIDS), also contributes to the considerable burden of infectious disease morbidity and mortality worldwide, causing 2.7 million new infections and 1.8 million deaths annually (WHO, 2010). As a chronic and immunosuppressive viral infection, HIV interacts with both tuberculosis and malaria, contributing to the burden due to each pathogen.

Of the 9.4 million new cases of active TB each year, approximately 15% are infected with HIV. HIV infected individuals are at greater risk of acquiring Mtb, developing active TB, and have an increased risk of death due to TB (Whalen, et al. 2000). Several pathologic mechanisms explain, at least in part, how HIV increases susceptibility to Mtb and exacerbates disease severity. The CD4+ T-lymphocyte and the macrophage, both essential for mycobacterial containment, are targets of HIV. HIV viral loads are elevated in the plasma of patients during acute infection with Mtb and are higher at the locus of mycobacterial infection, including bronchoalveolar lavage fluid cells in pulmonary TB, and pleural fluid in pleural disease (Nakata, K., et al. 1997). HIV produces a decrease in the total number of CD4+ T cells, and functional changes which impair specific antimycobacterial defenses. HIV causes a disruption of macrophage function and produces detrimental changes within granulomas (Diedrich, Flynn et al. 2011).

Acute malaria infection is estimated to produce a transient but clinically significant increase in the HIV viral load, which may increase transmission and fuel the spread of HIV in sub-Saharan Africa (Abu-Raddad, Patnaik et al. 2006). Quantitatively, malaria is estimated to produce a logarithmic increase in the HIV viral load of 0.82 log, lasting for 42 days. This increase in the viral load could result in a 2.0-fold increase in the odds of transmission per coital act, thereby potentiating the spread of HIV. On the other hand, acute malaria infection may be associated with decreased frequency of coital acts, which may function to reduce HIV transmission. The net effect has been estimated in transmission dynamic mathematical models which suggest that dual infection with HIV and malaria fuels the spread of both diseases in sub-Saharan Africa (Abu-Raddad, Patnaik et al. 2006).
Many areas of the world where malaria and tuberculosis are co-endemic also have a high prevalence of HIV. Given the chronic nature of HIV and tuberculosis, and the high frequency of malaria exposure in some hyperendemic zones, triple infection with these three pathogens may occur in individuals residing in zones of intense transmission. Our work in Chapter 3 of this thesis documented alterations in macrophage function due to malaria infection that affect intracellular mycobacterial expansion in vitro. As another pathogen of the macrophage, HIV may have an additive or synergistic detrimental effect on macrophage antimycobacterial defense programs. Likewise, granuloma dysfunction may be compounded in the presence of triple infection with HIV, malaria and mycobacteria. In epidemiologic studies of human populations, HIV will likely be an important effect modifier or confounding variable, and should be factored into the design of any observational trial.

10.4 Malaria-mycobacteria co-infection

10.4.1 Summary

Chapter 2 describes experiments pointing toward a biological interaction between malaria and mycobacteria in experimental systems in vitro and in vivo. Immune dysregulation in the context of acute malaria infection led to increased mycobacterial burden in infected mice and in murine macrophages. The mechanism appears to relate to hemozoin-loading of macrophages with consequent dysfunction in antimycobacterial defenses (nitric oxide production). These dysfunctional macrophages localize within granulomas, which may result in the loss of containment of mycobacteria at the locus of the chronic inflammatory response. These results add to the accumulating evidence supporting a biological basis for the interaction of malaria and tuberculosis. Previous murine models have demonstrated a bi-directional effect: malaria exacerbates M. tuberculosis infection (Scott, Kumar et al. 2004), and mycobacterial infection modulates the host response to malaria (Page, Jedlicka et al. 2005; Leisewitz, Rockett et al. 2008).

These findings lead to intriguing speculation about possible biological interactions between two of the world’s leading human pathogens. Tropical and/or developing countries carry the heaviest burden of both of malaria and tuberculosis, and co-infection is likely to occur in individuals in these zones of intense transmission. Might malaria explain, at least in part, why TB remains so
stably entrenched in Africa and Asia despite local and international control efforts, whereas it has been virtually eliminated in many malaria-free zones? Epidemiological interactions between the three major infectious disease are well-recognized: the resurgence of TB globally is largely driven by the HIV epidemic (Chaisson and Martinson 2008), and mathematical modeling studies have demonstrated that dual infection with malaria and HIV fuels the spread of both diseases in Africa (Abu-Raddad, Patnaik et al. 2006). However, malaria-tuberculosis interaction - the third relationship within this triad of globally important infections - has been explored less thoroughly.

Malaria and TB are still prevalent worldwide despite active international control efforts (Yamey 2004; Chaisson and Martinson 2008). Improved understanding of the interaction of these pathogens in human populations may illuminate new approaches for disease control. Possibly, integration of TB and malaria control programs could have synergistic effects on both diseases at a population level.

10.4.2 Future directions

Our experiments in animal model raise the question whether malaria may increase susceptibility to TB infection in humans, and conversely, whether TB may alter the course of malaria infection. This naturally leads to hypotheses testable with standard clinical epidemiology methods. As an initial step, a cross-sectional study of the point-prevalence of both diseases in a highly endemic area may point to an association between malaria and tuberculosis in human populations. Individuals latently infected with tuberculosis can be identified by standard - albeit imperfect - methods (tuberculin skin test positivity and/or IGRA). Simultaneously, malaria parasitemia can be determined by microscopy and/or RDT. The prevalence of each infection alone and in combination (malaria-tuberculosis co-infection) may point to a synergistic interaction between the pathogens at a population level. Such an association, if borne out empirically, would suggest that latent tuberculosis infection may influence malaria transmission by altering the pool of parasitemic individuals. For optimal statistical power, this association should be tested in a population where both diseases are highly endemic.

Beyond this logistically and conceptually simple cross-sectional study, case-control and prospective observational studies can be conceived which would provide stronger evidence of malaria-tuberculosis interactions at a population level. To test the hypothesis that malaria
exacerbates primary tuberculosis infection, as suggested by experiments outlined in Chapter 2, a case-control study in a pediatric population unlikely to have re-activation disease or previous exposure to tuberculosis may be informative. Retrospective diagnosis of malaria can be made using an HRP-2 based assay, which provides evidence of recent *P. falciparum* infection over the previous 28 days. Rates of recent malaria infection could be compared between cases with severe and disseminated primary tuberculosis (miliary tuberculosis and tuberculous meningitis) and exposed controls with no evidence of active infection (latent TB). Higher rates of HRP-2 positivity in patients with severe disseminated tuberculosis compared to controls with latent infection (controlled by host defenses) would lend support to the hypothesis that recent malaria infection exacerbates primary tuberculosis disease.

To test the hypothesis that malaria may promote clinically significant re-activation of *M. tuberculosis* from latent foci of infection, as suggested by results presented in Chapter 2, a similar case-control study could examine rates of recent malaria in adults with early classical apical cavitary tuberculosis compared to controls with: (1) latent TB; and (2) no evidence of latent or active TB. Increased rates of recent malaria infection among cases with re-activation disease would suggest an association between malaria infection and re-activation tuberculosis. Alternatively, cumulative exposure to malaria using serological methods could be used to investigate whether remote and/or recent malaria exposure is associated with re-activation tuberculosis.

A bi-directional interaction between malaria and tuberculosis is plausible, and the effect of chronic (latent) tuberculosis on malaria incidence and severity is also of interest. An appropriate clinical study design to test this hypothesis is a prospective cohort study comparing exposed (latently infected) with unexposed (uninfected individuals). Important outcomes would include the incidence of: (1) asymptomatic parasitemia or HRP-2 positivity; (2) symptomatic, parasitologically confirmed uncomplicated malaria; and (3) severe malaria. Comparing rates of malaria in TB exposed and unexposed individuals could provide evidence that TB modifies risk of malaria infection and/or severity.

We have outlined four potential clinical studies to examine a bi-directional effect of malaria-tuberculosis interaction. The classical designs (cross-sectional, retrospective case-control, and
prospective cohort studies) could provide increasingly compelling evidence of an interaction in human populations, which would have meaningful implications for public health. At the same time, increasing investment of resources would required to execute these studies, such that a step-wise approach, beginning with pilot data from a cross-sectional study should probably be sought before embarking on large and prolonged prospective studies.

10.5 CD36 and tuberculosis

10.5.1 Summary

One third of the world’s population is estimated to be infected with *Mycobacterium tuberculosis* (Bellamy, Beyers et al. 2000). A complex interaction of environmental and genetic factors causes the development of clinical tuberculosis. Environmental risk factors include diabetes, advanced age, alcohol abuse, HIV infection, and corticosteroid usage (Bellamy, Beyers et al. 2000). Evidence that genetic factors contribute to TB susceptibility and clinical disease include studies on racial variation in susceptibility to tuberculosis and twin studies (Bellamy, Beyers et al. 2000). Examples include the natural resistance-associated macrophage protein (NRAMP1) and vitamin D receptor (VDR) genes, which are associated with smear-positive pulmonary tuberculosis (Bellamy, Ruwende et al. 1998; Bellamy, Ruwende et al. 1999; Bellamy, Beyers et al. 2000). Chapter 3 adds to the expanding number of genetic factors that have been implicated in defense and pathogenesis of tuberculosis, identifying CD36 as a significant determinant of tuberculous disease in a murine model.

Chapter 3 outlined experiments designed to test the hypothesis that the scavenger receptor CD36 participates in the innate immune response to tuberculous infection in an animal model. In contrast to other experimental infections (Stuart, Deng et al. 2005; Patel, Lu et al. 2007), CD36 deficiency conferred protection against acute and re-activation mycobacterial disease, as indicated by reduced bacterial load in target organs (liver and spleen). Although further work will be necessary to elucidate the mechanism underlying this phenomenon, our experiments demonstrate that there is reduced intracellular growth of mycobacteria in CD36<sup>−/−</sup> murine macrophage monolayers *in vitro*, and that mycobacterial counts are reduced in CD36<sup>−/−</sup> macrophages in an experimental *in vitro* system recapitulating the cellular events of macrophage turnover early in granuloma formation.
These findings are intriguing from a biological point of view, extending the already broad spectrum of infections in which CD36 contributes to the host response, and highlighting the role of CD36 in numerous cellular processes that are engaged in innate immune defenses generally. The biological mechanisms implicated in antimycobacterial defenses include apoptosis, phagocytosis, cell migration, intracellular phagolysosome trafficking, and microbial killing. CD36 has an established role in several of these fundamental processes (Voll, Herrmann et al. 1997; Wintergerst, Jelk et al. 2000; Febbraio, Hajjar et al. 2001), and we have demonstrated that CD36 deficiency alters outcomes in a murine model of tuberculosis. These basic programs are engaged in the defense against a diverse range of pathogens, and CD36 has been implicated in the response to gram-positive (Hoebe, Georgel et al. 2005) and gram-negative bacteria, as well as parasites including *Plasmodium* species (Serghides, Smith et al. 2003; Patel, Lu et al. 2007). Thus, despite obvious differences between the *Plasmodium* parasite and the bacterium *M. tuberculosis*, CD36 contributes to the host-pathogen balance response in both infections (Table 10.1). On the other hand, whereas CD36 participates in the internalization of P. falciparum parasitized erythrocytes, we found no difference in the uptake and selected cytokine responses between CD36 deficient and wild type murine macrophages to mycobacterial challenge. It would appear that CD36, an evolutionarily conserved scavenger receptor from *Drosophila* to humans (Philips, Rubin et al. 2005), is exploited by the finely adapted mycobacterial pathogen, leading to higher burden of disease at early stages of the experimental infection. Similarly, recent seminal studies have linked mycobacterial virulence factors to the formation of granulomas, classically considered host-protective but now shown to promote bacterial expansion (Volkman, Clay et al. 2004).

If CD36 can be shown to affect susceptibility to tuberculosis in human populations, as it does in mice, several possible implications of these findings for human health can be conceived. As a marker of susceptibility, screening for CD36 might be a useful screening tool to target prevention strategies in susceptible populations. Pharmacologic manipulation of CD36 activity or expression might represent an as yet unexplored immunomodulatory strategy for treating mycobacterial diseases such as leprosy or tuberculosis.
10.5.2 Future directions

Additional experiments are warranted to further define the mechanism by which CD36 deficiency reduces intracellular mycobacterial replication *in vitro*, as well as mycobacterial loads *in vivo*. Translating these findings to human populations would also be of interest, and could take the form of an observational case-control study.

As an initial step in pinpointing mechanism for the restricted intracellular growth of mycobacteria within *Cd36*−/− macrophages, further imaging modalities may be useful. Electron microscopy studies may help as a preliminary step to visualize the subcellular events leading to restriction of mycobacterial replication in monolayers of *Cd36*−/− macrophages *in vitro*, and may provide clues for further experimentation. Confocal fluorescent microscopy using differentially labeled mycobacteria and phagolysosomes, as in (Tan, Lee et al. 2006), may also be informative to define potential differences in the in the intracellular fate of mycobacteria following internalization by *Cd36*+/+ and *Cd36*−/− macrophages.

To further dissect the *in vivo* phenomenon, further work might examine cellular migration events toward the site of infection (the peritoneum in our experimental system), and subsequent dissemination to reticuloendothelial tissues. CD36 mediates cell signaling pathways that alter cytoskeletal dynamics and inhibit macrophage motility. Previous studies have demonstrated that the CD36 ligand oxidized LDL downregulated *Cd36*+/+ but not *Cd36*−/− macrophage egress from inflamed peritoneum in a murine model. Moreover, *in vitro*, *Cd36*+/+ but not *Cd36*−/− macrophages exhibited decreased motility in response to oxidized LDL (oxLDL) in a modified Boyden Chamber assay (Park, Febbraio et al. 2009). Given its recognized role in cell migration, it may be of interest to examine, *in vitro* and *in vivo*, the kinetics of uninfected *Cd36*+/+ and *Cd36*−/− macrophage migration toward mycobacterial attractants (either whole bacterial or a cellular component), as well as the migration kinetics of *Cd36*+/+ and *Cd36*−/− mycobacteria-laden macrophages. CD36-dependent inhibition of macrophage motility in the context of tuberculous infection could explain, at least in part, impaired function of critical effector cells and increased burden of mycobacteria in *Cd36*+/+ as observed in our experiments. If CD36 plays a significant role in macrophage motility in the context of mycobacterial infection, this may be relevant to the pathogenesis of disseminated (miliary or meningitic) primary tuberculosis.
Another potential avenue of investigation is the foamy macrophage, a hallmark of atherosclerotic lesions, but also observed in clinical specimens from patients with tuberculosis. The foam cell develops as a result of excessive uptake of oxidized low density lipoprotein (oxLDL), which loads the macrophages with cholesterol to the point where natural efflux pathways are overwhelmed, generating abundant lipid-inclusion bodies. CD36 is a critical player in formation of foam cells, mediating the uptake of oxLDL, which contains lipids that serve as ligands or precursors of ligands for the nuclear hormone receptor PPARγ (Tontonoz, Nagy et al. 1998). PPARγ acts as a transcription factor that drives expression of many metabolic genes, including CD36 itself. The net effect is a positive feedback loop leading to increased surface expression of CD36, increased internalization of oxLDL, producing lipid-laden foamy macrophages (Silverstein, Li et al.). In the context of tuberculosis, foam cells appear to play a central role in sustaining persistent bacteria within the macrophage cytoplasm and contributing to the tissue pathology that leads to cavitation and the release of infectious bacilli (Russell, Cardona et al. 2009). Given their importance in TB pathogenesis and the known role of CD36 in their genesis, foamy macrophages may be of interest for further study. Comparison of tuberculous lesions in Cd36+/+ and Cd36−/− mice might reveal quantitative or qualitative differences in foam cells at the locus of infection. Absence or reduction in foam cell formation in CD36 deficient mice, resulting in fewer dysfunctional macrophages, might provide an explanation for relative resistance of Cd36−/− to mycobacterial challenge.

Certain human populations carry high rates of CD36 deficiency, including specific groups in Africa, Japan and Asia (Yamamoto, Ikeda et al. 1990; Curtis and Aster 1996). The CD36−/− genotype has been associated with susceptibility to a variety of metabolic diseases (Rac, Safranow et al. 2007), raising the question whether CD36 deficiency may represent a balanced polymorphism, conferring a simultaneous survival benefit. Recent population and family-based studies have not associated CD36 gene polymorphisms with severe malaria, and some authors have suggested that CD36 deficiency alleles may be maintained in human populations through selection pressure via a prevalent infection other than malaria (Aitman, Cooper et al. 2000; Fry, Ghansa et al. 2009). In light of our observations in a murine model, resistance to tuberculosis, a common and potentially lethal infectious disease, might explain the high prevalence of CD36 deficiency in populations where TB is prevalent, or was prevalent in recent human evolutionary history. This intriguing hypothesis could be tested using clinical epidemiology methods: a case-
control study examining CD36 gene polymorphisms in patients with primary TB, re-activation TB, or latent TB, compared to healthy controls might be informative if differences in CD36 alleles are observed between groups. This focused approach contrasts with genome-wide searches for tuberculosis-susceptibility genes which did not identify CD36 as a candidate gene (Bellamy, Beyers et al. 2000), but may have lacked sufficient power to detect any but the most potent genetic determinants.

10.6 Biomarkers of severe malaria

10.6.1 Summary

Chapters 4, 5, 6 and 7 outline retrospective observational case-control studies in five separate human populations that collectively demonstrate a robust association between dysregulation of endothelial function in the context of severe and fatal malaria, as outlined in Table 10.2. The study groups differ in age range (children as well as adults), geographic location (sub-Saharan Africa and Asia), and transmission intensity (seasonal, intermittent transmission in Thailand, moderate to high endemicity in Uganda (Idro, Aloyo et al. 2006) and Malawi), such that the consistent finding of elevated Ang-2 in severe and fatal malaria appears to be a uniform feature of malaria pathobiology.

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<thead>
<tr>
<th>Table 10.2. Biomarkers of malaria severity: summary of findings (Chapters 4-7)</th>
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<td>Country</td>
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<tr>
<td>Study Population</td>
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<tr>
<td>Adults (≥13 yr)</td>
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<td>25 UM</td>
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<td>25 CM</td>
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<td>SM severe malaria; CM cerebral malaria; UM uncomplicated malaria; HC healthy controls</td>
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These findings have broad implications for the development of novel tools to assist practitioners to triage, diagnose and manage patients presenting with malaria. The clinical syndrome of cerebral malaria may be difficult to differentiate from other causes of fever and CNS insult. A panel of endothelial-based biomarkers that distinguishes between clinical syndromes could be of benefit to clinicians treating patients with fever and CNS dysfunction. The prognostic value of biomarkers could also be harnessed for hospital triage, directing patients at greatest risk of adverse outcomes to intensive therapies and aggressive supportive care.

Research efforts can also be streamlined by using objective and quantitative biomarkers of malaria severity. For example, by identifying a group of patients with an elevated risk of mortality, biomarkers could help focus research studies that use death as a study endpoint. Enriching study populations with a high event rate (i.e., mortality) allows for efficient study design and smaller sample sizes, which may be appropriate for accelerating discovery at the proof-of-principle stage. Furthermore, biomarkers of malaria severity can be used themselves as surrogate endpoints in clinical trials. The use of objective and quantitative markers of disease severity may also allow for smaller sample sizes than binary outcomes such as mortality. We have adopted this strategy in the design of our clinical trial (Chapters 8 and 9). Ang-2 is the primary endpoint of our study examining the efficacy of adjunctive inhaled nitric oxide in children with severe malaria. Ang-2 serves as a surrogate endpoint, representing a validated, objective proxy of disease severity. The benefit for study design is a reduced sample size, allowing the hypothesis to be tested efficiently, without excessive resource utilization at this early stage of testing nitric oxide in the context of human malaria.

10.6.2 Future directions

The prognostic utility of endothelium-based biomarkers is demonstrated in multiple populations in these retrospective analyses, and could be further validated in prospective observational studies. Another approach would be to use an experimental design (prospective, randomized clinical trial) to examine the impact of biomarker assays on patient outcomes or cost-effectiveness. Conceptually, diagnostic, prognostic, or “theranostic” assays may influence patient outcomes by improving accuracy, changing treatment, or avoiding unnecessary admission, tests
or treatments relative to standard management without the assay. Current standard management of malaria involves using clinical and basic laboratory parameters to guide patient triage and treatment decisions. In resource-constrained settings where malaria is most prevalent, reliance on clinical information alone is commonplace. Furthermore, practitioners may have limited training, skill, and time to perform accurate clinical assessments and apply this information to patient management. While it stands to reason that improved prognostic information would be beneficial in this context, the added value of novel biomarkers of malaria severity in terms of improved patient outcomes or cost savings remains to be empirically demonstrated. Moreover, given the paucity of evidence-based adjunctive therapies available to supplement antimalarial therapy to date, there remains a need to demonstrate how the information provided by biomarkers can substantively alter patient management. The claim that prognostic information provided by biomarkers will improve patient outcomes or produce other benefits without adversely affecting patients deserves testing in clinical trials.

In principle, long-term randomized controlled trials of test-plus-treatment strategies compared to current best practice would offer ideal evidence of the benefits of introducing a new biomarker (Lord, Irwig et al. 2009). The testable hypothesis is that biomarkers may reduce mortality and morbidity by focusing resources on the sickest patients, or may provide a cost-savings by targeting resources to the small minority of patients at greatest risk of adverse outcomes. The proposed role of the biomarker test within the clinical decision-making framework first needs to be defined: biomarkers could be used as (1) replacement, (2) add-on, or (3) triage test to provide benefit to patients. In the first scenario, biomarkers could substitute for clinical judgment; for example, community health workers in remote regions with minimal medical or nursing training could utilize a simple biomarker-based diagnostic device to refer patients at greatest risk of adverse outcome to secondary and tertiary treatment centres. The utility of biomarkers in this setting could be assessed using a RCT design, assigning patients to biomarker-guided management versus clinical judgment alone. The second scenario would assess whether biomarkers can be used to enhance clinical acumen. This could be tested in a hospital-based setting, where physicians are provided with biomarker data or not in a randomized fashion, to determine if outcomes are affected in a cohort of hospitalized patients with malaria. The third scenario would employ biomarkers to triage patients for intensive monitoring and/or supportive care and could be compared to clinical triage without biomarkers to examine for effectiveness in
terms of patient outcomes. Each of these study designs might also examine patient management costs as a primary or secondary study outcome, to determine if biomarkers result in more efficient use of resources in the study cohort.

10.7 Nitric oxide for the adjunctive treatment of severe malaria

10.7.1 Summary

In Chapters 8 and 9, we outline the rationale, hypothesis and protocol for a randomized controlled trial to test the efficacy of inhaled nitric oxide for the adjunctive treatment of severe malaria in a pediatric population in a resource-constrained environment. In brief, the hypothesis rests on pre-clinical studies demonstrating the efficacy of inhaled nitric oxide and nitric oxide donor molecules in experimental cerebral malaria in a mouse model (Gramaglia, Sobolewski et al. 2006), as well as a study of Indonesian adults with severe malaria using a nitric oxide donor (Yeo, Lampah et al. 2007). To test this hypothesis, we are conducting a prospective, parallel arm, randomized, double-blind, placebo-controlled trial of adjunctive nitric oxide, in addition to intravenous artesunate, among Ugandan children age 1-10 years with severe malaria. The trial is currently underway in Jinja, Uganda. If inhaled nitric oxide can be shown to improve outcomes in severe malaria, this would represent a major advance in malaria therapeutics, with important implications for a leading threat to human health globally.

10.7.2 Future directions

Beyond the implementation of the clinical trial, future directions of this work include validation and translation of the findings into a practical intervention with clinical and commercial value. Within the stepwise framework of drug development, pre-clinical studies of iNO and animal models of disease that predate our trial have demonstrated its potential therapeutic efficacy. Phase 1 and phase 2a clinical trials of inhaled nitric oxide have demonstrated its safety profile in healthy volunteers and in diverse patient populations (neonates, older children and adults) in a range of clinical settings (examples include respiratory failure, tuberculosis, and sickle cell disease). Our study builds on this series of investigations, representing a Phase 2b-3a study of modest sample size with a surrogate endpoint (Ang-2) in a new disease context: severe malaria. Larger, multi-centre studies (Phase 3b) powered to detect a mortality benefit and/or a difference in neurocognitive outcome in trial participants may be warranted in the future. Additional studies
may also be undertaken to verify a therapeutic benefit of iNO in a different population, such as adults with severe malaria in Asia. Finally, phase 4 studies (post-marketing surveillance) should be conducted if the labeled indications for iNO are expanded to include severe malaria. Either active or passive case-finding could be employed to monitor for rare adverse events associated with iNO therapy in the context of severe malaria.

If found to be effective, and if independent clinical studies support the use of iNO in severe malaria, this potentially transformative treatment strategy should be rapidly brought to scale to maximize public health benefit. Given resource constraints in many malaria-endemic areas, cost-benefit analysis should be considered to inform public health policy. Public-private partnerships and collaboration with industry are reasonable strategies to pursue scale-up and knowledge translation to a clinically useful therapy.
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