BIOMARKERS OF SEVERE MALARIA:
COMPLEMENT ACTIVATION AND DYSREGULATED
ANGIOGENESIS IN PLACENTAL MALARIA AND
CEREBRAL MALARIA

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Laboratory Medicine and Pathobiology
University of Toronto

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Biomarkers of Severe Malaria:

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Andrea L. Conroy

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Abstract

Biomarkers measured in the blood can provide information about disease pathophysiology, diagnosis and prognosis. Pronounced proinflammatory responses are characteristic of severe malaria, and excessive activation of the immune system is central to the pathophysiology of both cerebral malaria and placental malaria. Severe malaria is characterized by cytoadherence of parasitized erythrocytes to the microvasculature; impaired tissue perfusion; dysregulated inflammatory responses; and activation of the complement system, mononuclear cells, and endothelium. Despite the availability of effective antimalarial drugs, the mortality rate in severe malaria remains unacceptably high. To glean further insight into malaria pathophysiology, we investigated host biomarkers of immune activation in the blood of subjects with different manifestations of severe disease. C5 has been identified as being necessary and sufficient for the development of experimental cerebral malaria. We hypothesized that C5a (a terminal component of the complement cascade with potent inflammatory properties) may mediate its action by inducing and exacerbating inflammatory processes in severe malaria, leading to endothelial activation and dysregulated angiogenesis. I tested this hypothesis in vitro, and found that C5a
interacted with malaria toxin \( Pf/GPI \) to drive deleterious inflammatory and anti-angiogenic responses. As C5a and anti-angiogenic factor sFlt-1 have been implicated in models of pathologic pregnancies, we asked whether increased levels of C5a in subjects with placental malaria were associated with altered angiogenesis and poor birth outcomes. Our results suggest that C5a impairs angiogenic remodelling in placental malaria leading to vascular insufficiency and fetal growth restriction. Further, altered profiles of inflammatory and angiogenic biomarkers in the periphery may identify occult placental malaria infections. We extended these observations to cerebral malaria where similar pathogenic pathways contribute to disease pathophysiology. In adults and children with cerebral malaria, altered profiles of angiogenic proteins were associated with disease severity and mortality and represent putative diagnostic and prognostic biomarkers in severe malaria.
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Dissemination of Work Arising from this Thesis

Chapter 2:


This article is available open access.

Chapter 3:


ALC contribution: Human data from Malawi, KLS contribution: Murine model

Chapter 4:

Silver K*, Conroy AL*, Leke R, Leke RJI, Gwanmesia P, Taylor DW, Molyneux ME, Rogerson SJ and Kain KC (2011) Circulating soluble endoglin levels in pregnant women in Cameroon and Malawi – associations with placental malaria and fetal growth restriction. Accepted: PLoS ONE. *These authors contributed equally to this manuscript

This article is available open access.

ALC contribution: Cross-sectional study from Malawi.

Chapter 5:

Chapter 6:


This article is available open access.

Chapter 7:

doi:10.1371/journal.pone.0015291

This article is available open access.

Chapter 8:

Additional Publications Arising During the Course of Graduate Studies


- This is the first report to demonstrate the importance of complement protein C5a in severe malaria. It was demonstrated using a P. berghei ANKA murine model of cerebral malaria with in vitro corroborating data.


- To further evaluate our initial findings of endothelial activation in cerebral malaria (which has been likened to systemic inflammatory response syndrome), we evaluated changes in angiopoietins in a longitudinal cohort of patients with sepsis.


- This study evaluated a panel of endothelium, coagulation, and inflammatory biomarkers in children with severe malaria (including severe malarial anemia) in Ugandan children and evaluated their prognostic ability. Combinatorial models were generated to demonstrate that biomarkers, when used in combination, can improve prediction of mortality.

- This Opinion piece features our hypothesis that C5a generated in placental malaria may lead to dysregulated angiogenesis and placental insufficiency. It was based on an earlier report showing that C5a synergistically induces anti-angiogenic factor sFlt-1 in the presence of PfGPI and other murine models of pathologic pregnancy.


- This protocol for Randomized Controlled Trial for inhaled nitric oxide in severe malaria arose from murine studies and human studies showing a decrease in bioavailable nitric oxide in severe malaria. In order to facilitate a well-powered but feasible study, Ang-2 is used as a surrogate biomarker of mortality and is the primary endpoint of the trial.


- This manuscript describes the scientific rationale behind the hypothesis that inhaled nitric oxide will improve outcome in severe malaria when used as an adjunctive treatment.
# Abbreviations

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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the (ROC) curve</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCS</td>
<td>Blantyre coma score</td>
</tr>
<tr>
<td>BRB</td>
<td>Blood retinal barrier</td>
</tr>
<tr>
<td>C3</td>
<td>Complement factor 3</td>
</tr>
<tr>
<td>C5</td>
<td>Complement factor 5</td>
</tr>
<tr>
<td>C5aR/CD88</td>
<td>C5a receptor</td>
</tr>
<tr>
<td>C5L2/GPR77</td>
<td>C5a receptor-like 2 / G protein-coupled receptor 77</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CD55, DAF</td>
<td>Decay accelerating factor</td>
</tr>
<tr>
<td>CD59</td>
<td>Protectin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CM</td>
<td>Cerebral malaria</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CM-N</td>
<td>Retinopathy negative cerebral malaria</td>
</tr>
<tr>
<td>CM-R</td>
<td>Retinopathy positive cerebral malaria</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CRT</td>
<td>Classification and regression tree</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin-sulfate A</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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</tbody>
</table>
Ct  Threshold cycle
DNA  Deoxyribonucleic acid
EDTA  Ethylenediaminetetraacetic acid
ELISA  Enzyme-linked immunosorbent assay
FACS  Fluorescence-activated cell sorting
FBS  Fetal bovine serum
FGR  Fetal growth restriction
G(#)  Gestational day
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GCS  Glasgow coma score
GPI  Glycosylphosphatidylinositol
Hb  Hemoglobin
HIV  Human immunodeficiency virus
HPRT  Hypoxanthine phosphoribosyltransferase 1
ICAM  Intercellular adhesion molecule
IFN  Interferon
IgG  Immunoglobulin
IL  Interleukin
IP-10  10kDa Interferon-induced protein
IPT(p/i/c)  Intermittent preventive therapy (pregnancy/infancy/childhood)
IQR  Interquartile range
IST  Intermittent screening and treatment
IUGR  Intrauterine growth restriction
IVS  Intervillous space
LBW  Low birth weight
LPS  Lipopolysaccharide
LR  Likelihood ratio
MAC  Membrane attack complex
MAPK  Mitogen activated protein kinase
MCP-1  Monocyte chemotactic protein-1
MG  Multigravidae
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Micro-CT</td>
<td>Microcomputed tomography</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>MiP</td>
<td>Malaria in pregnancy</td>
</tr>
<tr>
<td>MIP-1α/β</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NBW</td>
<td>Normal birth weight</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PbA</td>
<td><em>Plasmodium berghei</em> ANKA</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCT</td>
<td>Procalcitonin</td>
</tr>
<tr>
<td>PEs</td>
<td>Parasitized erythrocytes</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PfEMP1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein 1</td>
</tr>
<tr>
<td>PfGPI</td>
<td><em>Plasmodium falciparum</em> glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>PG</td>
<td>Primigravidae</td>
</tr>
<tr>
<td>PM</td>
<td>Placental malaria</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>PTD</td>
<td>Preterm delivery</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RDT</td>
<td>Rapid diagnostic test</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROC curve</td>
<td>Receiver operating characteristic curve</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>sEng</td>
<td>Soluble Endoglin</td>
</tr>
<tr>
<td>sFas</td>
<td>Soluble Fas</td>
</tr>
<tr>
<td>sFlt-1/sVEGFR-1</td>
<td>Soluble FMS like tyrosine kinase-1</td>
</tr>
<tr>
<td>SGA</td>
<td>Small-for-gestational-age</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SM</td>
<td>Severe malaria</td>
</tr>
<tr>
<td>SP</td>
<td>Sulfadoxine-pyrimethamine</td>
</tr>
<tr>
<td>sTFNR2</td>
<td>Soluble TNF receptor 2</td>
</tr>
<tr>
<td>sTREM-1</td>
<td>Soluble Triggering receptor expressed on myeloid cells 1</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Tie-2</td>
<td>TEK receptor tyrosine kinase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3,5,5-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UM</td>
<td>Uncomplicated malaria</td>
</tr>
<tr>
<td>uRBC</td>
<td>Uninfected RBC</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSA</td>
<td>Variant surface antigen</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>VWFpp</td>
<td>von Willebrand factor propeptide</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WPB</td>
<td>Weibel-Palade body</td>
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</table>
Chapter 1
Introduction

1.1 Biomarkers

Biomarkers are quantitative biological measures that can provide information about disease states. A biomarker may identify individuals at risk of developing a disease (e.g. *BRCA1/2* mutation to identify women at risk of developing breast cancer or sFlt-1 to predict the onset of preeclampsia) or may predict a poor outcome in the context of a known disease (e.g. death in children with cerebral malaria or the delivery of low birth weight infant in the context of placental malaria). Biomarkers can also be used for early diagnosis in situations where invasive or expensive tests are required or are unavailable (e.g. troponin for the emergency assessment of chest pain). As the field of molecular diagnostics evolves, the ability to develop rapid tests that can simultaneously provide quantitative assessment of multiple biomarkers is becoming feasible. Information from multiple biomarker tests can be combined to create “biosignatures” that improve the sensitivity and specificity of a test. Another area of considerable interest is the identification of surrogate biomarkers, or biomarkers that can be substituted for clinical endpoints. This is particularly relevant for rare outcomes (e.g. death or disability) where evaluating new treatments can be prohibitively expensive. Incorporation of biomarkers into existing trials along standard clinical and biochemical measures may enable a better understanding of disease processes and could identify important side-effects before they develop into clinical problems.

In this thesis, I will evaluate biomarkers from the endothelium and the complement system using severe malaria as a model system.

1.1.1 Angiogenesis and Endothelial Activation

Endothelial cells line the inner surface of blood cells and form a structural barrier between the blood and the rest of the body. During vasculogenesis (the formation of new blood vessels) and angiogenesis (the growth and remodelling of existing blood vessels), proteins produced by endothelial cells and their underlying mural cells are critical for the migration and apposition of endothelial cells to each other and their supporting cells. Two families that have been extensively
studied for their role in normal and pathologic angiogenesis are: vascular endothelial growth factor (VEGF) and its receptors Flt-1 (fms-like tyrosine kinase-1, VEGFR-1) and Flk-1 (VEGFR-2); and angiopoietin-1 and angiopoietin-2 and their cognate receptor Tie-2 (Risau 1997). Aberrations in these two families of proteins have also been the subject of considerable research in conditions of endothelial activation and dysfunction.

VEGF is a pro-vasculogenic, pro-angiogenic and pro-inflammatory protein that signals through two main receptors expressed on the endothelium, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). VEGFR-2 appears to the dominant signalling receptor necessary for inducing vascular permeability and vessel formation, whereas VEGFR-1 may serve to modulate VEGF signalling (Yancopoulos, Davis et al. 2000). VEGFR-1 can be alternatively spliced to generate a soluble version (sFlt-1), which is a potent anti-angiogenic factor (Kendall and Thomas 1993). VEGF is critical for vasculogenesis and angiogenesis and acts as a key destabilizing force during vessel remodelling. Another factor involved in the formation and regulation of vasculature is soluble endoglin (sEng), which is a coreceptor of transforming growth factor (TGF)-β and has anti-angiogenic properties.

The angiopoietins are another important family of angiogenic proteins that act in a context-dependent manner together with VEGF to regulate angiogenesis. Ang-1 is constitutively expressed by vascular mural cells (pericytes and vascular smooth muscle cells) and signals through its cognate receptor, Tie-2, to promote vascular stability and quiescence (Fiedler and Augustin 2006). Ang-2 predominantly acts as an antagonist to Ang-1 signalling, though it can induce phosphorylation of Tie-2 when Ang-1 is absent (Yuan, Khankin et al. 2009). Ang-2 is contained within Weibel-Palade bodies (WPB) in the endothelium and can be rapidly mobilized and released upon endothelial activation, alongside other WPB products such as von Willebrand factor (VWF) and its propeptide (VWFpp) (Fiedler, Scharpfenecker et al. 2004). Ang-2 is an important factor involved in sensitizing the endothelium to inflammatory stimuli and has been shown to induce the expression of ICAM-1 and VCAM-1 at sub-saturating concentrations of TNF (Fiedler, Reiss et al. 2006).

**1.1.2 The Complement System**

The complement system is composed of over 30 cell-surface and fluid-phase proteins that exist in an inactive form under normal physiologic conditions and can be activated by a series of
proteolytic events to modulate immune activation (Figure 1.1). The complement system is an integral component of the innate immune system and it is activated by three main pathways: classical, alternative or lectin. These three pathways converge at the level of C3 and lead to the formation of C3a and C3b and the downstream activation of C5. Activation of C5 results in the production of anaphylatoxin C5a and C5b which forms the membrane attack complex (MAC) with C6-9. C5 can also be activated directly by endogenous or pathogen proteases resulting in functional C5a (Ward and Hill 1970; Maruo, Akaike et al. 1997; Vogt 2000; Huber-Lang, Younkin et al. 2002; Huber-Lang, Sarma et al. 2006; Fukuoka, Xia et al. 2008). Complement factor C5a represents a key downstream activation product of the complement system, which is a potent inflammatory protein with noted deleterious effects when inadequately regulated.

C5a has been implicated in pathologic inflammatory responses in both infectious and non-infectious models of systemic-inflammatory response syndromes including sepsis (Huber-Lang, Younkin et al. 2002), rheumatoid arthritis (Grant, Picarella et al. 2002; Woodruff, Strachan et al. 2002), fetal rejection (Girardi, Yarilin et al. 2006) and cerebral malaria (Patel, Berghout et al. 2008). C5a mediates its biological functions through two receptors: C5aR (CD88) and C5L2 (GPR77) (Monk, Scola et al. 2007). The relative contribution of each receptor to C5a-induced biological effects is controversial, but C5a-C5aR signaling appears to be the dominant pathway in a number of models. Blockade of C5a activity has been shown to prevent multi-organ injury and improve survival in animal models of disease (Czermak, Sarma et al. 1999; Huber-Lang, Sarma et al. 2001; Huber-Lang, Riedeman et al. 2002; Patel, Berghout et al. 2008; Rittirsch, Flierl et al. 2008).

Complement activation is well-described in malaria and there is evidence of classical, lectin, and alternative pathway activation (reviewed in (Silver, Higgins et al. 2010)), while C3-independent mechanisms of complement activation have not been investigated. In malaria, complement has been implicated in opsonic clearance of parasitized erythrocytes (PEs), rosetting (adherence of PEs to uninfected red blood cells), parasite invasion and inflammation (Turrini, Ginsburg et al. 1992; Rowe, Moulds et al. 1997; Patel, Berghout et al. 2008; Spadafora, Awandare et al. 2010).
Figure 1.1 An overview of the complement system

The classical, lectin and alternative pathways converge at C3 resulting in the production of anaphylatoxin C3a and opsonin C3b. The formation of the C5 convertase then results in the cleavage of C5 to generate the potent anaphylatoxin C5a and C5b, which forms the membrane attack complex (MAC) alongside complement proteins C6, C7, C8, C9. Regulation of the complement activation is modulated, in part, by cell-surface bound complement regulatory proteins (e.g. decay accelerating factor, DAF; membrane cofactor protein, MCP; and Protectin). C5 can also be cleaved independently of normal proteolytic cascades through products released from activated leukocytes and other proteases. C5a has diverse biologic roles and is important in inflammation, coagulation and angiogenesis.
1.2 Overview of Malaria Pathogenesis

While there have been considerable public health efforts directed at malaria treatment and control worldwide, malaria remains the most important parasitic disease globally with almost one-quarter of the world’s population at risk and over 250 million infections annually (Hay, Guerra et al. 2009) (Figure 1.2). Malaria is a parasitic disease of the blood caused by an infection of red blood cells with the protozoan parasite Plasmodium. There are five species of Plasmodium that infect humans (falciparum, vivax, ovale, malariae and knowlesi), and malaria can be transmitted by over 30 species of Anopheline mosquitoes. Plasmodium falciparum is the predominant species in sub-Saharan Africa where over 90% of all malaria deaths occur (WHO 2010). P. falciparum results in a number of different pathologies associated with specific organ systems, including the brain (known as cerebral malaria, CM) and the placenta (known as placental malaria, PM).

The remainder of this chapter introduces important aspects of malaria, focusing on severe manifestations of disease and emphasizing specific pathologies associated with cerebral malaria and placental malaria. I will describe how perturbations in key innate immune pathways can contribute to the pathophysiology of disease. Finally, I outline and provide specific objectives for each chapter.
Figure 1.2 The distribution of malaria transmission and sulfadoxine-pyrimethamine resistance. Adapted from: (WHO 2005)
1.2.1 Life Cycle

The clinical manifestations of *P. falciparum* are associated with the life cycle of the parasite, which is reviewed in Figure 1.3. Briefly, a female Anopheles mosquito infected with malaria injects sporozoites into the host while taking a blood meal. These sporozoites rapidly travel to the liver where they can migrate through several cells before infecting a hepatocyte and initiating a process of asexual and asymptomatic reproduction known as exo-erythrocytic schizogony. Following the growth and maturation phase within hepatocytes (generally lasting 7-14 days), thousands of merozoites are released into the circulation and begin the disease-causing blood stage of malaria infection. During the blood stage of infection, *P. falciparum* parasites undergo a process of invasion (merozoites), intracellular growth (trophozoites), multiplication (schizont) and reinvasion of host erythrocytes over a 48 hour period. Some intraerythrocytic parasites take a different developmental path and produce male and female gametocytes to begin the sexual stage of the malaria life cycle within the mosquito midgut. Disease in *P. falciparum* is related to the latter half of the erythrocytic cycle, where: i) schizonts express parasite proteins that mediate the cytoadherence of PEs in the microvasculature; and ii) schizonts rupture releasing infective merozoites and other parasite-derived bioactive products such as *PfGPI*. These two processes are considered fundamental aspects of disease pathogenesis and are reviewed below.
Figure 1.3 A simplified life cycle of *Plasmodium falciparum* within the human host

The life cycle of *P. falciparum* is initiated when a female *Anopheles* mosquito injects sporozoites into the human host which migrate to the liver, and following a stage of replication within hepatocytes, thousands of merozoites are released into the circulation. (B) During the blood stage: (1) merozoites attach and invade host erythrocytes; (2) metabolically active trophozoites digest host hemoglobin; (3) mature schizonts express *P. falciparum* erythrocyte membrane protein 1 and adhere to the microvasculature (C); (4) schizonts rupture releasing 12-24 merozoites. (D) A portion of merozoites will form gametocytes which (E) infect a mosquito during a blood meal and (F) undergo sexual reproduction within the mosquito midgut.
1.2.2 General Pathophysiology

The classical fever-like symptoms of malaria occur upon parasite rupture from the host red blood cell, as a number of inflammatory products are released that activate components of the innate immune system and lead to the production of pyrogens (reviewed below). When an infection is synchronous, the coordinated rupture of PEs leads to the prototypical malarial fevers. While this feature is common among all species of malaria that infect humans, the timing of clinical symptoms depends on the maturation phase within the erythrocyte (between 24 - 72 hours). Often, it can take several erythrocytic cycles for patent parasitemia to develop synchronized febrile cycles (White, Chapman et al. 1992). Non-immune individuals will often seek treatment from non-specific malarial symptoms (including fever, headache, malaise, vomiting and diarrhea) prior to developing malarial paroxysms. Thus, it can be difficult to differentiate malaria febrile illness from fevers of other causes, especially in areas of high malaria endemicity where incidental parasitemia is common and diagnostic capacity is limited.

Cytoadherence of PEs in the microvasculature and dysregulated inflammatory responses have been implicated in the pathogenesis of severe falciparum malaria. These pathogenic processes occur in both cerebral malaria and placental malaria, and will be reviewed independently below.

*P. falciparum* expresses parasite proteins (including *Plasmodium falciparum* erythrocyte membrane protein 1, PfEMP1) on the surface of the infected red blood cell that promotes the cytoadherence of PEs in the microvasculature (Baruch, Pasloske et al. 1995; Su, Heatwole et al. 1995). PfEMP1 is a large protein that spans the erythrocyte membrane and enables the parasite to interact with receptors present on the vascular endothelium to avoid detection and clearance by the spleen. PfEMP1 is a highly variable protein, encoded by approximately 60 *var* genes, which enable the parasite to avoid recognition by the host immune system. *var* genes are generally expressed on the surface of red blood cells in a mutually exclusive fashion—thus, it can take years for an individual in malaria endemic areas to acquire clinical immunity against malaria. The most well-described endothelium receptors for PfEMP1 are ICAM-1 in the brain, CD36 outside of the brain, and chondroitin-sulfate A (CSA) in the placenta; although binding to other receptors, including thrombospondin, PECAM-1, P-selectin, E-selectin, and VCAM has been reported (Roberts, Sherwood et al. 1985; Rock, Roth et al. 1988; Ockenhouse, Tegoshi et al. 1992; Fried and Duffy 1996; Udomsangpetch, Taylor et al. 1996; Newbold, Warn et al. 1997; Treutiger,

### 1.2.3 Immunity

In non-immune individuals, malaria has the potential to undergo rapid growth causing severe anemia and death. However, severe disease develops in only 1-2% of clinical cases. Despite the availability of effective antimalarial drugs, the mortality rate in severe falciparum malaria remains high. Evidence from *in vitro* studies, murine models, and *P. falciparum* infections in malaria-naïve individuals highlight the importance of innate immunity in the ability to limit the initial phase of parasite replication, providing the host time to develop protective adaptive responses (reviewed in (Stevenson and Riley 2004)). There are multiple innate immune pathways involved in the recognition and clearance of PEs, including the complement system and pattern recognition receptors (e.g. toll-like receptors, TLRs) (Erdman, Finney et al. 2008; Silver, Higgins et al. 2010). These pathways must occur in a tightly regulated manner to control rising parasitemia levels without leading to immunopathology.

#### 1.2.3.1 Inflammation

An important part of the innate immune response to malaria is the coordinated production of proinflammatory cytokines, especially IFN-γ and IL-12, which are required to control the early parasitemia in murine models of malaria (van der Heyde, Pepper et al. 1997; Su and Stevenson 2000; Su and Stevenson 2002). Further, IFN-γ and IL-12 are produced in a coordinated manner at the time of parasite emergence from the liver in experimental *P. falciparum* infections and are rapidly produced by peripheral blood mononuclear cells (PBMCs) *in vitro* following exposure to *P. falciparum* PEs (Scragg, Hensmann et al. 1999; Hermsen, Konijnenberg et al. 2003).

Inflammation is necessary for early control of parasitemia; however, excessive or dysregulated production of inflammatory mediators can lead to immunopathology (Grau, Taylor et al. 1989; Kwiatkowski, Hill et al. 1990).

One component of the innate immune response that plays a critical role in the recognition of pathogen ligands and initiates appropriate effector responses is the family of pattern recognition receptors, to which the TLRs belong. Three TLRs have been implicated in malaria infection: TLR2, TLR 4 and TLR9. TLR2 and to a lesser extent TLR4 recognize *P. falciparum*
glycosylphosphatidylinositol (PfGPI) (Krishnegowda, Hajjar et al. 2005) and TLR9 recognizes parasite DNA bound to the heme polymer hemozoin (Parroche, Lauw et al. 2007). Although GPls are ubiquitously expressed by eukaryotes as membrane anchors, the GPls expressed by parasitic protozoa differ considerably from human GPls and exhibit immunostimulatory activity (Gowda 2007). When parasite components engage TLRs, they lead to an inflammatory response through MAPK and NF-κB that result in the production of cytokines (TNF, IL-1β, IL-6, IL-12), chemokines and type I interferons (reviewed in (Gowda 2007)). Excessive production of these cytokines has been implicated in immunopathology in both murine models of severe malaria and in human disease (Artavanis-Tsakonas, Tongren et al. 2003; Schofield and Grau 2005). TLRs have also been implicated in the pathogenesis of human and murine malarial disease (Mockenhaupt, Hamann et al. 2006; Erdman, Finney et al. 2008), and there is evidence that engagement of multiple TLR pathways may be necessary to mediate pathology (Finney, Lu et al. 2010). While inflammation generated from TLRs can be detrimental to the host if inappropriately regulated, TLRs also serve another function: to cooperate with other innate scavenger receptors to enhance clearance of PEs (Erdman, Cosio et al. 2009).

### 1.2.3.2 Phagocytic-Clearance of Parasitized Erythrocytes

The monocyte/macrophage is an important innate effector cell early in infection because of its ability to phagocytose PEs in the absence of cytophilic antibodies- through scavenger receptor CD36 (McGilvray, Serghides et al. 2000). This non-opsonic clearance of PEs via CD36 occurs in a non-inflammatory way (Erdman, Cosio et al. 2009), thus limiting the bystander damage produced. Complement deposition on PEs and antibody recognition of PEs also contribute to the removal of PEs (Turrini, Ginsburg et al. 1992).

### 1.3 Cerebral malaria in Non-Immune Individuals: Children in Sub-Saharan Africa and Children and Adults in Southeast Asia

CM is defined by the detection of *P. falciparum* parasites and unrousable coma (Blantyre Coma Score, BCS ≤2 in children <5; Glasgow Coma Score, GCS≤8 in children ≥5) with no other identifiable cause for fever or loss of consciousness. The most recent estimates of CM mortality are 18% in pediatric populations in Africa and 30% in mainly adult populations from South East Asia (Dondorp, Nosten et al. 2005; Dondorp, Fanello et al. 2010). CM is a syndrome under the
umbrella of severe malaria, and often occurs with other manifestations of severe disease, including renal failure and pulmonary edema in adults (Robinson, Mosha et al. 2006), and severe anemia and respiratory distress in children (Marsh, Forster et al. 1995). Therefore, it is difficult to discuss the pathophysiology of cerebral disease without also considering other aspects of severe malaria.

1.3.1 Clinical Presentation

CM is characterized by a diffuse encephalopathy that frequently presents with convulsions. In children, most deaths occur within the first 24-48 hours and comas resolve quickly, whereas the duration of illness is longer in adults (Dondorp, Nosten et al. 2005; Dondorp, Fanello et al. 2010). CM in children can lead to long-term cognitive (~25%) and neurologic (1-4%) impairment in survivors (van Hensbroek, Palmer et al. 1997; Boivin 2002; Boivin, Bangirana et al. 2007; Birbeck, Molyneux et al. 2010). While there have been no systematic evaluation of neurologic and cognitive function in adults following CM, there has been a report documenting neuropsychological outcomes in adults following CM (Roze, Thiebaut et al. 2001).

The prevalence *P. falciparum* parasitemia can be very high in some areas of sub-Saharan Africa (e.g. up to 70% from one study in Tanzania (Smith, Charlwood et al. 1993)); therefore, it is important that other causes for loss-of-consciousness are considered (such as hypoglycemia, meningitis or post-ictal state) in a comatose child with parasitemia. A study from Kenya examining a large cohort of children with impaired consciousness showed that the mortality rate was higher in smear-negative “CM” patients than those that were smear-positive (Berkley, Mwangi et al. 1999), due to the failure to treat other illnesses. As countries work to extend availability of the more expensive but efficacious artemisinin-based drugs (oral artemisinin-based combination treatments (ACTs) and intravenous artesunate) as the first-line therapy for uncomplicated and severe malaria respectively, the importance of accurate diagnoses will become more important than ever.

1.3.2 Diagnosis

Malaria is diagnosed by detection of PEs in the blood. The gold standard for diagnosis is microscopy: using a combination of thick and/or thin smears for detection of parasites and species identification. Alternatively, malaria can be detected using rapid diagnostic tests to
identify parasite antigen (pLDH, HRP2, aldolase) when microscopy is not available. In areas of high malaria endemicity, it can be difficult to ascertain whether malaria is the true cause of illness because the clinical presentation can overlap with other common childhood febrile illnesses. In CM, where the reference standard for diagnosis may be considered to be post-mortem confirmation of PEs sequestered in the brain microvasculature, there are difficulties in assessing the true burden of disease. In a study of Malawian children with fatal CM, 23% of children had an alternative non-malarial cause of death identified by post-mortem examination (Taylor, Fu et al. 2004). Currently, the diagnosis of CM relies on the exclusion of other causes of coma, which is difficult to do in any setting, but is especially challenging in resource-constrained settings. Attempts to identify a set of signature signs capable of differentiating CM from comas of other causes have been made. Malaria retinopathy is one tool that has been used to improve identification of patients of cerebral malaria, particularly in research settings (Beare, Taylor et al. 2006; Lewallen, Bronzan et al. 2008).

1.3.2.1 Retinopathy

Malaria retinopathy consists of 3 signs: hemorrhage, retinal whitening and vessel discolouration, shown in Figure 1.4 (Lewallen, Bronzan et al. 2008). Papilloedema may also occur in CM, but it is no longer considered sufficient for a definition of malaria retinopathy (Lewallen, Bronzan et al. 2008). Retinal changes can be observed by direct and/or indirect ophthalmoscopy. Indirect ophthalmoscopy is preferred, as it is more sensitive to retinal changes (Maude, Sayeed et al. 2010) but it requires the use of mydriatic drops to dilate pupils and is more difficult for non-ophthalmologists to perform. The ability of non-specialists to perform direct ophthalmoscopy at the bedside makes this technique more amenable to routine use (Maude, Sayeed et al. 2010).

The identification of malaria retinopathy in a patient with suspected CM improves the likelihood that the coma is attributable to malaria. It is difficult to assess the true sensitivity and specificity of retinopathy for CM since there is no reference standard in life; however, children with clinical CM but normal retinal findings have a lower mortality rate and shorter duration of coma (Lewallen, Bronzan et al. 2008). These findings support the idea that children without retinopathy that are classified as having CM have an alternative cause of coma or less severe disease.
Figure 1.4 A Fundus photograph of retinopathy in a pediatric patient with cerebral malaria.

This photograph includes examples of all components of malaria retinopathy: several haemorrhages (mostly white-centred), patchy whitening (arrow heads) and orange discolouration of vessels (arrow). From: (White, Lewallen et al. 2009).
There have been fewer systematic studies undertaken to examine retinal findings in adults with severe malaria. Early studies in adults in Papua New Guinea, Thailand and India reported haemorrhages in 25%, 15% and 9% of CM patients respectively, using predominantly direct ophthalmoscopy (Davis, Vaterlaws et al. 1982; Looareesuwan, Warrell et al. 1983; Kochar, Shubhakaran et al. 1998). Retinal whitening (previously referred to as retinal edema) and papilloedema (Looareesuwan, Warrell et al. 1983; Kochar, Shubhakaran et al. 1998) occurred less frequently. However, more recent reports from Bangladesh describe hemorrhages in 41% of CM cases using indirect ophthalmoscopy and in 55% of CM cases using high-definition portable retinal photography (Maude, Beare et al. 2009; Sayeed, Maude et al. 2011). However, the utility of retinopathy as a diagnostic tool in adults with CM is of questionable value, since a diagnosis of CM is less likely to be confounded by incidental parasitemia.

Because the brain and retina are derived from the same embryonic tissue and have analogous blood-tissue-barriers, retinopathy has also been a useful tool to improve our understanding of disease pathogenesis. The retina is considered a “window into the brain” and the importance of this statement with regards to malaria pathophysiology is supported by the finding that haemorrhages seen in the retina correlate to those observed in the brain (White, Lewallen et al. 2001). Studies using fluorescein angiography to investigate blood flow and tissue perfusion have topographically mapped areas of pathology in fundoscopic images to areas of altered perfusion in angiographic images to gain critical insight into the pathophysiology of retinopathy (Beare, Harding et al. 2009). Whitening is associated with areas of non-perfusion and hypoxia and is often found surrounding areas of vessel changes (Beare, Harding et al. 2009). Vessel changes are thought to correspond to sequestered parasites that result in either partial (orange) or complete (white) occlusion of the vessel (Beare, Harding et al. 2009). Thus, retinopathy may represent a putative diagnostic tool for CM but it also provides critical insights into the pathogenesis of disease.
1.3.3 Immunopathology

The pathogenesis of severe disease in CM is thought to arise from the ability of *P. falciparum*-infected erythrocytes to sequester in the microvasculature leading to impaired microcirculatory flow and metabolic disturbances. In addition, CM is characterized by a systemic inflammatory response, characterized by high levels of proinflammatory molecules, endothelial activation and blood-brain-barrier dysfunction. It is likely a combination of these processes that contribute to the morbidity and mortality in severe malaria.

1.3.3.1 Sequestration and Reduced Microcirculatory Flow

Sequestration of mature PEs in the microvasculature is characteristic of all *P. falciparum* infections, including asymptomatic infections. Therefore, sequestration alone is not sufficient to lead to severe disease. Rather, the distribution and density of parasite sequestration is likely a determinant of disease. Quantification of parasite antigen in post-mortem tissues, histological assessment of parasitized vessels from post-mortem studies, and *in vivo* imaging of rectal mucosa show dense parasite sequestration in organs (particularly brain, intestines, and skin) (MacPherson, Warrell et al. 1985; Seydel, Milner et al. 2006; Dondorp, Ince et al. 2008). This microvascular obstruction can lead to impaired tissue perfusion. Obstruction of vessels occurs directly through the interaction of parasite ligands (e.g. PfEMP1) to endothelial receptors; and indirectly through reduced deformability of uninfected erythrocytes, PEs bound to other PEs (via hemagglutination or platelet-mediated clumping) and PEs bound to uninfected erythrocytes (rosetting) (Roberts, Craig et al. 1992; Carlson 1993; Ringwald, Peyron et al. 1993; Rowe, Obeiro et al. 1995; Dondorp, Angus et al. 1997; Roberts, Pain et al. 2000; Pain, Ferguson et al. 2001; Dondorp, Nyanoti et al. 2002; Chotivanich, Sritabalsri et al. 2004; Arman, Raza et al. 2007).

Using intravital imaging to measure microvascular obstruction in the rectal mucosa, investigators were able to show a positive correlation between the percentage of blocked capillaries and lactate concentrations in Bangladeshi adults (Dondorp, Ince et al. 2008). Thus, sequestration likely precipitates the tissue hypoxia and metabolic acidosis that is well described in severe malaria (Taylor, Borgstein et al. 1993; Waller, Krishna et al. 1995; Newton, Valim et al. 2005). These findings support a critical role for the endothelium in mediating parasite adhesion and hypoxia in severe disease.
1.3.3.2 Endothelial Activation

Endothelial cells line the inner surface of blood cells and form a structural barrier between the blood and the rest of the body. The endothelium is a dynamic organ system that regulates the transport of proteins and cells into the parenchyma and is actively involved in immunosurveillance. Endothelial activation has been described in malaria through pathology reports, studies measuring endothelium-receptor expression on tissues, and soluble mediators released from-or cleaved from- the endothelium, and through in vitro and ex vivo experimentation (Turner, Morrison et al. 1994; Turner, Ly et al. 1998; Combes, Taylor et al. 2004; Taylor, Fu et al. 2004; Tchinda, Tadem et al. 2007; Wassmer, Moxon et al. 2011).

Upregulation of constitutively expressed molecules such as ICAM-1, which is the predominant receptor involved in PEs cytoadherence in the brain, and the release of soluble ICAM-1 (sICAM-1) from the endothelium have been documented in malaria and are positively correlated with disease severity (Jakobsen, Morris-Jones et al. 1994; Turner, Ly et al. 1998). Soluble versions of other receptors expressed on the endothelium have also been associated with disease severity in malaria, including sVCAM-1, E-selectin, sFlt-1, sTie-2, sEng and sFas (Jakobsen, Morris-Jones et al. 1994; Turner, Ly et al. 1998; Tchinda, Tadem et al. 2007; Jain, Armah et al. 2008; Dietmann, Helbok et al. 2009; Conroy, Phiri et al. 2010; Erdman, Dhabangi et al. 2011). Other measures of endothelial activation include soluble factors released from the endothelium or underlying mural cells, such as: angiogenic markers Ang-2, Ang-1 and VEGF, (Jain, Armah et al. 2008; Yeo, Lampah et al. 2008; Conroy, Lafferty et al. 2009; Lovegrove, Tangpukdee et al. 2009); and procoagulant molecules like von Willebrand factor (VWF) and its propeptide (VWFpp) (Hollestelle, Donkor et al. 2006; Larkin, de Laat et al. 2009; Conroy, Phiri et al. 2010).

1.3.3.3 Blood-Brain-Barrier Dysfunction

The blood brain barrier (BBB) is a structural and functional barrier that restricts the transport of substances between the blood and central nervous system. The main physical barrier consists of continuous complexes of tight junctions between adjacent endothelial cells; and the functionality of the BBB is regulated, in part, by pericytes. Pericytes are vascular mural cells that share the basement membrane with endothelial cells and form direct attachments with endothelial cells. The formation of the BBB is dependent on the recruitment of pericytes to the vascular wall (Daneman, Zhou et al. 2010). Studies from mice lacking pericytes from vessel walls in the BBB
show disorganized alignment of tight junctions between adjacent cells, increased vessel permeability, and increased transcytosis of molecules across the endothelium (Armulik, Genove et al. 2010; Daneman, Zhou et al. 2010). In addition, studies have shown that the pericyte: endothelial cell density is inversely related to the permeability of vessels, with the highest density being in the retina and central nervous system (1:1 ratio vs. 1:100 ratio in skeletal muscle) (Shepro and Morel 1993; Armulik, Genove et al. 2010; Daneman, Zhou et al. 2010).

The concept of BBB dysfunction in cerebral malaria was first proposed over 60 years ago in an account of the pathological features of malaria (Maegraith 1948). A number of studies have now published evidence supporting the concept of focal BBB disruption in CM. The most striking example of BBB disruption is the presence of hemorrhages, which have been noted in the white matter of fatal CM (Riganti, Pongponratn et al. 1990; White, Lewallen et al. 2001; Patankar, Karnad et al. 2002; Taylor, Fu et al. 2004). Studies from adults in Vietnam and children in Malawi have shown decreased expression of tight-junction proteins in fatal CM (Brown, Chau et al. 2000; Brown, Rogerson et al. 2001) and PEs and soluble parasite factors have been shown to reduce endothelial barrier integrity in vitro (Tripathi, Sullivan et al. 2007). These results are supported by findings of increased cerebrospinal fluid: blood albumin ratio (indicative of increased BBB permeability) observed in select Vietnamese adults and Malawian children with CM in vivo (Brown, Chau et al. 2000; Brown, Rogerson et al. 2001). Increased fluorescein leakage has also been observed in both adults and children with retinopathy and may be considered a surrogate for increased BBB permeability based on the similarities between the blood-retinal-barrier and BBB (Davis, Suputtamongkol et al. 1992; Beare, Harding et al. 2009).

Based on these pathophysiologic findings (reviewed in Figure 1.5), adjunctive strategies that could reduce endothelial activation, limit parasite sequestration in the brain microvasculature, preserve BBB integrity and thus reduce the mortality and neurologic after-effects of CM would represent a major advance.
In cerebral malaria, parasitized erythrocytes (PEs) expressing *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) adhere to the cerebral microvasculature via endothelial receptors such as ICAM-1. The rupture of PEs results in the release of inflammatory byproducts (e.g. PfGPI, hemozoin) that lead to increases in proinflammatory cytokines and chemokines, the recruitment of monocyte (in pediatric CM), endothelial activation (upregulation of adhesion molecules, release of microparticles, WPB release) and platelet deposition (which can act with VWF as a putative bridge to bind PEs via CD36). Combined, these pathways likely contribute to tissue hypoxia and metabolic acidosis.
1.3.4 Adjunctive Therapies for Cerebral Malaria

Investigations into the pathophysiology of severe malaria highlight the importance of the host immune response in determining the outcome of infection. With mortality rates of 15-30% in CM, adjunctive therapies that target the host response could have clinical utility. A number of adjunctive therapies have been tested in severe and cerebral malaria (see Table 1.1 for an overview of selected adjunctive studies). Adjunctive therapies have attempted to modulate deleterious inflammatory responses, reduce iron availability through chelation, reduce oxidative stress and prevent coagulation (reviewed in (John, Kutamba et al. 2010)). Efforts have also been made to intervene in clinical endpoints through exchange transfusion, correction of acidosis, reduction of edema, and prevention of seizures (John, Kutamba et al. 2010). Albumin and pentoxyfylline are two adjunctive therapies tested that showed some efficacy, with decreases in mortality in one study with albumin (severe malaria) and decreases in coma duration with a trend towards reduced mortality in two studies of pentoxyfylline (Di Perri, Di Perri et al. 1995; Das, Mishra et al. 2003; Maitland, Pamba et al. 2005). Efforts to reproduce consistent findings have had limited success. A recent report examining boluses of either albumin or saline as fluid resuscitation in children with severe febrile illness with impaired perfusion (where 57% had malaria) found a significant increase in 48-hour mortality in the bolus groups compared to controls (Maitland, Kiguli et al. 2011).

It is important to note that most of the trials published to date have been underpowered to detect differences in mortality (and the populations, age groups, and clinical syndromes targeted have been variable). Thus, failures to reproduce significant results or to identify effective adjunctive therapies should be interpreted with caution (Enwere 2005). Inclusion of mortality as the primary endpoint in trials testing adjunctive therapies would be prohibitively expensive for early efficacy trials (requiring enrolment of ~3000 participants to show a 20% mortality benefit (i.e. 20% to 16%) with 80% power and alpha=0.05). Therefore, robust and reliable measures of disease severity that could be used as a surrogate measures of disease severity would make testing adjunctive therapies in severe malaria a more feasible pursuit (from both a safety and efficacy standpoint). This could facilitate smaller trials that would be less expensive and faster in order to evaluate promising targets in the pipeline.
<table>
<thead>
<tr>
<th>Study Design</th>
<th>Population</th>
<th>Sample size</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
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<tr>
<td><strong>Interventions with detrimental effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Randomized, double blind, placebo controlled trial</td>
<td>Thailand, adults</td>
<td>100</td>
<td>Increase in coma duration and complications (pneumonia, gastrointestinal bleeding) in treatment group</td>
</tr>
<tr>
<td>Intravenous Immunoglobulin from semi-immune donors</td>
<td>Randomized, double blind, placebo controlled trial</td>
<td>Malawi, children</td>
<td>31*</td>
<td>Trend toward increased mortality and neurologic sequelae in treatment group</td>
</tr>
<tr>
<td>Anti-TNF monoclonal antibody</td>
<td>Randomized, double blind, placebo controlled trial</td>
<td>The Gambia, children</td>
<td>610</td>
<td>Increased risk of neurologic sequelae in treatment group</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Randomized, placebo controlled trial</td>
<td>Kenya, children</td>
<td>340</td>
<td>Two-fold increased in morality in treatment group</td>
</tr>
<tr>
<td><strong>Interventions with no or modest effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td>Randomized, double blind, placebo controlled trial</td>
<td>Uganda, children</td>
<td>156</td>
<td>No difference in mortality, coma recovery time, or any other outcome measures</td>
</tr>
<tr>
<td>Pentoxifylline‡</td>
<td>Randomized, controlled trial</td>
<td>India, adults</td>
<td>52</td>
<td>Trend to lower mortality, faster resolution time, lower TNF in treatment group</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Burundi, children</td>
<td>56</td>
<td>Trend to lower mortality, shorter coma duration</td>
</tr>
</tbody>
</table>

* Trial stopped for lack of efficacy
‡ Two studies in adults from Germany and Thailand with severe malaria observed no effect on outcome
1.3.5 Prognosis

A number of studies have evaluated prognostic markers in malaria in order to define features associated with poor outcome. If an effective adjunctive therapy were identified that could improve outcome in a subset of children with severe disease, prognostic indicators could be used to stratify a subset of individuals most likely to benefit from adjunctive treatment, while minimizing cost and unnecessary treatment (and potential side-effects) in those likely to do well. Prognostic indicators could also be useful to identify children in need of referral or more intensive therapy.

Clinical parameters are the most well investigated prognostic measures and are often the fastest, least expensive and easiest measures to assess. The most prominent clinical features that have been associated with poor outcome in severe malaria are prostration, decreased consciousness or coma, respiratory distress, convulsions, poor peripheral circulation (cold periphery, delayed capillary refill) and jaundice (Molyneux, Taylor et al. 1989; Marsh, Forster et al. 1995; Waller, Krishna et al. 1995; Jaffar, Van Hensbroek et al. 1997; Evans, May et al. 2006). Other prognostic measures that can often be assessed within a reasonable time-frame to inform patient care are hypoglycemia, severe anemia, and parasitemia (Molyneux, Taylor et al. 1989; Marsh, Forster et al. 1995; Waller, Krishna et al. 1995; Jaffar, Van Hensbroek et al. 1997). The availability of laboratory and biochemical measures can differ between sites; however, in sites with adequate laboratory support, measurement of lactate, leukocyte counts and assessment of renal function can be useful to predict mortality (White, Warrell et al. 1985; Molyneux, Taylor et al. 1989; Taylor, Borgstein et al. 1993; Waller, Krishna et al. 1995; Agbenyega, Angus et al. 1997; Jaffar, Van Hensbroek et al. 1997; Ladhani, Lowe et al. 2002; Medana, Hien et al. 2002; Newton, Valim et al. 2005). Retinopathy has been associated with poor clinical outcome in some studies but it may be difficult to assess in practice because it is dependent on the availability of specialized equipment and trained personnel (Lewallen, Bakker et al. 1996; Olumese, Adeyemo et al. 1997; Beare, Southern et al. 2004; Sayeed, Maude et al. 2011). Other prognostic indicators that have been endorsed in severe malaria are circulating pigmented leukocytes and reduced blood cell deformability (Dondorp, Nyanoti et al. 2002; Kremsner, Valim et al. 2009).

Additional prognostic measures (such as host biomarkers) that can be incorporated into affordable point-of-care devices could improve triage and referral of individuals with severe
disease. Currently, it is not possible to measure host biomarkers for bedside use in Africa. However, efforts are being made to develop rapid, affordable and robust point-of-care tests that could be used in resource constrained settings (Lee, Kim et al. 2010). Most prognostic biomarkers evaluated in severe malaria are involved in inflammatory responses and originate from activated leukocytes and/or the endothelium. Select markers include: TNF, sTNFR2, IP-10, sTREM-1, PCT, sFas, Ang-1, Ang-2, sICAM-1, and sFlt-1 (Kwiatkowski, Hill et al. 1990; Jain, Armah et al. 2008; Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009; Erdman, Dhabangi et al. 2011). More studies are needed to systematically evaluate the predictive performance of candidate biomarkers in order to identify the most suitable candidates, if any, for commercialization. To date, no single measure (parasite/clinical/biomarker) has been identified and validated that has sufficient sensitivity and specificity to act as a stand-alone prognostic or predictive measure.

Over the last 20 years, researchers have been working on the premise that multiple measures can be combined to generate scores with improved prognostic performance. Starting with 8 clinical parameters in a bedside prognostic index (Molyneux, Taylor et al. 1989), scores have been gradually refined until a score with just 3 clinical parameters was developed (Helbok, Kendjo et al. 2009). Acidosis (assessed using plasma base excess or lactate) is a consistent feature in both children and adults with severe malaria (White, Warrell et al. 1985; Waller, Krishna et al. 1995; Agbenyega, Angus et al. 1997; Newton, Valim et al. 2005), and point-of-care devices exist that are suitable for use in the developing world. Despite these early findings, a multicentre trial in Africa found that base excess and lactate did not improve prediction of mortality when used in combination with clinical parameters (Newton, Valim et al. 2005). Identification of biomarkers that could improve prediction alongside existing measures could be transformative, particularly if an effective adjunctive therapy was available.
1.4 Malaria in Pregnancy: Immunologic Changes During Pregnancy and the Emergence of Novel Parasites

Pregnant women represent another high-risk group for severe malaria. Every year, an estimated 85 million pregnancies occur in areas of *P. falciparum* transmission, with almost 55 million of these occurring in areas of stable malaria transmission (Dellicour, Tatem et al. 2010). In areas of high malaria transmission, malaria infection in pregnancy (MiP) leads to increased risk of adverse outcomes including severe maternal anemia, and intrauterine and perinatal fetal death (WHO 2004). In contrast, an infection during pregnancy in areas of low transmission (where women often lack clinical immunity against malaria) can lead to severe manifestations of disease, including coma, hypoglycemia and respiratory distress (WHO 2004). The increased susceptibility to disease has been attributed to general immunologic changes in pregnancy and the emergence of a dominant CSA binding parasite in pregnancy capable of sequestering in the placental intervillous space (IVS). The presence of CSA-binding PEs in the IVS (termed ‘placental malaria’ (PM)) can lead to impaired fetal growth and preterm delivery.

1.4.1 Clinical Presentation and Special Considerations of Malaria in Pregnancy

One of the major clinical challenges with PM is the propensity for infections to be asymptomatic or paucisymptomatic (Huynh, Fievet et al. 2011). Signs and symptoms (when present) are non-specific, such as anemia, fever, headache and chills. Primigravidae represent the group at highest risk of infection (Brabin 1983; McGregor, Wilson et al. 1983), and the risk gradually declines over subsequent pregnancies as women acquire protective antibodies that block the cytoadherence of PEs to the IVS and enhance the phagocytic clearance of PEs (Fried, Nosten et al. 1998; Keen, Serghides et al. 2007). Thus, multigravidae (≥3 pregnancies) are generally at reduced risk of adverse clinical outcomes due to PM than primigravidae, unless immunocompromised (e.g. HIV-1 infection). HIV-positive pregnant women fail to acquire protective immunity to CSA-binding PEs and thus all HIV-positive pregnant women are at increased risk of malaria- and HIV- related adverse pregnancy outcomes (Keen, Serghides et al. 2007).
1.4.1.1 Intermittent Preventive Treatment to Reduce Disease Burden

One approach to limit the burden of malaria on populations at risk of developing severe disease is the administration of a curative treatment dose of an efficacious antimalarial drug at predefined intervals to individuals at high-risk of complications from severe malaria regardless of infection status. This is termed intermittent preventive treatment (IPT) of malaria. Currently, IPT is recommended by the WHO for use in pregnancy (IPTp; see Figure 1.5 for WHO recommended dosing schedule of IPTp). This policy has been adopted in 33 countries in sub-Saharan Africa, two in the Eastern Mediterranean (Sudan, Somalia) and one in the Western Pacific (Papua New Guinea) (WHO 2010). Trials have been underway to test the feasibility and utility of IPT programs in infants (IPTi) and children (IPTc) in areas of high malaria transmission (reviewed in (Aponte, Schellenberg et al. 2009; Wilson 2011)), but currently no country has adopted an IPT program outside of pregnancy (WHO 2010).

The ideal IPT drug would be safe for use in pregnancy and young children; require the administration of a single treatment dose so it could be incorporated into routine clinical care; and have a long half life to provide a lasting prophylactic effect. To date, the drug that has fulfilled all of these criteria is sulfadoxine-pyrimethamine (SP). A systematic review of two-dose SP-IPTp studies in Africa has shown that a two-dose regimen of SP at antenatal visits was effective at reducing placental malaria, low birth weight and maternal anemia (ter Kuile, van Eijk et al. 2007). However, increasing resistance to SP is compromising the utility in SP in IPT programs, especially in areas of high malaria endemicity and HIV prevalence (See Figure 1.1 for a map of SP resistance), and more frequent dosing may be required until a suitable replacement is available (ter Kuile, van Eijk et al. 2007). IPTp represents one strategy to reduce the burden of malaria and it should be integrated with the use of insecticide-treated nets and appropriate case management of malaria to achieve optimal effect.
The fetal growth velocity was calculated based on fetal nomograms derived from a reference Congolese population (Landis, Ananth et al. 2009). The WHO recommends 4 antenatal visits during pregnancy, with 3 visits after quickening (detection of fetal movement). At least two doses of antimalarials (sulfadoxine-pyrimethamine, SP) are recommended at the second and third antenatal visit, with an optional third dose at the final visit (WHO 2004). In HIV-positive women, an additional IPTp dose (third dose) is recommended (WHO 2004). SP is both effective at treating existing malaria infections and providing long-term prophylaxis. Pharmokinetic data suggest drug resistance against pyrimethamine can result in a one month reduction in the post-treatment prophylactic effect of SP (reduced from 60 days to 30 days) (White 2005). With a two-dose regimen, women in SP-resistant areas would be relatively unprotected from malaria from 30 weeks gestation onwards (Rogerson, Mwapasa et al. 2007), which corresponds to the peak of fetal growth velocity.
1.4.2 Diagnosis

PM may be a silent disease since parasites have a tendency to preferentially accumulate in the placenta (and can be undetectable by peripheral smear). Therefore, it can be difficult to accurately identify women with occult PM infections. A study in Cameroon of over 1,000 pregnant women found that 20% of women with placental malaria detected by light microscopy at delivery were negative by peripheral smear (Leke, Djokam et al. 1999); whereas a study in Ghana found the number to be almost 50% (Mockenhaupt, Bedu-Addo et al. 2006). Because traditional malaria diagnostics are unreliable to detect PM, malaria management in pregnancy had focused on prevention using long-lasting insecticide treated nets, IPTp and presumptive treatment of febrile illness.

Rapid diagnostic tests (RDTs) have been evaluated as an alternative to microscopy to diagnose PM in pregnancy because they do not rely on the presence of circulating parasites, but rather parasite antigen. However, few studies have specifically addressed how well RDTs detect occult PM (Leke, Djokam et al. 1999; Mankhambo, Kanjala et al. 2002; Mockenhaupt, Ulmen et al. 2002). Attempts have also been made to develop rapid and field-applicable PCR based assays; however, it remains unclear whether sub-microscopic infections lead to clinically significant outcomes with some, but not all, studies showing an effect on infant birth weight or maternal anemia (Mockenhaupt, Rong et al. 2000; Mockenhaupt, Ulmen et al. 2002; Adegnika, Verweij et al. 2006; Mockenhaupt, Bedu-Addo et al. 2006). Finally, investigators have asked whether proteins elevated in the maternal peripheral blood are associated with PM in the absence of circulating peripheral parasites (Mockenhaupt, Rong et al. 2000; Adegnika, Verweij et al. 2006; Thevenon, Zhou et al. 2010), but this area is remains largely unexplored.

Unlike non-pregnant adults where clinically silent infections may be harmless (but contribute to malaria transmission), asymptomatic infections are common in pregnancy and can contribute to adverse outcomes if left untreated (Fleming 1989; Huynh, Fievet et al. 2011). Therefore, there is an urgent need to develop better diagnostic tools to identify women at high-risk of adverse pregnancy outcomes due to malaria so they can receive targeted treatment with effective antimalarials.
1.4.3 Immunopathology

Malaria in pregnancy is estimated to cause up to 200,000 infant deaths every year (Guyatt and Snow 2001) as a result of low birth weight deliveries. Thus, low birth weight (LBW, <2500g) is considered an important endpoint in studies of placental malaria. LBW can occur as a result of preterm delivery (PTD, <37 weeks gestation) and/or intrauterine growth restriction (IUGR). The most well defined pathologic correlate of LBW in placental malaria is the infiltration of mononuclear cells in the IVS (Rogerson, Pollina et al. 2003). However, the mechanisms leading to fetal growth restriction in utero remain incompletely understood.

Pregnancy constitutes a unique physiologic state where the maternal immune system must protect against infection while preventing rejection of the semi-allogenic fetus. In infection, activated macrophages secreting high levels of inflammatory cytokines can disrupt the balance of cytokines at the maternal-fetal interface leading to poor fetal outcomes (Hunt 1989; Marzi, Vigano et al. 1996). Monocyte accumulation in the IVS may be massive in PM and contribute to a polarized inflammatory state (Fried, Muga et al. 1998; Fievèt, Moussa et al. 2001; Rogerson, Pollina et al. 2003). PEs sequestered in the placenta induce the secretion of β-chemokines from maternal mononuclear cells (Abrams, Brown et al. 2003; Chaisavaneeyakorn, Moore et al. 2003; Suguitan, Leke et al. 2003) and by fetal syncytiotrophoblast (Abrams, Brown et al. 2003; Lucchi, Koopman et al. 2006) resulting in further recruitment of monocytes to the placenta. The production of macrophage inhibitory factor in the IVS acts to retain mononuclear cells in the IVS (Chaisavaneeyakorn, Lucchi et al. 2005; Lucchi, Peterson et al. 2008). Thus, proinflammatory cytokines and chemokines act in concert to recruit, retain and activate monocytes. A number of studies have demonstrated associations between mononuclear cell infiltrates, their inflammatory products, and malaria associated LBW (Fried, Muga et al. 1998; Rogerson, Brown et al. 2003; Rogerson, Pollina et al. 2003; Kabyemela, Fried et al. 2008).

There remain critical gaps in our understanding of the pathogenic processes downstream of mononuclear cell accumulation. Increased production of proinflammatory cytokines (such as TNF and IL-1β) could impair the transplacental passage of amino acids (Carbo, Lopez-Soriano et al. 1995; Thongsong, Subramanian et al. 2005), thus providing a potential mechanism for impaired fetal growth in PM. A recent report demonstrated altered levels of insulin-like growth factors in placental malaria with mononuclear cell infiltrates (Umbers, Boeuf et al. 2011); this is
consistent with the concept that malaria may impair nutrient transport by altering amino acid transport across the trophoblast. Further, the thickening of the trophoblast basement membrane, syncytial knotting and fibrinoid necrosis described in malaria-infected placentas could also impair the uptake of essential nutrients (Galbraith, Fox et al. 1980; Walter, Garin et al. 1982; Yamada, Steketee et al. 1989).

Malaria could also lead to altered angiogenic remodeling in the context of the PM. This is supported by reports describing tortuous and irregular villous arborization and increased number and diameter of fetal blood vessels in human placentas infected with malaria (Walter, Garin et al. 1982; Leke 2002). Further, physiologic changes to fetal and maternal blood flow have been reported in PM (Dorman, Shulman et al. 2002; Arbeille, Carles et al. 2003). Studies from Tanzania and Cameroon have shown perturbations in two major families of angiogenic factors (vascular endothelial growth factor and angiopoietins) (Muehlenbachs, Mutabingwa et al. 2006; Muehlenbachs, Fried et al. 2008; Silver, Zhong et al. 2010).
Figure 1.7 Immunopathogenesis of Placental Malaria

The accumulation of CSA-binding PEs in the placental intervillous space can activate the syncytiotrophoblast and resident macrophages leading to the production of proinflammatory cytokines and chemokines. This leads to the further recruitment, retention and activation of mononuclear cells in the IVS and poor birth outcomes.
A number of tools have been used to investigate PM pathogenesis. These include: histologic examination of malaria-infected placentas; assessment of immune cell inflammatory mediators present in infection; transcriptional analysis of infected vs. uninfected placentas; and in vitro and ex vivo studies using syncytiotrophoblast explants, cell lines, and placental mononuclear cells (Galbraith, Fox et al. 1980; Walter, Garin et al. 1982; Yamada, Steketee et al. 1989; Fried and Duffy 1996; Fried, Muga et al. 1998; Fried, Nosten et al. 1998; Moore, Nahlen et al. 1999; Moormann, Sullivan et al. 1999; Abrams, Brown et al. 2003; Chaisavaneeyakorn, Moore et al. 2003; Rogerson, Brown et al. 2003; Rogerson, Pollina et al. 2003; Suguitan, Cadigan et al. 2003; Suguitan, Leke et al. 2003; Chaisavaneeyakorn, Lucchi et al. 2005; Muehlenbachs, Fried et al. 2007; Lucchi, Peterson et al. 2008). An underutilized tool for assessing pathogenic processes in PM is animal models, which are available and share common features with human disease.

1.4.4 Murine Model

*Plasmodium berghei* ANKA is widely used with rodents (mice) to model pathogenesis of both CM and PM (reviewed in (de Souza, Hafalla et al. 2010) and (Hviid, Marinho et al. 2010)). Strain dependent susceptibility of mice to either CM or PM has enabled researchers to gain insight into the molecular mechanisms of disease. In pregnancy, *Plasmodium berghei* ANKA (PbA) infection of susceptible mice shows an accelerated course of disease that is uniformly fatal (Oduola, Holbrook et al. 1982; Hioki, Hioki et al. 1990). When mice are infected with PbA early in pregnancy, they have been shown to abort, while infections later in pregnancy lead to decreased fetal viability and impaired fetal growth. This model recapitulates many critical features of PM including the inflammatory environment generated by infection (shown in Table 1.2).
Table 1.2 Similarities between the key features of placental malaria in the *P. berghei* ANKA murine model and human disease

<table>
<thead>
<tr>
<th></th>
<th>Murine Model</th>
<th>Human disease</th>
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<tr>
<td></td>
<td><em>(P. berghei ANKA)</em></td>
<td><em>(P. falciparum)</em></td>
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<tr>
<td>Sequestration of PEs to CSA</td>
<td>(Marinho, Neres et al. 2009)</td>
<td>(Fried and Duffy 1996)</td>
</tr>
<tr>
<td>Mononuclear cell infiltration</td>
<td>(Neres, Marinho et al. 2008)</td>
<td>(Walter, Garin et al. 1982; Rogerson, Pollina et al. 2003)</td>
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<tr>
<td>Parity-related effect</td>
<td>(van Zon, Eling et al. 1985; Marinho, Neres et al. 2009)</td>
<td>(McGregor and Smith 1952)</td>
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<tr>
<td>Placental inflammation</td>
<td>(Neres, Marinho et al. 2008)</td>
<td>(Fried, Muga et al. 1998; Moormann, Sullivan et al. 1999)</td>
</tr>
<tr>
<td>Changes to villous architecture</td>
<td>(Neres, Marinho et al. 2008)</td>
<td>(Walter, Garin et al. 1982; Leke 2002)</td>
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1.5 Research Objectives

There are limited tools to identify malaria-infected individuals at risk of poor outcomes. The purpose of this work was to examine how host biomarkers can improve our understanding of severe malaria pathogenesis and may serve as diagnostic or prognostic biomarkers. Ultimately, it is our hope that these biomarkers may identify individuals likely to benefit from novel interventions (anti-malarial, supportive or adjunctive).

In chapter 2, we examine the interaction between complement C5a (previously identified as an important mediator of disease pathology in a *P. berghei* ANKA model of murine cerebral malaria) and malaria bioactive product *Pf*GPI using an *in vitro* system and human peripheral blood mononuclear cells (PBMCs). The objectives of this work were:

1. To determine the effect of *P. falciparum* parasites and parasite by-product *Pf*GPI on complement C5 activation and C5a receptor expression.

2. To examine possible interactions between C5a and *Pf*GPI on inflammatory responses.

3. To evaluate whether C5a levels are elevated in placental malaria.

We found that cultured CSA or E8B-binding parasites were capable of activating the complement system leading to the production of C5a. Further, expression of the C5a receptor was induced on monocytes following exposure of PBMCs to *Pf*GPI. Together, C5a and *Pf*GPI interacted *in vitro* to synergistically enhance the production of cytokines, chemokines and the anti-angiogenic factor sFlt-1. We could abrogate this response by specific antibody blockade of the C5a receptor. Finally, we measured levels of C5a in primigravid women from Kenya and found elevated C5a levels women with placental malaria compared to those without.

We extend these observations in Chapter 3, where C5a levels were measured at delivery in pregnant women from Malawi. In this large case-control study of placental malaria, we were able to ask whether changes in C5a were associated with poor birth outcomes (e.g. fetal growth restriction). We were able to model the role of C5a in placental malaria, focusing specifically at angiogenesis using a murine model and structural equation modeling. The objectives for this work were:
1. To confirm and extend the observations from Kenya in an independent population of women with placental malaria.

2. To explore the relationship between C5a and angiogenic factors

3. To examine the role of C5a and angiogenic factors on adverse birth outcomes in the context of placental malaria.

In this study, we show elevated C5a in women with placental malaria, irrespective of gravidity. Elevated C5a levels were associated with increased odds of delivering a small-for-gestational-age infant. In addition, we show altered levels of angiogenic factors (increased Ang-2, sEng, sFlt-1, and decreased Ang-1) in placental malaria. We were able to posit how C5a may affect placental angiogenesis and test our hypothesis using structural equation modeling. The results from the statistical modeling were then confirmed using a murine model of placental malaria and showing a role for C5a in modulating angiogenic responses to malaria through genetic and pharmacologic manipulation of C5a signalling.

Anti-angiogenic factor sEng has been identified as a putative biomarker in preeclampsia (which shares features with placental malaria) and in severe malaria in children. However, levels of sEng have not been measured in the context of malaria in pregnancy. As an extension to the previous study looking at sEng levels alongside other angiogenic proteins in the placenta, in Chapter 4, we examined maternal peripheral levels of sEng in two populations of pregnant women in malaria endemic areas. The objectives were:

1. To examine how sEng levels change over the course of gestation and in different gravidae.

2. To determine whether peripheral sEng has utility as a biomarker in placental malaria.

3. To examine the relationship between sEng and poor fetal outcomes.

In this study, we show that sEng levels are highest in primigravidae and change over the course of gestation. While there were increases in maternal sEng during malaria infection, the dynamic range was not sufficient to justify use of sEng as a biomarker for PM. However, increases in
sEng were associated with increased risk of fetal growth restriction, suggesting that sEng may be involved in the pathophysiology of PM.

*P. falciparum* parasites have a tendency to accumulate in the placental intervillous space, sometimes leading to occult or ‘hidden’ infections. In Chapter 5, we asked whether proteins detectable in the maternal peripheral blood would be associated with placental malaria infections, in the absence of circulating peripheral parasites. Our objectives were:

1. To identify whether any peripheral blood biomarkers were associated with the presence of placental malaria infections (in the absence of circulating peripheral parasites)

2. To evaluate the diagnostic accuracy of the biomarkers to detect occult PM.

We identified 3 putative biomarkers (sFlt-1, leptin, CRP) that were significantly associated with occult PM and had areas under the ROC curve >0.70. By combining the biomarkers together into a simple score and integrating the biomarkers with easy-to-measure clinical parameters, we could improve identification of occult PM compared to individual biomarkers or a clinical score. These data suggest that peripheral blood biomarkers may be able to improve detection of occult PM.

Moving away from biomarkers in placental malaria to severe and cerebral malaria in non-pregnant individuals, in Chapter 6 we measured levels of angiogenic factors in whole blood from a population with *P. falciparum* malaria from Thailand. Earlier reports from our lab, and others, suggested that angiopoietins may be good biomarkers of disease severity in severe and cerebral malaria in both South East Asian adults and in Ugandan children (Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009). The objectives of this work were:

1. To determine whether Ang-1 and Ang-2 could be reliably measured in lysed whole blood.

2. To evaluate the relationship between Ang-1 and Ang-2 to disease severity in *P. falciparum* malaria.

In this work, we show that Ang-1 and Ang-2 are robust biomarkers that can be readily measured in lysed whole blood and have good discriminatory ability between uncomplicated and severe disease. This effect was independent of covariates (age, sex, ethnicity and parasitemia). Further, we show that Ang-2 was associated disease severity using a score of cumulative organ injury.
To confirm and extend the results from the Thai adults with severe malaria, in Chapter 7 we measured Ang-1 and Ang-2 alongside other proposed biomarkers of severe malaria in a pediatric population from Malawi. The children included in this study had uncomplicated malaria, well-defined cerebral malaria, or fever with decreased consciousness not attributable to malaria (CNS controls). In addition, biomarker levels were tested at follow-up (day 28) for the children in the CM group. This study is unique because it evaluates biomarkers in a group of children with clinically defined CM, of whom some had the ‘hallmark’ retinal findings of CM, and some did not. The objectives of this study were:

1. To determine whether children with retinopathy positive cerebral malaria differed from children with clinically defined CM without retinopathy.

2. To compare the ability of different biomarkers to differentiate between different clinical groups (uncomplicated malaria, cerebral malaria, CNS controls).

3. To examine the difference in biomarker levels at admission and convalescence in cerebral malaria.

In this study, we measured soluble Tie-2 as a novel biomarker of the angiopoietin-Tie-2 system and found it was the best discriminator between retinopathy positive and retinopathy negative CM. Further, we confirmed earlier reports that Ang-1 was the best single biomarker at differentiating between uncomplicated malaria or CNS controls and cerebral malaria, and outperformed other biomarkers in the field (VWF, sICAM-1). Ang-2, on the other hand, was a better biomarker of retinopathy in CM and had moderate ability to differentiate between CNS controls and CM. Both Ang-1 and Ang-2 (and the ratio of Ang-2:Ang-1) showed significant and uniform changes between admission and convalescence in CM.

Finally, in Chapter 8 we examined a larger population of Malawian children with cerebral malaria and evaluated how Ang-1, Ang-2, and sTie-2 relate to retinal changes and mortality. In this population, detailed assessment of the retinal changes was available including a semi-quantitative score of severity. There was also detailed clinical data available for all children. The objectives for this study were:
1. To investigate the relationship between biomarkers and specific aspects of malaria retinopathy

2. To evaluate the prognostic ability of the biomarkers.

3. To explore how combinations of biomarkers and/or clinical or laboratory parameters could be integrated to improve prognostic and predictive accuracy in children with cerebral malaria.

In this study, we found significant relationships between all three biomarkers and retinopathy and mortality. The most striking changes were observed in Ang-2 and sTie-2 and these parameters remained significant predictors of mortality when included in multivariable models. Inclusion of Ang-2 alone into a clinical model to predict mortality improved the predictive accuracy compared to clinical parameters alone. Using a complementary approach of classification analysis, we were able to show that Ang-2 was the best single predictor of mortality among all variables, and it could be integrated with clinical judgment to improve classification of fatal cases.
Chapter 2
C5a Enhances Dysregulated Inflammatory and Angiogenic Responses to Malaria In Vitro: Potential Implications for Placental Malaria

2.1 ABSTRACT

2.1.1 Background

Placental malaria (PM) is a leading cause of maternal and infant mortality. Although the accumulation of parasitized erythrocytes (PEs) and monocytes within the placenta is thought to contribute to the pathophysiology of PM, the molecular mechanisms underlying PM remain unclear. Based on the hypothesis that excessive complement activation may contribute to PM, in particular generation of the potent inflammatory peptide C5a, we investigated the role of C5a in the pathogenesis of PM in vitro and in vivo.

2.1.2 Methodology and Principal Findings

Using primary human monocytes, the interaction between C5a and malaria in vitro was assessed. CSA- and CD36-binding PEs induced activation of C5 in the presence of human serum. *Plasmodium falciparum* GPI (*Pf*GPI) enhanced C5a receptor expression (CD88) on monocytes, and the co-incubation of monocytes with C5a and *Pf*GPI resulted in the synergistic induction of cytokines (IL-6, TNF, IL-1β, and IL-10), chemokines (IL-8, MCP-1, MIP1α, MIP1β) and the anti-angiogenic factor sFlt-1 in a time and dose-dependent manner. This dysregulated response was abrogated by C5a receptor blockade. To assess the potential role of C5a in PM, C5a plasma levels were measured in malaria-exposed primigravid women in western Kenya. Compared to pregnant women without malaria, C5a levels were significantly elevated in women with PM.

2.1.3 Conclusions and Significance

These results suggest that C5a may contribute to the pathogenesis of PM by inducing dysregulated inflammatory and angiogenic responses that impair placental function.
2.2 INTRODUCTION

Placental malaria (PM) is a major determinant of maternal and infant health in the developing world. PM, especially in primigravidae, can have profound maternal and fetal health consequences, including anemia, stillbirth, preterm delivery, intrauterine growth restriction (IUGR) and delivery of low birth weight (LBW) infants (Gilles, Lawson et al. 1969; Diagne, Rogier et al. 1997). The accumulation of *Plasmodium falciparum* parasitized erythrocytes (PEs) within the placenta is believed to be an essential step in the pathogenesis of PM (Fried and Duffy 1996). A subpopulation of PEs that express novel variant surface antigens (VSA-PM), specifically adhere to glycosaminoglycan CSA in the placental intervillous space (Fried and Duffy 1996; Buffet, Gamain et al. 1999; Duffy, Byrne et al. 2005).

PM is also characterized by the infiltration of the placenta with maternal mononuclear cells (mφ). The sequestered PEs release bioactive molecules including those with associated glycosylphosphatidylinositol anchor molecules (*Pf*GPI) that can stimulate maternal mononuclear cells (Abrams, Brown et al. 2003; Chaisavaneeyakorn, Moore et al. 2003; Suguitan, Leke et al. 2003; Krishnegowda, Hajjar et al. 2005; Nebl, De Veer et al. 2005) and fetal syncytiotrophoblast (Abrams, Brown et al. 2003; Lucchi, Koopman et al. 2006; Lucchi, Peterson et al. 2008) to produce inflammatory cytokines, such as TNF and IFN-γ, and β-chemokines including macrophage-inflammatory protein (MIP)-1α, MIP-1β, monocyte chemoattractant protein-1 (MCP-1), and macrophage migration inhibitory factor (MIF). These cytokines and chemokines further recruit, retain, and activate mononuclear cells in the placenta (Abrams, Brown et al. 2003; Chaisavaneeyakorn, Moore et al. 2003; Suguitan, Leke et al. 2003; Chaisavaneeyakorn, Lucchi et al. 2005; Lucchi, Peterson et al. 2008). The resultant accumulation of activated mononuclear cells is believed to contribute to adverse birth outcomes (Fried, Muga et al. 1998; Rogerson, Brown et al. 2003; Rogerson, Pollina et al. 2003). Although PEs and mononuclear cells collect in the placenta, how they may contribute to fetal and placental injury is unknown.

The maintenance of regulated cytokine responses in the placenta is essential in order to prevent rejection of the semiallogenic fetoplacental unit and ensure appropriate immunological responses to infection (Caucheteux, Kanellopoulos-Langevin et al. 2003). This involves a generalized physiological adaptation that results in a bias towards cytokines promoting humoral immunity at
the expense of cell-mediated immunity (Bowen, Chamley et al. 2002). PM is associated with heightened $T_{H}^{1}$-type responses that disrupt the balance of cytokines at the maternal-fetal interface (Hunt 1989; Marzi, Vigano et al. 1996; Fried, Muga et al. 1998; Rogerson, Brown et al. 2003). However, the molecular basis underlying PM-associated cytokine dysregulation, placental immunopathology, and adverse birth outcomes is incompletely understood.

The complement system is an essential component of the innate immune response to a number of infectious agents. The complement cascade can be activated by four distinct pathways, three of which converge at the level of the C3 component, leading to the cleavage of C3 and C5 to their activated forms, C3a and C5a, as well as the formation of the terminal membrane attack complex (Guo and Ward 2005; Huber-Lang, Sarma et al. 2006). Recently, a pathway of C5a generation occurring independently of C3 was described (Huber-Lang, Younkin et al. 2002; Huber-Lang, Sarma et al. 2006). Several lines of evidence have implicated excessive activation of the complement system, notably generation of the potent proinflammatory peptide C5a, in mediating deleterious innate host responses to bacterial and fungal infections and contributing to the development of sepsis (reviewed in (Guo and Ward 2005)) (Chenoweth and Hugli 1978; Riedemann, Guo et al. 2003; Ward 2004; Huber-Lang, Sarma et al. 2006). Sepsis, similar to severe malaria, is a clinical syndrome characterized by systemic inflammation and endothelial activation in response to infection (Aird 2003; Pober and Sessa 2007). Elevated C5a levels in sepsis have been implicated in adverse clinical outcomes and death. Blockade of C5a-C5aR activity in animal models of sepsis prevents end-organ injury and improves survival (Smedegard, Cui et al. 1989; Czermak, Sarma et al. 1999; Huber-Lang, Sarma et al. 2001).

Excessive C5a generation has also recently been identified as a critical mediator of placental and fetal injury in a non-infectious mouse model of spontaneous miscarriage and IUGR, via dysregulation of angiogenic factors required for normal placental development (Girardi, Yarilin et al. 2006). Activated complement components were shown to directly induce the release of soluble fms-like tyrosine kinase-1 (sFlt-1, sVEGFR-1), a potent anti-angiogenic factor that prevents signaling of vascular endothelial growth factor and placental growth factor (Charnock-Jones and Burton 2000). Excessive levels of sFlt-1 inhibit placental differentiation and are thought to play a direct role in preeclampsia (Zhou, McMaster et al. 2002; Levine, Maynard et al. 2004; Lam, Lim et al. 2005) and C5a-associated IUGR and pregnancy loss (Girardi, Yarilin et al.
2006). Of note, Muehlenbachs et al. have reported elevated plasma levels of sFlt-1 in primigravid women with PM (Muehlenbachs, Mutabingwa et al. 2006).

Based on the hypothesis that C5a may play a role in PM by generating angiogenic factors that mediate IUGR and fetal loss, we determined whether malaria parasites can activate C5 and if C5a potentiates the induction of sFlt-1, inflammatory cytokines and chemokines associated with adverse pregnancy outcomes. Here we show that CSA- and CD36-binding PEs activate C5, that C5a and PfGPI cooperate to induce an amplified inflammatory and anti-angiogenic response to P. falciparum malaria in vitro, and that primigravid women with PM have elevated circulating C5a levels in vivo.

2.3 METHODS

2.3.1 P. falciparum Culture

The laboratory isolates CS2 (CSA-binding) and E8B (ICAM-1 and CD36 binding) were cultured in vitro as described (Patel, Serghides et al. 2004; Serghides, Patel et al. 2006). Cultures were routinely treated with mycoplasma removal agent (ICN), and were tested and found negative for mycoplasma by PCR.

2.3.2 Mononuclear Cell Isolation

PBMCs were isolated from healthy volunteers using a Ficoll gradient as previously described (McGilvray, Serghides et al. 2000). PBMCs were counted using a hemocytometer and cell viability was determined by Trypan blue exclusion test. Cells were plated at a concentration of 2.0x 10^6 cells/mL (48 well plate) in RPMI 1640 medium supplemented with 10% heat inactivated FBS and gentamycin (R10G). Cells were cultured at 37ºC in 5% CO2 for the time indicated.

2.3.3 Isolation and Purification of GPIs from P. falciparum

HPLC-purified P. falciparum GPI (PfGPI) was isolated (Naik, Branch et al. 2000), conjugated to gold beads and used at a concentration of 300ng/ml as previously described (Krishnegowda, Hajjar et al. 2005). Unconjugated gold beads alone were added as controls to wells containing media, or C5a (endotoxin-free; Biovision, USA). All preparations of purified PfGPIs were tested for endotoxin by Limulus amebocyte lysate assay prior to use.
2.3.4 Complement Assays

PEs, uninfected red blood cells (uRBCs), or media control were incubated in the presence of 30% serum from malaria naïve donors. After 30 min, supernatants were collected and analyzed for C5 activation. C5a levels in culture supernatants and human plasma were measured by ELISA (R&D Systems).

2.3.5 Cytokine and Chemokine Assays

PBMCs were cultured with or without recombinant human C5a in addition to *P. falciparum* culture supernatants (1:10 dilution) and R10G alone as media control, or HPLC-purified GPI (300ng/mL) and unconjugated gold beads as a control. C5a concentrations ranging from 1 nM to 100 nM were selected representing a spectrum of C5a levels ranging from physiologically normal levels (<10 nM) to those associated with sepsis (10-100 nM) (Solomkin, Jenkins et al. 1981; Ward 2004). For receptor blockade studies *PfGPI*, the C5aR blocking antibody (Serotec, S5/1, 5 µg/mL) or an isotype control (eBioscience, eBM2a, 5 µg/mL) was added to culture medium just prior to stimulation with 50 nM C5a. Culture supernatants were collected aseptically at times indicated. IL-6, TNF, IL-1β, and IL-10 were measured by Cytometric Bead Array (CBA) (BD Biosciences, human inflammation kit). IL-8, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α and - β (MIP-1α and MIP-1β) and sFlt-1 (sVEGFR-1) were measured by ELISA (R&D Systems).

2.3.6 Flow Cytometry

PBMCs (2.0 x 10^6) were blocked in 20% serum for 20 min at 4°C. Cells were then stained for CD14-APC (eBioscience, 61D3, 100 uL/mL) and CD88-PE or isotype IgG1-PE (BD Biosciences, D53-1473, 150 uL/mL and eBioscience, P3, 150uL/mL) for 30 min at 4°C. Cells were fixed in 1% paraformaldehyde/PBS overnight and analyzed on a Becton Dickinson FACS Calibur flow cytometer. Data was analyzed using FlowJo software. A minimum of 50,000 events were collected. Positive background PE staining in isotype samples was subtracted from respective CD88-PE stained samples and then reported as specific CD88 staining.

2.3.7 Statistical Analysis

Data are expressed as means ± SEM, unless otherwise noted. For continuous variables a Student’s t test or one-way ANOVA with Bonferronni post-tests was used to compare means.
Non-parametric tests (Mann-Whitney or Kruskal-Wallis) were used to compare groups where parametric test conditions were not met. Synergism was determined by the interaction term of a two way ANOVA (C5a*GPI). Synergism in the time course was determined using the cumulative cytokine levels after 48 hours by performing a two way ANOVA and examining the interaction term. Significance in the C5a receptor blockade experiments was assessed by repeated measures ANOVA to determine whether levels of cytokines differed between cells treated with PfGPI alone, or anti-CD88 plus C5a and PfGPI, or student’s t-test by comparing the isotype and anti-CD88 co-treated (C5a and PfGPI) groups after correcting for the background levels observed in media controls. In cases where C5a receptor blockade resulted in values of “0”, a one sample t-test was used to determine whether the isotype C5a and PfGPI co-treated groups differed from a hypothetical value of “0”. Correlation of peripheral parasitemia to placental parasitemia was determined using Spearman correlation. Experiments were performed in duplicate or triplicate and repeated as indicated in the figure legends. Statistical analysis of experimental replicates is found in the supporting information files (S1-S4). Differences with p<0.05 were considered significant. Statistical analysis was performed using GraphPad Prism and SPSS.

2.3.8 Study Participants

Plasma samples were collected at time of delivery from the peripheral blood and placental blood of primigravid women, living in an area of holoendemic malaria transmission in Western Kenya, with and without placental malaria infection as described previously (Chaisavaneeyakorn, Moore et al. 2003). Malaria infection was evaluated by examination of Giemsa-stained peripheral and placental thick and thin blood smears by light microscopy. Percent parasitemia was estimated by counting a minimum of 1000 erythrocytes and calculated using the following formula: (# infected erythrocytes/total # erythrocytes) X 100. Women who tested positive for HIV were excluded. This study was approved by the institutional review boards of the University of Georgia, the Kenya Medical Research Institute and the Centers for Disease Control and Prevention, Atlanta, Georgia, United States. Written informed consent was obtained from all participants.
2.4 RESULTS

2.4.1 CSA- and CD36-binding PEs Activate C5 and PfGPI Induces C5a Receptor Expression

The proinflammatory anaphylatoxin C5a has been implicated in mediating deleterious host responses to bacterial and fungal agents (Ward 2004; Guo and Ward 2005). To confirm that CSA-binding PEs were capable of activating the complement system and thus generating C5a, serum from malaria-naïve donors was added to mature stage CS2 PEs or uRBCs. Compared to uRBCs or controls, C5 activation was only observed in the presence of PEs (Figure 2.1A) (mean C5a levels: CS2 PEs 24.77 ng/mL [95% confidence interval (CI) 2.229-47.30], E8B PEs 26.19 ng/ml [95% CI -10.44-62.82], uRBCs 7.114 ng/mL [95% CI 5.922-8.306], control 7.242 ng/mL [95% CI 4.116-10.37]; CS2 PEs vs. uRBCs p<0.001 Student’s t-test). Non-PM PEs (E8B) also activated C5 (E8B PEs vs. uRBCs p<0.001 Student’s t-test).

In order to respond to activated C5, cells must express a C5a receptor. Previous studies report that C5a responsiveness increases following cellular stimulation (Monk, Scola et al. 2007). To determine the effect of PfGPI on monocyte C5aR (CD88) expression, PBMCs were incubated as above with PfGPI or media control for 24 or 48 hours, and C5aR and CD14 expression (used to define monocytes) was determined by flow cytometry. Compared to media only control cells, a significant increase in the number of monocytes expressing C5aR (Figure 2.1B) was observed (mean: media 1.2% [95% CI –0.2-2.7] vs. PfGPI 3.0 [95% CI 1.5-4.4] p=0.0279 by Student’s t-test). The density of the C5aR, as determined by the mean fluorescent intensity on monocytes already expressing the receptor, was unchanged (data not shown).
Figure 2.1 Parasitized erythrocytes (PEs) induce C5 activation and PfGPI upregulates C5aR expression on monocytes. (A) Serum from malaria-naïve donors was added to mature stage CS2 (CSA binding) PEs or E8B (ICAM-1 and CD36 binding), uninfected red blood cells (uRBCs) or media for 30min and supernatants were assayed for C5a by ELISA. Increased C5a levels were observed in the supernatants of PEs compared to uRBC or control, (Student’s t-test: uRBCs vs. Mature PEs ***p<0.001). Data are presented as means ± SEM and are representative of four independent experiments for CS2 parasites and two independent experiments for CD36-binding parasites. (B) Human PBMCs were stimulated with PfGPI or unconjugated gold beads alone as a control for 24h. Expression of C5a receptor was determined by flow cytometry on monocytes (CD14). The percentage of monocytes expressing C5aR was significantly higher for PfGPI-treated versus media control cells, (Student’s t-test: *p=0.0279). Data are presented as means ± SEM and are representative of two independent experiments.
2.4.2 C5a Potentiates *Pf*GPI-Induced Inflammatory Responses

C5a has been shown to enhance TLR-mediated proinflammatory responses to microbial products such as LPS, contributing to sepsis syndromes (Albrecht and Ward 2004; Ward 2004; Guo and Ward 2005; Zhu, Krishnegowda et al. 2005). The parasite product *Pf*GPI is recognized by TLR2 and is believed to be a mediator of the inflammatory response that characterizes severe malarial syndromes (Albrecht and Ward 2004; Krishnegowda, Hajjar et al. 2005; Zhu, Krishnegowda et al. 2005; Niederbichler, Hoesel et al. 2006). To assess whether C5a would amplify *Pf*GPI-induced proinflammatory responses, we treated PBMCs with increasing concentrations of recombinant human C5a (0, 1, 5, 10, 50, and 100nM) with or without *Pf*GPI for 24 hours and determined the production of IL-6 and TNF by ELISA. We observed a dose-dependent potentiation of *Pf*GPI-induced IL-6 and TNF production by C5a (*Figure 2.2A and B*; two-way ANOVA (C5a*GPI), p<0.0001).

![Figure 2.2 C5a potentiates *Pf*GPI-induced inflammatory cytokines in a dose-dependent manner.](image)

*Figure 2.2 C5a potentiates *Pf*GPI-induced inflammatory cytokines in a dose-dependent manner.* Human PBMCs were stimulated with varying concentrations of recombinant human C5a (0, 1, 5, 10, 50, 100nM) for 24 hours, with or without *Pf*GPI. Culture supernatants were collected and assayed for IL-6 (A) and TNF (B) by ELISA. Data were analyzed by two-way ANOVA and demonstrate an interaction effect (C5a*Pf*GPI) for IL-6 (**p<0.0001) and for TNF (**p<0.0001). Data are presented as means ± SEM of three independent experiments.
2.4.3 C5a Receptor Blockade Inhibits Enhanced Cytokine Responses

Elevated levels of T\textsubscript{H}\textsuperscript{1} cytokines have been implicated in PM pathogenesis and adverse pregnancy outcomes (Fried, Muga et al. 1998; Moormann, Sullivan et al. 1999; Rogerson, Brown et al. 2003). In order to determine the effect of C5a on the kinetics of Pf/GPI-induced cytokine production, we treated PBMCs with C5a (50nM) and Pf/GPI (HPLC-purified, 300ng/mL) for 48 hours. Supernatants were collected at baseline, 1, 2, 4, 6, 8, 12, 24 and 48 hours and assayed for inflammatory cytokines using a cytometric bead array. To examine the role of C5a-C5aR interactions in the observed inflammatory responses, we also performed parallel experiments including monoclonal antibody blockade of the C5a receptor. Pf/GPI-stimulated cytokines, including IL-6, TNF, IL-1β and IL-10, were synergistically induced in the presence of C5a (Figure 2.3A-D: two-way ANOVA on cumulative cytokine levels (C5a*GPI); p<0.0001 for IL-6, p=0.0124 for TNF, p=0.0418 for IL-1β and p<0.0001 for IL-10). A significant reduction in cytokine production was observed in experiments using anti-C5aR blocking antibody compared to its isotype control for all cytokines measured (Figure 2.3E-H: repeated measures ANOVA). Further analysis (repeated measures ANOVA) was performed to determine whether C5aR blockade returned cytokine production to levels for cells treated with Pf/GPI alone. In the presence of anti-C5aR, production of IL-6, TNF, and IL-1β was returned to levels that were not significantly different from Pf/GPI alone (p>0.05). However, this was not true for IL-10 (p= 0.0101).
Figure 2.3 C5a potentiates PfGPI-induced inflammatory cytokines and this effect is abrogated by C5a receptor blockade.

Human PBMCs were cultured with unconjugated gold beads (media control), unconjugated gold beads and C5a (50nM), PfGPI (300ng/mL), or a combination of C5a and PfGPI. (A-D) Supernatants were assayed for IL-6 (A), TNF (B), IL-1β (C), and IL-10 (D). A significant interaction effect between C5a and PfGPI was observed for all cytokines tested. p<0.0001 for IL-6, p=0.0124 for TNF, p=0.0418 for IL-1β and p<0.0001 for IL-10 (by two-way ANOVA on cumulative cytokine production over 48h). Data are representative of three independent experiments. (E-H) Human PBMCs were treated with an α-CD88 mAb (5µg/mL) to block the C5a receptor or an appropriate isotype control and exposed to either unconjugated gold beads (media control) or a combination of C5a (50nM) and PfGPI (300ng/mL) for 48h. Supernatants were assayed for IL-6 (E), TNF (F), IL-1β (G) and IL-10 (H). Cytokine production was significantly reduced upon C5a receptor blockade; p=0.0005 for IL-6, p=0.0028 for TNF, p=0.0479 for IL-1β and p=0.0191 for IL-10 (repeated measures ANOVA comparing cytokine levels between isotype control mAb and α-CD88 mAb groups co-treated with C5a and PfGPI). Values shown are corrected for background levels observed in the media controls, and are presented as means ± SEM. Data are representative of two independent experiments.
2.4.4 C5a Potentiates Secretion of Chemokines and the Angiogenesis Inhibitor sFlt-1

The infiltration of monocytes into the placenta during PM is associated with adverse birth outcomes (Moore, Nahlen et al. 1999; Menendez, Ordi et al. 2000; Rogerson, Pollina et al. 2003). Furthermore, elevated levels of the anti-angiogenic factor sFlt-1 (sVEGFR-1) have been causally implicated in placental dysfunction, growth restriction and fetal loss (Girardi, Yarilin et al. 2006; Muehlenbachs, Mutabingwa et al. 2006). In order to determine whether C5a could potentiate the induction of chemokines involved in the recruitment of monocytes and the anti-angiogenic factor sFlt-1, we incubated PBMCs with C5a, PfGPI, or media controls with or without C5aR blocking antibody or an isotype control antibody and measured IL-8 (CXCL8), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), and sFlt-1 (sVEGFR-1) by ELISA (Figure 2.4). In the C5a and PfGPI co-treated group, synergistic induction of all factors was observed by two-way ANOVA (C5a*GPI; p=0.0265 for IL-8, p=0.0010 for MCP-1, and p<0.0001 for MIP-1α, MIP-1β and sFlt-1). Blocking C5aR inhibited the augmented secretion of all chemokines with the exception of IL-8 (Figure 2.4A-E: Student’s t-test p=0.0388 for MCP-1, p=0.0005 for MIP1-α, p=0.0002 for MIP1-β). Blockade of the C5aR reduced levels of sFlt-1, but the differences did not consistently reach statistical significance.
Figure 2.4 C5a potentiates secretion of Pf/GPI-induced chemokines and the anti-angiogenic factor sFlt-1.

Human PBMCs were treated with unconjugated gold beads (media control), unconjugated gold beads and C5a (50nM), Pf/GPI (300ng/mL), or a combination of C5a and Pf/GPI for 48h. Where indicated PBMCs were treated with either 5µg/mL of α-CD88 mAb to block the C5a receptor or an appropriate isotype control and were exposed to C5a and Pf/GPI. Supernatants were assayed for IL-8 (A), MCP-1 (B), MIP-1α (C), MIP-1β (D) and sFlt-1 (E). A significant interaction effect between C5a and Pf/GPI was observed; *p=0.0265 for IL-8, **p=0.0010 for MCP-1, * p=0.0177 for sFlt-1, and ***p<0.0001 for MIP-1 α, and MIP-1β (by two-way ANOVA). C5a receptor blockade significantly reduced the levels of MCP-1 (* p=0.0388), MIP-1α (*** p=0.0005), and MIP-1β (*** p=0.0002) (by student’s t-test). Data are presented as means ± SEM and are representative of three independent experiments.
2.4.5 C5a is Increased in PM and is Associated with Higher Placental Parasite Burdens

In order to extend our observations to the in vivo setting, we examined C5a levels in malaria-exposed women (Table 2.1 and 2.2). We analyzed C5a levels in the peripheral and placental plasma of HIV negative primigravid women with or without PM from Kisumu, Kenya. Peripheral C5a levels were significantly increased in women with PM (Figure 2.5A: mean C5a levels; PM negative: 98.85 ng/mL [95% CI 84.14-113.6] vs. PM positive: 147.1 ng/mL [95% CI 96.81-197.3] p=0.01 by Student’s t-test two-tailed test on log transformed data). Furthermore, C5a was significantly elevated in the placental plasma of women with PM (Figure 2.5B: mean C5a levels; PM negative: 69.07 ng/mL [95% CI 58.17-79.98] vs. PM positive: 104.0 ng/mL [95% CI 73.10-135.0] p=0.0264 by Student’s t-test two-tailed test on log transformed data). No significant differences were observed between the ages of PM positive vs. PM negative women; however, primigravid women with PM had significantly lower hemoglobin levels, consistent with the destruction of maternal erythrocytes associated with malarial infection (Table 2.1 and 2.2).
Figure 2.5 Primigravid women with placental malaria (PM) have increased levels of C5a in peripheral and placental plasma.

C5a levels were assayed in peripheral and placental plasma by ELISA. Samples were obtained from HIV negative primigravid women at time of delivery in Kisumu, western Kenya. C5a levels were significantly higher in (A) peripheral plasma of placenta malaria positive (PM+, n=21) vs. placenta malaria negative (PM-, n=45) women, *p=0.01 by two-tailed Student’s t-test on log transformed data; and (B) in placenta plasma of placenta malaria positive (PM+, n=24) vs. placenta malaria negative (PM-, n=47) women, *p=0.0264 by Student’s t-test two-tailed test on log transformed data.
2.5 DISCUSSION

This study provides the first evidence implicating excessive activation of the complement component C5 and the induction of anti-angiogenic factors in the pathogenesis of PM. We demonstrate that PEs and parasite products (PfGPI) activated C5 and induced C5a receptor expression (Figure 2.1). Further, C5a and PfGPI induced synergistic production of inflammatory cytokines, chemokines and an anti-angiogenic factor associated with adverse pregnancy outcomes (Figures 2.2, 2.3 & 2.4). A role for C5a-C5aR was confirmed by inhibiting inflammation with C5aR blockade (Figures 2.3 & 2.4). These findings were extended to the in vivo setting, where primigravid women with PM were shown to have elevated C5a levels in both peripheral and placental plasma (Figure 2.5).

The generation of C5a is normally tightly controlled and, under physiological conditions, C5a enhances the effector function of macrophages and neutrophils, contributing to effective innate responses to microbial pathogens (Guo and Ward 2005). However, detectable systemic C5a suggests a loss of regulation of complement activation. Elevated C5a levels are associated with a number of deleterious impacts on host innate defence, including defects in phagocyte and endothelial cell function, potentiated chemokine and cytokine secretion to bacterial components such as LPS, and lymphoid apoptosis (reviewed in (Ward 2004; Guo and Ward 2005)).

There are several mechanisms by which malaria may induce excessive complement activation, thereby contributing to the pathophysiologic mechanisms underlying PM. We observed that mature stage PEs are capable of generating C5a and a bioactive product of parasite rupture, PfGPI can result in an increase in monocytes expressing the C5aR. These observations suggest the potential for an autocrine loop, resulting in augmented inflammatory and angiogenic responses that may act in concert to mediate or amplify placental and fetal injury.

Our observations of malaria-induced C5a are consistent with studies of severe malaria and experimental malaria challenge models that report activation of the complement system (Yamada, Steketee et al. 1989; Wenisch, Spitzauer et al. 1997; Roestenberg, McCall et al. 2007). In experimental human malaria infection, complement activation was observed prior to microscopic detection of parasitemia (Roestenberg, McCall et al. 2007). Similarly, a recent study
of *P. berghei* ANKA implicated C5a as an early mediator of experimental cerebral malaria (Patel, Berghout et al. 2008). In the latter study, C5a levels were elevated prior to the onset of symptoms suggesting a role for C5a as an initiating or amplifying factor in malaria pathogenesis. Our observations are further supported by the findings of a recent genome-wide analysis of PM, where selected complement genes as well as C5aR were shown to be upregulated (Muehlenbachs, Fried et al. 2007).

During sepsis the excess activation of C5 has been associated with enhanced secretion of proinflammatory mediators in response to TLR ligands such as LPS (Riedemann, Guo et al. 2004). Similarly *PfGPI* has been shown to induce inflammatory mediators in a TLR2-dependant manner (Krishnegowda, Hajjar et al. 2005; Patel, Lu et al. 2007). Additional studies will be needed to determine if the synergistic induction of inflammatory and angiogenic factors observed with *PfGPI*-C5a are also TLR-dependent.

In our *in vitro* model, C5aR blockade abrogated the potentiated inflammatory responses that are believed to be central to the pathogenesis of PM. However, a different response was observed for IL-10. While IL-10 production was reduced by C5aR blockade, the levels did not return to those observed with *PfGPI* alone. We speculate that this may be attributable to the expression of the alternate C5a receptor, C5L2, which has been shown to promote T_{H2} skewing (Hawlisch, Wills-Karp et al. 2004) as well as have inflammatory effects in experimental sepsis (Rittirsch, Flierl et al. 2008). Future studies will be required to investigate the role of *PfGPI* and other parasite products on the expression of C5L2. When cells were treated with *PfGPI* and C5a, IL-10 was induced earlier than with *PfGPI* alone (Figure 2.3D). This may have important implications *in vivo* since TGF-β and IL-10 regulate inflammatory responses. Early IL-10 may impair T_{H1}-mediated parasite clearance during malaria infection and increased IL-10 levels have been reported in PM (Kabyemela, Muehlenbachs et al. 2008).

Monocyte infiltration into the placental intervillous space has been associated with poor birth outcomes including the risk of low birth weight infants (Leopardi, Naughten et al. 1996; Ordi, Ismail et al. 1998; Moore, Nahlen et al. 1999; Menendez, Ordi et al. 2000; Rogerson, Pollina et al. 2003). Chemokines function as specific chemoattractants for leukocytes. IL-8 plays a role in neutrophil and monocyte migration, while the β-chemokines are involved in macrophage migration. High levels of macrophage-derived IL-8 during PM have been associated with IUGR
(Moormann, Sullivan et al. 1999). Elevated levels of MIP-1α, MIP-1β and MCP-1 have been observed in PM and it is likely that these chemokines are derived from both maternal (leukocyte) (Abrams, Brown et al. 2003; Suguitan, Leke et al. 2003) and fetal (syncytiotrophoblast) sources (Lucchi, Koopman et al. 2006; Lucchi and Moore 2007). As with cytokines, C5a and PfGPI synergistically induced chemokines, and this effect was abrogated for the β-chemokines by blocking the C5aR (Figure 2.4 A-D). No significant difference for IL-8 production was found between cells treated with anti-C5aR versus an isotype control due to the variation in the latter group. However, there was no difference between cells treated with PfGPI alone or with anti-C5aR plus C5a and PfGPI, indicating that blocking the C5aR inhibits the effect attributable to C5a.

Peak prevalence of malaria infection during pregnancy occurs between 13-20 weeks gestation and gradually falls as gestation increases (Brabin 1983). This coincides with the second wave of trophoblast invasion of the maternal spiral arteries (16-18 weeks). It is hypothesized that PM may impair transformation of the maternal vasculature leading to placental dysfunction (Dorman, Shulman et al. 2002; Jansson and Powell 2007). One protein that may play a role in impaired angiogenesis is the anti-angiogenic factor sFlt-1. This alternatively spliced soluble variant of VEGFR-1 binds and sequesters placental growth factor and vascular endothelial growth factor. Excess sFlt-1 has been shown to inhibit placental cytotrophoblast differentiation and invasion (Charnock-Jones and Burton 2000). Increased sFlt-1 levels have been implicated in the pathogenesis of placentation associated with preeclampsia and IUGR (Zhou, McMaster et al. 2002; Levine, Maynard et al. 2004; Lam, Lim et al. 2005) and have been shown to predict the development of preeclampsia (Lam, Lim et al. 2005). sFlt-1 can be produced extra-placentally by monocytes treated with C5a (Girardi, Yarilin et al. 2006). Therefore, we determined whether C5a and PfGPI would potentiate sFlt-1 production from mononuclear cells. We show that sFlt-1 was synergistically induced by co-stimulation with these molecules and that this effect was reduced but not consistently inhibited by C5aR blockade (Figure 2.4E). We postulate this may be due to different PBMC donors, as FLTI genotype has been associated with differing sFlt-1 levels in the peripheral blood of primigravid women, as well as PBMC responsiveness to LPS in vitro (Muehlenbachs, Fried et al. 2008). Furthermore, the study by Muehlenbachs et al., provides the first evidence that both fetal and maternal genotype may contribute to immune responses to PM
(Muehlenbachs, Fried et al. 2008). Taken together, these data suggest a putative mechanism for impaired angiogenesis and placental dysfunction during PM.

In order to extend these observations to human infection, we measured C5a levels in the peripheral blood of a cohort of malaria-exposed primigravid Kenyan women and we measured C5a levels in the placental blood of a similar cohort of malaria-exposed primigravid women at time of delivery (Table 2.1-2) Significantly increased levels of C5a were found in peripheral blood (Figure 2.5A) and the placental blood (Figure 2.5B) of pregnant women with PM compared to those without. These results provide the first evidence that C5a levels are elevated in women with PM. However, these findings will need to be confirmed in larger prospective clinical studies. It will be of interest to also examine how C5a levels change with respect to other disease parameters, including parasitemia and mononuclear cell infiltrates in larger populations.

In summary, we have demonstrated a role for C5a in the enhanced cytokine and chemokine responses that characterize PM and in the induction of sFlt-1, an anti-angiogenic factor associated with abnormal placental development and poor birth outcomes (Figure 2.6). Our findings that disruption of the C5a-C5aR interaction inhibits inflammatory and anti-angiogenic responses to malaria suggest anti-C5a-C5aR strategies as potential therapeutic approaches for PM (Hillmen, Young et al. 2006).
Figure 2.6 Proposed mechanisms for C5a-mediated dysregulation of the placental environment in PM.

Mature schizonts activate C5 and rupture releasing parasite components containing GPI that induce expression of C5aR and activate macrophages. The concomitant expression of C5aR and generation of C5a represent a potential autocrine mechanism by which C5a can amplify inflammation in *P. falciparum* malaria resulting in the excessive cytokine and chemokine responses that characterize PM. The dysregulated production of cytokines, chemokines and the anti-angiogenic factor sFlt-1 contribute to placental dysfunction, intrauterine growth restriction and low birth weight infants.
Table 2.1: Characteristics of women tested for C5a in peripheral blood

<table>
<thead>
<tr>
<th></th>
<th>Age mean ± SD</th>
<th>Hemoglobin mean ± SD</th>
<th>% Parasitemia</th>
<th>Hemozoin (% Score)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Peripheral mean ± SD</td>
<td>Placental mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Control (n=47)</td>
<td>19.04 ± 2.64</td>
<td>11.89 ± 2.92</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Malaria (n=24)</td>
<td>18.48 ± 2.48</td>
<td>9.95 ± 2.37†</td>
<td>1.54 ± 1.90</td>
<td>5.70 ± 10.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 ± 0.00</td>
<td>7.07 ± 8.47</td>
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Abbreviations: Hemoglobin (g/dL), % parasitemia (% infected: non-infected erythrocytes), Hemozoin % Score (# pigment containing WBC/ 300WBC)

† p<0.001 (Student’s t-test)

Table 2.2: Characteristics of women tested for C5a in placental blood

<table>
<thead>
<tr>
<th></th>
<th>Age mean ± SD</th>
<th>Hemoglobin mean ± SD</th>
<th>% Parasitemia</th>
<th>Hemozoin (% Score)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peripheral mean ± SD</td>
<td>Placental mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Control (n=45)</td>
<td>18.58 ± 2.74</td>
<td>12.36 ± 2.75</td>
<td>NA</td>
<td>0.05 ± 0.34</td>
</tr>
<tr>
<td>Malaria (n=21)</td>
<td>18.13 ± 2.03</td>
<td>10.36 ± 3.09†</td>
<td>1.41 ± 1.79</td>
<td>7.95 ± 11.98</td>
</tr>
</tbody>
</table>

Abbreviations: Hemoglobin (g/dL), % parasitemia (% infected: non-infected erythrocytes), Hemozoin % Score (# pigment containing WBC/ 300WBC)

† p<0.001 (Student’s t-test)
Chapter 3

Complement Activation, Placental Vascular Insufficiency and Fetal Growth Restriction in Placental Malaria

3.1 ABSTRACT

3.1.1 Background

Placental malaria is a major cause of fetal growth restriction and low birth weight, yet the underlying mechanism is unclear. We hypothesized that excessive activation of complement component C5 alters the expression of angiogenic factors important for placental remodeling.

3.1.2 Methods

We conducted a case-control study of 492 Malawian women with placental malaria, and analyzed the relationship of plasma levels of C5a and angiogenic factors to birth outcomes. These data were examined by structural equation modeling to test the hypothesis that C5a initiates angiogenic dysregulation and fetal growth restriction. To provide direct experimental evidence that C5a mediates placental insufficiency and adverse birth outcomes, we used a model of placental malaria.

3.1.3 Results

Elevated systemic C5a levels were associated with an increased risk of delivering a small-for-gestational-age (SGA) infant (adjusted OR=2.8; 95% CI, 1.5 to 5.1). Placental C5a was significantly increased in placental malaria, was negatively correlated with angiopoietin-1, and positively correlated with angiopoietin-2, soluble endoglin and vascular endothelial growth factor. Women who delivered SGA infants had altered angiogenic factor levels regardless of gravidity. A structural model indicating how C5a may mediate fetal growth restriction through angiogenic dysregulation fit the human data well [chi-square=381.2; df=282; P<0.001; rmsea=0.027 (0.019-0.033)]. In a mouse model of placental malaria, genetic or pharmacological blockade of C5a or its receptor resulted in greater fetoplacental vessel development, reduced placental vascular resistance and improved fetal growth.
3.1.4 Conclusions

These observations provide evidence that C5a drives fetal growth restriction in placental malaria through dysregulation of angiogenic factors and placental vascular insufficiency.
3.2 INTRODUCTION

Over 125 million pregnant women are at risk of malaria infection every year (Dellicour, Tatem et al. 2010). A *Plasmodium falciparum* infection in pregnancy doubles the risk of delivering a child that is low birth weight (LBW) (Desai, ter Kuile et al. 2007). Malaria-related LBW is responsible for up to 200,000 infant deaths each year (Guyatt and Snow 2001). Sequestration of infected erythrocytes and accumulation of mononuclear cells in the placental intervillous space, are histopathological hallmarks of placental malaria associated with LBW (Rogerson, Pollina et al. 2003). Chemokines that promote monocyte infiltration and inflammatory cytokines secreted by activated macrophages have been associated with malaria-related LBW (Fried, Muga et al. 1998; Abrams, Brown et al. 2003; Suguitan, Leke et al. 2003). However, the precise mechanism by which parasite accumulation and placental inflammation result in fetal growth restriction and LBW remains poorly understood.

The complement system is a central component of innate host defense and is activated in malaria-infected non-pregnant individuals (reviewed in (Silver, Higgins et al. 2010)). Multiple complement activation pathways converge upon cleavage of component C5 to C5a, which has recognized roles in induction of inflammation and initiation of acquired immune response (Guo and Ward 2005). Levels of maternal plasma C5a and placental mRNA encoding the C5a receptor, C5aR, are increased with placental malaria infection (Muehlenbachs, Fried et al. 2007; Conroy, Serghides et al. 2009). C5a has been shown to influence angiogenesis (Langer, Chung et al. 2010) and has been implicated as a mediator of poor fetal outcomes in non-infection-based animal models of fetal loss and growth restriction (Girardi, Yarilin et al. 2006). Based on these observations we hypothesized that C5a may cause fetal growth restriction associated with placental malaria by creating an imbalance in the angiogenic factors required for normal placental vascular development.

3.3 METHODS

3.3.1 Participants and Specimens

Between 2001-2006, pregnant women delivering a live singleton newborn at Queen Elizabeth Central Hospital, Blantyre, Malawi were recruited into a case-control study. Cases were defined
by the presence of *P. falciparum* asexual parasites in the placental blood, as assessed by smear. For each case, two age (±2 years) and gravidity-matched controls negative for malaria parasites by both peripheral and placental smear were enrolled. The study was approved by the ethics committee of The College of Medicine, Blantyre, Malawi, and written informed consent was obtained from all participants.

At delivery, birth weight was recorded, gestational age assessed (Ballard, Novak et al. 1979), and maternal peripheral and placental EDTA plasma samples were collected and stored at -80°C. The following were considered adverse birth outcomes: low birth weight (LBW, <2500g), preterm delivery (<37 weeks of gestation) and fetal growth restriction, defined as small-for-gestational-age (SGA, <10th percentile for growth in sub-Saharan African populations (Landis, Ananth et al. 2009)). SGA accounts for ~50% of LBW outcomes in placental malaria (Steketee, Wirima et al. 1996; Umbers, Aitken et al. 2011).

### 3.3.2 Mouse Model of Placental Malaria

Eight- to ten-week-old BALB/c mice (wild type or C5aR−/−) were obtained from Jackson Laboratories (Bar Harbor, ME). Cryopreserved *P. berghei* ANKA (MR4; Manassas, VA) was thawed and passaged through male BALB/c mice. Naturally mated pregnant mice were infected on gestational day (G)13 with 10⁶ *P. berghei*-infected erythrocytes in RPMI medium via injection into the lateral tail vein. Control (uninfected) pregnant mice were injected with the same volume of RPMI medium alone. Parasitemia was monitored daily by thin blood smear stained with modified Giemsa stain (Protocol Hema3 Stain Set, Sigma, Oakville, ON). For blocking experiments, polyclonal rabbit antiserum raised against C5aR, polyclonal rabbit antiserum raised against C5L2, or polyclonal goat antiserum raised against rat C5a (Riedemann et al. *J Clin Invest* 2002), or preimmune control serum (Sigma) was administered in two 250μL doses, at 2-3 hours prior to malaria infection and 3 days post infection, via injection into the lateral tail vein.

### 3.3.3 Fetal Outcomes

Pregnant female mice were euthanized by CO₂ on G19 (i.e., 6 days post infection/control injection). Yolk sacs were dissected from uteri, fetuses were removed and weighed, and placenta was snap frozen and stored at -80 °C until analyzed. Fetal viability was determined by
assessing pedal withdrawal reflex. Non-viable fetuses (i.e., lacking the pedal withdrawal reflex) were considered aborted.

### 3.3.4 Placental Transcript Analysis

RNA was extracted from snap-frozen placentas after homogenization in TRIzol (1 mL/100 mg tissue; Invitrogen, Burlington, ON) according to manufacturer’s protocol. Extracted RNA (2 μg per sample) was treated with DNase I (Ambion, Streetsville, ON) and reverse transcribed to cDNA with SuperScript III (Invitrogen, Burlington, ON) in the presence of oligo (dT)18 primers (Fermentas, Burlington, ON). Residual RNA was degraded with RNase H (Invitrogen, Burlington, ON). Sample cDNA was amplified in triplicate with SYBR Green master mix (Roche, Laval, QC) in the presence of 1 μM both forward and reverse primers in a Light Cycler 480 (Roche, Laval, QC). Transcript number was calculated based on Ct as compared to a standard curve of mouse genomic DNA included on each plate by Light Cycler 480 software (Roche, Laval, QC), and normalized to geometric average of GAPDH and HPRT expression levels. Primer sequences (5’–3’):

- **Ang-1:** F- cctctgttgaatattggcttggga, R- agcatgtactgcctgactgttg
- **Ang-2:** F- agagtactgctggccatagtt, R- ttccagttcctgctggttctt
- **sFlt-1:** F- aggtgagcactgcggca, R- atgagtcctttaatgtttgac
- **VEGF-A:** F- catcttaacggcctgctgtg, R- actccagggtctctggttaca
- **C5aR:** F- atcctgtgctgctgctcactacattg; R- tctgacaccagatgctgccagaca
- **GAPDH:** F- tccaagcaactccactcttcca, R- ttgctaggagacacagccagcc
- **HPRT:** F- ggagctctgtgagttgctgca, R- gggacgcaacttgcaattctta

### 3.3.5 Fetoplacental Vasculature Analysis

In preparation for micro-CT scanning a radio-opaque silicone rubber contrast agent (Microfil; Flow Technology, Carver, MA) was perfused via the umbilical artery using methods previously described. {Rennie, 2011 #113} Briefly, uteri were collected from pregnant mice at G18 into ice-cold PBS. Fetuses were individually exposed and warmed with saline to initiate cardiac function and placental blood flow. Placentas were perfused (2% xylocaine, 100 IU/mL heparin in saline) via umbilical artery cannulation. Contrast agent was perfused until it reached the capillaries at which point the umbilical vessels were tied off to maintain pressure. Perfused placentas were left
to set for at least one hour then separated from the fetuses, fixed in 10% phosphate-buffered formalin and mounted in 1% agar in 10% phosphate-buffered formalin. Mounted placentas were scanned with a MS-9 micro-CT scanner (GE Medical Systems, London, ON) Amira visualization software (TGS, Berlin, Germany) was used to obtain measurements of vascular volume, surface area, span, depth, and umbilical artery diameter from isosurface renderings. To obtain information on vascular geometry and resistance, datasets underwent an automated vascular segmentation process, as previously described (Rennie, Detmar et al. 2011), which generates a tubular model of the data for which the lengths, diameters, and connectivity of each vessel segment are known, and from which resistance can be calculated. Analysis was performed on placentas from wild type ($n_{uninfected} = 7$ from 4 litters; $n_{infected} = 7$ from 4 litters) and C5aR$^{-/-}$ ($n_{uninfected} = 5$ from 3 litters; $n_{infected} = 8$ from 3 litters) mice. One dataset (C5aR$^{1/}$ uninfected) for which the umbilical vessel was not present due to the umbilical cord being tied off too close to the chorionic plate during the perfusion process was eliminated from hemodynamic modeling analyses.

### 3.3.6 Biomarker ELISAs

C5a, angiopoietin (Ang)-1, Ang-2, soluble Tie-2 (sTie-2), soluble endoglin (sEng), vascular endothelial growth factor (VEGF), and soluble Fms-like tyrosine kinase-1 (sFlt-1) levels (DuoSets) and C3a [354113 and purified polyclonal goat IgG] (R&D Systems, Minneapolis, MN) were measured by ELISA (Conroy, Phiri et al. 2010). All samples were tested consecutively, on the first thaw, with the investigator blinded to group and outcome.

### 3.3.7 Statistical Analysis

Continuous variables were analyzed by Mann-Whitney U test unless noted (SPSS). Categorical data were analyzed by Pearson’s chi-square test or Fisher’s exact test, as appropriate. Adjusted odds ratios were obtained using logistic regression. In order to test our hypothesis within a multivariate framework, we employed structural equation modeling (AMOS) to simultaneously examine the relationships between multiple dependent and independent variables (Calis, Phiri et al. 2008), as well as latent concepts and their multiple indicators. Model estimates were generated using maximum-likelihood estimation and fitness was assessed via the likelihood ratio test statistic (Byrne 2010).
3.4 RESULTS

3.4.1 A CASE-CONTROL STUDY OF PLACENTAL MALARIA IN MALAWI

3.4.1.1 Placental Malaria Infection is Associated with Poor Birth Outcomes

We enrolled 606 women at delivery, of whom 492 had live birth outcomes, placental histological examination, available plasma samples and were eligible for the study (Figure 3.1). The majority (n=305) of women were primigravidae.

Consistent with previous reports (Ismail, Ordi et al. 2000; Rogerson, Pollina et al. 2003), women with peripheral parasitemia were more likely to be febrile, anemic and have histopathological evidence of acute or chronic placental malaria infection (Table 3.1). Placental malaria infection was associated with increased risk of LBW (adjusted odds ratio, 2.0; 95% confidence interval (CI), 1.2-3.5) following adjustment for age and gravidity. Primigravidae with placental malaria had a median 200 g reduction in birth weight as compared to primigravidae without placental malaria (P=0.001).
The flow chart in panel A shows the samples included in our study as cases (women who were positive for *Plasmodium falciparum* malaria by smear) or controls (negative for malaria parasites by smear). Panel B illustrates the breakdown of birth outcomes as low birth weight (<2500g), preterm (<37 weeks gestation), or small-for-gestational-age (SGA, <10th centile for growth).

**Figure 3.1 Description of Study Population and Birth Outcomes**
Table 3.1 Characteristics Associated with Placental Malaria and Small-for-Gestational-Age Infants in Primigravidae and Multigravidae

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Placental Malaria †</th>
<th>Small-for-Gestational-Age‡</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Primigravidae</td>
<td>Multigravidae</td>
</tr>
<tr>
<td></td>
<td>Yes (n=92)</td>
<td>No (n=213)</td>
</tr>
<tr>
<td>Age- years</td>
<td>18 (17-20)</td>
<td>19 (18-20)</td>
</tr>
<tr>
<td>Height- cm</td>
<td>154 (150-157)</td>
<td>154 (150-158)</td>
</tr>
<tr>
<td>Fetal Sex- no. (%)</td>
<td>60.9</td>
<td>47.1</td>
</tr>
<tr>
<td>Weight of Baby- g</td>
<td>2700 (2500-3000)</td>
<td>2900 (2600-3200)</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin- g/dL</td>
<td>10.9 (9.6-12.0)</td>
<td>12.5 (11.0-13.7)</td>
</tr>
<tr>
<td>Temperature- °C</td>
<td>37.0 (36.2-37.4)</td>
<td>36.2 (36.0-36.6)</td>
</tr>
<tr>
<td>Blood pressure &gt;140/90- no. (%)</td>
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<td>2.8</td>
</tr>
<tr>
<td>Febrile symptoms a</td>
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<td>5.6</td>
</tr>
<tr>
<td>Histological</td>
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<tr>
<td>Monocyte Count</td>
<td>30 (12-47)</td>
<td>4 (0-15)</td>
</tr>
<tr>
<td>Monocyte Pigment Score b</td>
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</tr>
<tr>
<td>Fibrin Pigment Score</td>
<td>0 (0-2)</td>
<td>1 (0-3)</td>
</tr>
<tr>
<td>----------------------</td>
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<tr>
<td><strong>Placental markers of complement activation and angiogenesis</strong></td>
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</tr>
<tr>
<td>C5a–ng/mL</td>
<td>85.7</td>
<td>71.3</td>
</tr>
<tr>
<td>64.5- (52.0- 111.3)</td>
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</tr>
<tr>
<td>C3a–µg/mL</td>
<td>3.3</td>
<td>3.8</td>
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<tr>
<td>(2.3-5.5-2.5-6.5)</td>
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<tr>
<td>Angiopoietin-1–ng/mL</td>
<td>28.2</td>
<td>36.6</td>
</tr>
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<td>(17.4- (22.2-49.4)</td>
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<tr>
<td>Angiopoietin-2–ng/mL</td>
<td>15.2</td>
<td>11.8</td>
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<td>(8.3- (6.5-31.2)</td>
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<tr>
<td>sTie-2–ng/mL</td>
<td>38.1</td>
<td>35.7</td>
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<tr>
<td>(30.8- (29.5-45.6)</td>
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<tr>
<td>Endoglin–ng/mL</td>
<td>64.5</td>
<td>47.2</td>
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<td>(36.2- (32.2-122.2)</td>
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<tr>
<td>sFlt-1–ng/mL</td>
<td>144.2</td>
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<td>(46.3- (40.8-244.4)</td>
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<tr>
<td>VEGF–pg/mL</td>
<td>82.5</td>
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</tbody>
</table>

Placental markers measured by ELISA on placental EDTA plasma collected at delivery. Values represented as median (interquartile range). Dichotomous variables analyzed using Pearson chi-square; continuous variables analyzed using Mann-Whitney U test (non-parametric).

† Defined as a positive placental smear
3.4.1.2 Elevated C5a Levels are Associated with Fetal Growth Restriction

There are no published longitudinal data on maternal C5a levels over the course of healthy or growth-restricted pregnancies. One cross-sectional study found an association of increased C5a with preeclampsia, with or without growth restriction (Soto, Romero et al. 2010). We measured and categorized C5a levels as elevated (≥100 ng/mL) or normal (<100 ng/mL) and compared them to birth outcome. A cut-off of 100 ng/mL (~10 nM) was used because above this concentration, C5a can synergistically induce inflammation, cause monocyte release of the anti-angiogenic factor sFlt-1 in the presence of parasite factors (Conroy, Serghides et al. 2009), and alter coagulation and white blood cell function (Ward 2004). Elevated levels of C5a in the maternal peripheral blood were associated with an increased risk of delivering a SGA infant following adjustment for maternal age and gravidity (adjusted odds ratio, 2.8; 95% CI, 1.5-5.1).

3.4.1.3 C5a and Angiogenic Factors are Altered in Placental Malaria

The median placental levels of C5a were also higher in both primigravidae (P<0.001) and multigravidae (P=0.002; Table 3.1) with placental malaria than in those without. Median angiopoietin (Ang)-1 levels in both primigravid (P=0.006) and multigravid (P=0.024) cases were lower than in controls. Conversely, there were higher median levels of Ang-2, sFlt-1 and soluble endoglin (sEng) in primigravidae (Ang-2, P=0.039; sFlt-1, P=0.045; sEng, P=0.057) and multigravidae (Ang-2, P=0.011; sFlt-1, P=0.020; sEng, P=0.001) with placental malaria than in controls.

In primigravidae, C5a levels were negatively correlated with Ang-1 (Spearman’s rho, -0.27; P<0.001) and positively correlated with Ang-2 (0.38; P<0.001), sEng (0.46; P<0.001), vascular endothelial growth factor (VEGF; 0.32; P<0.001) and sFlt-1 (0.42; P<0.001). Multigravidae showed the same relationships between C5a and angiogenic factors, except for sFlt-1, where the correlation was weaker (Spearman’s rho, 0.19; P=0.009).
3.4.1.4 Dysregulated Placental Angiogenic Factors and Fetal Growth Restriction

Ang-1 levels were lower in women who delivered SGA infants than those who delivered normal-for-gestational-age infants (P=0.043). Additionally, Ang-2, sEng and sFlt-1 were significantly increased in women who delivered SGA infants compared to those who delivered normal-for-gestational-age infants (Table 3.1). A trend towards increased VEGF was observed in primigravidae and multigravidae delivering SGA infants (P=0.076 and P=0.073, respectively).

3.4.1.5 Structural Equation Modeling Supports a Role for C5a in Initiating Dysregulated Angiogenesis and Fetal Growth Restriction in Placental Malaria

We applied structural equation modeling to test our hypothesis that C5a mediates fetal growth restriction by altering the expression of angiogenic factors in the placenta (Figure 3.2). Based on the proposed pathogenesis of placental malaria (Rogerson, Hviid et al. 2007), we generated a model where each path represents a significant relationship after adjustment for all other covariates, and we were able to assess how well the model as a whole corresponds to the data using standard measures of fit. The case-control data fits a model with a sequence of events where malaria parasites sequester in the placenta and lead to the accumulation of mononuclear cells. We generated a latent variable “mononuclear cell infiltrates” composed of mononuclear cell counts and semi-quantitative assessments of monocyte and fibrin pigment that indicate the chronicity of infection (Figure 3.2A). In this model, mononuclear cell infiltration is inversely related to maternal hemoglobin levels, and maternal hemoglobin level is a major determinant of preterm delivery and fetal growth restriction. We also constructed the latent concept “dysregulated angiogenesis” to reflect several measured variables–pro-angiogenic factor Ang-2 and anti-angiogenic factors sFlt-1 and sEng–that change with similar patterns (Figure 3.2B).

A novel component of our model is the axis focusing on C5a and fetal growth restriction (Figure 3.2C). Our human data fit a model where mononuclear cells are upstream of, and contribute to, the generation of C5a, and the resultant increase in C5a initiates dysregulated angiogenic responses (Figure 3.2C). In this model C5a also modulates angiogenesis by reducing Ang-1 levels and increasing VEGF. Together, the dysregulated angiogenic factors promote fetal growth
restriction and SGA infants. The data were not consistent with a model where C5a initiates placental monocyte recruitment.

Figure 3.2 Structural Equation Model for Complement Activation, Dysregulated Placental Angiogenesis and Birth Outcomes in Primigravidae with Placental Malaria
Positive associations are indicated by black lines; inverse associations, by red lines. Standardized regression coefficients (range, -1.0 to +1.0) of factors associated with placental malaria infection and poor birth outcomes. All lines represent significant relationships (P<0.05). (A) The latent variable “Mononuclear Cell Infiltrates” is comprised of the mononuclear cell count, and a semi-quantitative score of pigmented mononuclear cells and fibrin pigment (0-none to 4-large amounts of pigment in every field at 40x objective), as assessed by histology. (B) The latent variable “Dysregulated Angiogenesis” is comprised of measures of pro-angiogenic factor Ang-2, anti-angiogenic factor sFlt-1 (which sequesters VEGF), and anti-angiogenic factor sEng. (C) A structural equation model was generated positing how malaria may contribute to adverse birth outcomes through complement activation and dysregulated angiogenesis. Risk factors for adverse outcomes in pregnancy were included in exploratory models, and through a process of trimming, were subsequently removed if insignificant (P>0.05; e.g. number of antenatal visits, number of antimalarial doses). This model includes fetal sex, maternal height, and the latent variable “febrile symptoms”, composed of self-reported fever, headache or chills one week prior to delivery (omitted from figure for clarity). All estimates were generated using maximum-likelihood estimation and expressed as chi-square and the root mean square error of approximation (rmsea), where a low value (<0.05) and a narrow confidence interval indicate a good fit. The overall fit of the model was good (chi-square=381.2; df=282, P<0.001; rmsea=0.027 (90% CI: 0.019-0.033)). We show results for primigravidae since the greatest burden of LBW occurs in this population; however multigravidae yielded a very similar model (Supplemental Fig. 3.1).
3.4.2 AN EXPERIMENTAL PLACENTAL MALARIA MODEL

3.4.2.1 Interruption of C5a-C5aR Signaling Improves Fetal Outcomes

To provide direct experimental evidence of the central role of C5a in malaria-induced fetal growth restriction, we used a mouse model that recapitulates characteristics of placental malaria in primigravidae (Neres, Marinho et al. 2008). Briefly, these include placental accumulation of parasitized erythrocytes and monocytes, and decreased fetal viability and growth (Neres, Marinho et al. 2008). Infection of naturally mated pregnant mice at gestational day (G) 13 (beginning of the third trimester of a normal 20-day mouse gestation) with the rodent malaria parasite, *Plasmodium berghei ANKA* (PbA), lead to fetal growth restriction as measured by a significant reduction in the weight of viable fetuses at G19 (Figure 3.3; Supplemental Data). PbA infection of pregnant mice was associated with an increase in placental levels of mRNA encoding the C5a receptor (C5aR, CD88) (Supplemental Figure 3.2A). Genetic deficiency of C5aR (C5aR−/−) partially rescued developing fetuses from the growth restricting effects of malaria: the mean weight of fetuses from infected C5aR−/− mice was significantly higher than that of fetuses from infected wild type (C5aR+/+) mice (P<0.001; Figure 3.3A). Additionally, C5aR deficiency increased fetal viability at G19 by 50% (P=0.021; Supplemental Figure 3.2C).

A significant improvement in fetal growth was also achieved by blocking C5a – C5aR signaling with antiserum to C5a or C5aR during malaria infection in C5aR+/+ mice (Figure 3.3B). In contrast, blocking the alternative C5a receptor, C5L2, did not further improve fetal weight in infected C5aR−/− mice (Figure 3.3B). Peripheral parasitemia was comparable in both genotypes and with all treatments (Supplemental Figure 3.2B).
Figure 3.3 C5a-C5aR Mediates Angiogenic Dysregulation, Placental Insufficiency and Fetal Growth Restriction in a Mouse Model of Placental Malaria.

In panel A, C5aR deficiency (C5aR^{-/-}) reduced the fetal growth restriction observed in viable fetuses of wild type (C5aR^{+/+}) mice infected with *P. berghei ANKA*. Viable fetuses were weighed
at gestational day (G) 19, six days after G13 pregnant mice received an injection of medium alone (uninfected) or $10^6$ *P. berghei ANKA* infected erythrocytes in medium (infected). Bars represent group medians and dots represent individual viable fetuses. ***, $P<0.001$ by Dunn’s post-test of Kruskal-Wallis analysis. 10-18 litters per group were assessed.

Panel B shows that G19 fetal growth restriction was similarly rescued with administration of C5aR or C5a antiserum to infected pregnant C5aR$^{+/+}$ mice. Blocking the alternative C5a receptor, C5L2, with antiserum did not provide any further protection against fetal growth restriction. **, $P<0.01$; ***, $P<0.001$ by Bonferonni post-test of one-way ANOVA. 3-15 litters per group were assessed.

In panel C, infected placentas associated with viable fetuses had increased expression of mRNA encoding Ang-2 and decreased expression of mRNA encoding Ang-1 in comparison to uninfected placentas (Supplemental Fig 2D). This is reflected by an overall increase of the Ang-2:Ang-1 mRNA ratio, regardless of C5aR genotype. In contrast, the ratio of VEGF to sFlt-1 mRNA copy numbers were unchanged in infected placentas associated with viable C5aR$^{+/+}$ fetuses but significantly elevated in infected C5aR$^{-/-}$ placentas. This appears to be the result of a decrease in sFlt-1 mRNA copy numbers in infected C5aR$^{-/-}$ placentas ($P=0.022$ compared to infected C5aR$^{+/+}$; Supplemental Fig 2D). **, $P<0.01$; ***, $P<0.0001$.

Panel D shows representative micro-CT imaging of G18 fetoplacental arterial vasculature from infected C5aR$^{+/+}$ or C5aR$^{-/-}$ mice color-coded by vessel diameter. Infected C5aR$^{+/+}$ placentas had increased cumulative vessel numbers when compared to either uninfected C5aR$^{-/-}$ placentas ($P<0.001$) or infected C5aR$^{+/+}$ placentas ($P<0.001$; post-test of repeated measures ANOVA). Plot depicts the number of fetoplacental arterial vessels larger than the threshold diameter. Lines represent mean and standard error of mean. This increase in vessel number in C5aR$^{-/-}$ placentas allowed for the arterial resistance to remain low with infection. 5-8 placentas from 3-4 litters were analyzed.
3.4.2.2 Placental Angiogenic Factor Transcription is Altered with Malaria Infection

Using quantitative real-time PCR, we observed a change in placental Ang transcript levels in favor of Ang-2 in infected placentas (Figure 3.3C). This increase in Ang-2/Ang-1 ratio was observed regardless of C5aR genotype.

Placental VEGF mRNA expression was also increased with infection regardless of genotype (Supplemental Figure 3.2D). In C5aR+/+ mice, however, the increase was accompanied by a proportional increase in sFlt-1 transcription, which resulted in a consistent VEGF/sFlt-1 ratio (Figure 3.3C). In contrast, C5aR deficiency resulted in a significant increase in placental VEGF/sFlt-1 ratio with infection (Figure 3.3C), mainly due to a decrease in sFlt-1 transcription in the absence of C5a-C5aR signaling (Supplemental Figure 3.2D).

3.4.2.3 C5a-C5aR Signaling Modulates Placental Vasculature During Malaria Infection

To examine the effect of infection on placental vessel structure, we imaged contrast-agent-perfused G18 fetoplacental arterial vasculature with micro-computed tomography (Rennie, Detmar et al. 2011). At G18, all fetuses were viable and those from infected mice were the same weight as those from uninfected mice (Supplemental Table 3.2). Therefore any changes in the placental vasculature we observed at this point precede the poor fetal outcomes observed beginning at G19.

Analysis of the fetoplacental arterial vasculature revealed a greater number of vessels with malaria infection (P=0.017), which was most notable in vessels of 50-100 μm diameter. Infected C5aR−/− placentas had significantly more vessels as compared to C5aR+/+ placentas (Figure 3.3D). Increased placental arterial vascular resistance was observed with infection and abrogated with C5aR deficiency (Figure 3.3D). Total length, surface area, volume and span of the fetoplacental vasculature were also increased in infected C5aR−/− placentas as compared to infected C5aR+/+ placentas (Supplemental Table 3.2).
3.5 DISCUSSION

The biological processes that differentiate healthy from pathological pregnancy outcomes remain poorly defined. Our findings provide the first evidence of a direct connection between innate responses to infection (C5 activation) and altered placental vascular remodeling that results in poor birth outcomes. A critical role for C5a in the pathogenesis of placental malaria and LBW is supported by several observations: First, we show that C5a levels are increased in women of all gravidities with placental malaria. Second, high systemic C5a levels are associated with increased odds of delivering a SGA infant. Third, structural equation modeling of data from our case-control study supports a model in which C5a initiates angiogenic dysregulation and fetal growth restriction. Finally, using a mouse model of placental malaria, we provide direct experimental evidence that C5a could achieve its effect on fetal viability and growth through its effects on placental vascular development.

Remodeling of uterine and placental vasculature is essential for normal placental function and fetal growth. Placental vascular development and remodeling are controlled primarily through the highly regulated actions of angiogenic factors from the VEGF and angiopoietin families (Geva, Ginzinger et al. 2002). Ang-1 has a major role in the stabilization and maturation of newly formed vessels, whereas Ang-2 provides the destabilizing effect necessary to initiate vascular remodeling (Yancopoulos, Davis et al. 2000). VEGF is necessary for early vessel formation whereas its soluble receptor, sFlt-1, can inhibit angiogenesis through its ability to bind VEGF and placental growth factor (Levine, Qian et al. 2006). VEGF, which is both proinflammatory and pro-angiogenic, requires tight regulation during fetal development since either high or low levels result in embryonic lethality (Carmeliet, Ferreira et al. 1996; Hiratsuka, Minowa et al. 1998; Miquerol, Langille et al. 2000). Perturbation of these angiogenic factors have also been reported in preeclampsia and fetal growth restriction associated with inadequate placental vascularization (Kaufmann, Black et al. 2003).

Placental malaria is believed to lead to placental insufficiency, a progressive deterioration in placental function and inability to sustain fetal growth, resulting in LBW infants at increased risk of perinatal mortality (Umbers, Aitken et al. 2011). Previous reports are consistent with the hypothesis that placental malaria may influence angiogenesis and vascular development. Histological and ultrasound studies of placental malaria suggest that malaria infection alters the
placental vascular structure (Dorman, Shulman et al. 2002; Leke 2002; Arbeille, Carles et al. 2003). Altered levels of angiopoietins, VEGF, sFlt-1 or C5a have been shown in several populations of African women with placental malaria at delivery (Conroy, Serghides et al. 2009; Silver, Zhong et al. 2010). However, it has been unclear whether these changes are a cause or consequence of fetal growth restriction, and, if the former, where they are positioned in the sequence of events leading to LBW.

Structural equation modeling provides an opportunity to model putative hierarchal relationships in complex datasets. In our case-control study, we observed a shift in angiopoietin levels favoring the pro-angiogenic Ang-2, which supports the premise that malaria induces angiogenic remodeling of the placenta. At the same time, C5a-C5aR signaling can inhibit angiogenesis by inducing sFlt-1 (Girardi, Yarilin et al. 2006; Langer, Chung et al. 2010). In this study, increased protein levels of anti-angiogenic factor sFlt-1 correlated with elevations in C5a in all gravidae with placental malaria and in primigravidae who delivered SGA infants. High sFlt-1 levels may result in less free VEGF available to stimulate placental vessel growth (McKeeman, Ardill et al. 2004). Applying structural equation modeling to this case-control dataset confirmed the relationship of known risk factors for LBW, including maternal anemia, and provided a putative sequence of events linking C5a and the observed changes in placental angiogenic factors. Our data fit a model where C5 activation is an initiating event upstream of altered angiogenic factors including angiopoietins, VEGF and sFlt-1, which then contribute to SGA and LBW infants. The model further illustrates that placental mononuclear cells might be directly cleaving C5 or amplifying C5a production in placental malaria (Huber-Lang, Sarma et al. 2006).

Direct experimental evidence to support these observations was obtained in a murine model of placental malaria. Ablation of C5a-C5aR signaling in this model was characterized by an increase in the placental ratio of VEGF/sFlt-1 mRNA levels, and enabled compensatory vascular remodeling and more optimal fetal growth and survival. These findings are in agreement with our previous report that sFlt-1 secretion in response to in vitro stimulation with malaria bioactive products is decreased when C5a-C5aR signaling is blocked by antibody treatment (Conroy, Serghides et al. 2009), and with the association of sFlt-1 levels with predicting adverse birth outcomes in placental malaria and preeclampsia (Levine, Maynard et al. 2004; Levine, Lam et al. 2006; Muehlenbachs, Fried et al. 2008). Moreover, we show upregulation of C5aR in infected
placentas, similar to findings in placental malaria in Tanzanian women (Muehlenbachs, Fried et al. 2007). While blocking C5a-C5aR interactions improved fetal growth, blockade of C5L2 did not improve fetal outcome beyond that of C5aR deficiency, suggesting that this alternative C5a receptor (Ward 2009; Bamberg, Mackay et al. 2010) is not involved in mediating pathogenic responses in the mouse model of placental malaria.

Collectively our findings suggest that in placental malaria, C5a dysregulates angiogenic factors, counteracts angiopoietin-mediated attempts to compensate for placental malaria and inflammation (Conroy, McDonald et al. 2011; Umbers, Aitken et al. 2011), and contributes to fetal growth restriction through functional placental vascular insufficiency.

Like other evolutionarily ancient systems, the complement system has diverse effector functions. Through C5a production, the complement system can rapidly amplify an inflammatory response. The association of inflammation with poor birth outcomes has been well described in placental malaria (Rogerson, Brown et al. 2003; Rogerson, Pollina et al. 2003; Umbers, Aitken et al. 2011). Our findings do not exclude a contribution of inflammation in adverse outcomes of placental malaria, but rather suggest that inflammatory and angiogenic pathways may mediate deleterious outcomes through a shared component, C5a.

The link we have established between C5a, angiogenic factor dysregulation and fetal growth restriction in placental malaria suggests potential biomarkers to predict women at risk of adverse birth outcomes, such as LBW, and new targets for intervention to prevent these complications.
Supplemental Figure 3.1 Structural Equation Model for Complement Activation, Dysregulated Placental Angiogenesis and Birth Outcomes in Multigravidae with Placental Malaria

Standardized regression coefficients (range, -1.0 to +1.0) of factors associated with placental malaria infection and poor birth outcomes in multigravidae. Positive associations are indicated by black lines; inverse associations, by red lines. All lines represent significant relationships (P<0.05). This model includes fetal sex, maternal height, and the latent variable “febrile symptoms”, composed of self-reported fever, headache or chills one week prior to delivery (omitted from figure for clarity). All estimates were generated using maximum-likelihood estimation. The overall fit of the model was good (chi-square=381.2; df=282, P<0.001; rmsea=0.027 (90% CI: 0.019-0.033)).
Supplemental Figure 3.2: Role of C5aR in Mouse Model of Placental Malaria.
(A) C5aR mRNA transcript levels increase in G19 placentas associated with viable fetuses of WT mice infected with *P. berghei ANKA* at G13. **, P<0.01.

(B) Parasitemia is the same between pregnant WT and C5aR\textsuperscript{−/−} mice. Parasitemia was determined by microscopy of modified Giemsa stained thin blood smear.

(C) Viability of fetuses is increased in C5aR\textsuperscript{−/−} as compared to WT mice infected with malaria. *, P=0.021.

(D) Placental mRNA transcript levels of angiogenic factors are altered with malaria and C5aR deficiency. Infection increases placental Ang-2, Ang-1 and VEGF transcript levels (2-way ANOVA P<0.05 for Ang-2, VEGF; P<0.001 for Ang-1). Placentas from infected C5aR\textsuperscript{−/−} mice have lower sFlt-1 transcript levels. *, P=0.027.

(E) Uninfected C5aR\textsuperscript{−/−} placentas have an increased number of fetoplacental arterial vessels (P<0.01, post-test of repeated measures ANOVA). Plot depicts the number of fetoplacental arterial vessels larger than the threshold diameter.
## Supplemental Table 3.1 Non-standardized Coefficients for the Structural Equation Model

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<th>Multigravidae‡</th>
</tr>
</thead>
<tbody>
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<td>Standard error</td>
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<td>Maternal hemoglobin → preterm delivery</td>
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</tr>
<tr>
<td>Preterm delivery → LBW</td>
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<tr>
<td>Maternal hemoglobin → SGA</td>
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<td>0.014</td>
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<td>Mononuclear cell infiltrates → placental C5a levels</td>
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<td>Placental C5a levels → VEGF</td>
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<td>0.001</td>
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<tr>
<td>Placental C5a → Ang-1</td>
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<td>0.038</td>
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<tr>
<td>Placental C5a → dysregulated angiogenesis</td>
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<td>0.022</td>
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<td>Ang-1 → dysregulated angiogenesis</td>
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<td>Dysregulated angiogenesis → SGA</td>
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<tr>
<td>SGA → LBW</td>
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<td>0.036</td>
</tr>
</tbody>
</table>

† Model shown in Figure 3.2
‡ Model shown in Supplemental Figure 3.1
Supplemental Table 3.2 Gross Measurements of Fetoplacental Vascular Trees from WT or C5aR−/− Gestational Day 18 Placentas Scanned by Micro-CT.

<table>
<thead>
<tr>
<th></th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>C5aR−/−</td>
</tr>
<tr>
<td>Fetus weight —mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>683</td>
<td>784</td>
</tr>
<tr>
<td></td>
<td>(591 - 774)</td>
<td>(714 - 853)</td>
</tr>
<tr>
<td>Span —mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.62</td>
<td>6.95</td>
</tr>
<tr>
<td></td>
<td>(6.46 - 6.79)</td>
<td>(6.74 - 7.16)</td>
</tr>
<tr>
<td>Depth —mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.43</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>(1.22 - 1.64)</td>
<td>(1.41 - 1.56)</td>
</tr>
<tr>
<td>Umbilical Artery Width —mm</td>
<td>0.47</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>(0.43 - 0.51)</td>
<td>(0.43 - 0.51)</td>
</tr>
<tr>
<td>Total Arterial Tree Length —mm</td>
<td>345†</td>
<td>444‡</td>
</tr>
<tr>
<td></td>
<td>(279 - 411)</td>
<td>(359 - 530)</td>
</tr>
<tr>
<td>Surface Area —mm²</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>111†</td>
<td>131‡</td>
</tr>
<tr>
<td></td>
<td>(91 - 131)</td>
<td>(115 - 148)</td>
</tr>
<tr>
<td>Volume —mm³</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.89†</td>
<td>2.14‡</td>
</tr>
<tr>
<td></td>
<td>(1.60 - 2.18)</td>
<td>(1.88 - 2.40)</td>
</tr>
</tbody>
</table>

Values presented as mean (95% CI)
* P < 0.05, ** P < 0.01; Bonferonni post-test of 2-way ANOVA compared to infected WT
† Significant (P<0.01) increase in total arterial tree length, surface area and volume due to C5aR deficiency (2-way ANOVA)
Chapter 4
Circulating Soluble Endoglin Levels in Pregnant Women in Cameroon and Malawi – Associations with Placental Malaria and Fetal Growth Restriction

4.1 ABSTRACT

4.1.1 Background

Placental infections with *Plasmodium falciparum* are associated with fetal growth restriction resulting in low birth weight (LBW). The mechanisms that mediate these effects have yet to be completely described; however, they are likely to involve inflammatory processes and dysregulation of angiogenesis. Soluble endoglin (sEng), a soluble receptor of transforming growth factor (TGF)-β previously associated with preeclampsia in pregnant women and with severe malaria in children, regulates the immune system and influences angiogenesis. We hypothesized that sEng may play a role in development of LBW associated with placental malaria (PM).

4.1.2 Methods and Findings

Plasma levels of sEng were measured in women (*i*) followed prospectively throughout pregnancy in Cameroon (n=52), and (*ii*) in a case-control study at delivery in Malawi (n=479). The relationships between sEng levels and gravidity, peripheral and placental parasitemia, gestational age, and adverse outcomes of PM including maternal anemia and LBW were determined. In the longitudinal cohort from Cameroon, 28 of 52 women (54%) experienced at least one malaria infection during pregnancy. In Malawi we enrolled two aparasitemic gravidity-matched controls for every case with PM. sEng levels varied over the course of gestation and were significantly higher in early and late gestation as compared to delivery (P<0.006 and P<0.0001, respectively). sEng levels were higher in primigravidae than multigravidae in both Cameroon and Malawi, irrespective of malarial infection status (p<0.046 and p<0.001, respectively). Peripheral parasitemia in Cameroonian women and PM in Malawian women were each associated with elevated sEng levels following correction for gestational age and gravidity (p=0.006 and p=0.033, respectively). Increased sEng was also associated with the delivery of LBW infants in
primigravid Malawian women (p=0.017); the association was with fetal growth restriction
(p=0.003) but not pre-term delivery (p=0.286).

4.1.3 Conclusions

Increased maternal systemic sEng levels are associated with *P. falciparum* infection in pregnancy
and with fetal growth restriction in primigravidae with PM.
4.2 INTRODUCTION

Low birth weight (LBW) is a well documented effect of malaria infections in pregnancy and increases the risk of death in the first two years of life (MacDorman and Atkinson 1999; Guyatt and Snow 2001). This effect is most highly associated with *Plasmodium falciparum* infections of the placenta (McGregor, Wilson et al. 1983) referred to as placental malaria (PM). Malaria infection during pregnancy also increases the risk of moderate or severe maternal anemia, which is another risk factor leading to LBW (Steketee, Nahlen et al. 2001). Since over 85 million pregnant women are at risk of *P. falciparum* infection every year (Dellicour, Tatem et al. 2010), there is a need to better understand the mediators of poor clinical outcomes associated with PM. Such information should lead to better diagnosis and treatment of mothers who are at risk of having LBW babies.

LBW resulting from malaria is associated with placental inflammation. This includes monocyte infiltration of the placental intervillous space (Menendez, Ordi et al. 2000; Rogerson, Pollina et al. 2003) and secretion of cytokines and chemokines (Fried, Muga et al. 1998; Moormann, Sullivan et al. 1999; Rogerson, Brown et al. 2003). In addition, there is a growing body of evidence that dysregulated angiogenic factors contribute to LBW in the context of PM (Muehlenbachs, Mutabingwa et al. 2006; Silver, Zhong et al. 2010). Inflammation and angiogenesis are not mutually exclusive processes, but have complementary functions in the control of infection and injury and the limitation of detrimental tissue neo-vascularization (Fiedler and Augustin 2006; Pober and Sessa 2007).

Soluble endoglin (sEng) is involved in both regulation of immune function and angiogenesis. It exerts these effects by binding and interfering with the activity of transforming growth factor (TGF)-β, which is a regulatory cytokine with multiple isoforms (e.g., TGF-β1, TGF-β3) and pleiotropic effects (Li, Wan et al. 2006). Specifically, sEng has been shown to reduce TGF-β1 bioavailability to membrane-bound TGF-β receptors and co-receptor Eng (Venkatesha, Toporsian et al. 2006). Eng is expressed on the membranes of endothelial cells in adult and fetal vessels, activated monocytes and macrophages, and placental syncytiotrophoblast (Gougos and Letarte 1988; Gougos, St Jacques et al. 1992; Lastres, Bellon et al. 1992). The syncytiotrophoblast is also a source of sEng (Venkatesha, Toporsian et al. 2006). By binding
TGF-β, sEng may inhibit the regulatory effects of TGF-β on the immune system. sEng also prevents angiogenic processes such as capillary formation and vascular permeability, and inhibits nitric oxide-mediated vasodilation (Venkatesha, Toporsian et al. 2006) – processes that could affect the placental vascularization required for efficient growth of the developing fetus.

Elevation of plasma sEng levels has been shown to precede the onset of preeclampsia (Levine, Lam et al. 2006; Venkatesha, Toporsian et al. 2006), a pathological pregnancy condition that also increases the risk of preterm birth, fetal growth restriction and LBW. Elevated concentrations of circulating sEng have been associated with increased severity of malaria infection in children in Gabon (Dietmann, Helbok et al. 2009). To date, no published studies have reported on the relationship between PM, LBW and sEng levels. The purpose of this study was to determine whether maternal sEng levels are increased with malaria in pregnancy and PM, and whether increases are associated with delivery of LBW infants. We measured maternal peripheral plasma levels of sEng in two study populations: women prospectively followed over the course of pregnancy in Cameroon, and women studied at the time of delivery in Malawi. We report that increased sEng levels are associated with malaria infection and fetal growth restriction in a gravidity-dependent manner.

## 4.3 METHODS

### 4.3.1 Ethics Statement

The prospective study in Cameroon was approved by the Institutional Review Board, Georgetown University and the National Ethical Committee, Ministry of Public Health, Cameroon. The cross-sectional study in Malawi received ethical approval from The College of Medicine Research and Ethics Committee in Blantyre, Malawi (COMREC). Informed consent was obtained from each participant of both studies.

### 4.3.2 Participants and Specimens

During 2001-2004, a cohort of pregnant women was prospectively followed over the course of pregnancy in Yaoundé and Ngali II, Cameroon (Thevenon, Leke et al. 2009; Leke, Bioga et al. 2010). The transmission of *P. falciparum* in Yaoundé is low with ~13 infectious bites / person / year (Manga, Robert et al. 1992). Ngali II, which is 30km north-east of Yaoundé, is
hyperendemic for malaria with approximately 265 infectious bites per person per year (Thevenon, Leke et al. 2009) (Leke, Bioga et al. 2010). Last menstrual period was used to calculate gestational age at enrolment. Peripheral plasma samples were collected; thin and thick blood smears were prepared and examined for the presence of *P. falciparum* at monthly visits (up to seven visits per woman) spanning all three trimesters of pregnancy. Women who were blood-smear positive for malaria were prescribed antimalarial treatment according to the Cameroon Ministry of Health’s policy. Peripheral plasma samples, thin and thick blood smears, intervillous space (IVS) blood and placental impression smears were collected at delivery, and infant weight was recorded. Levels of sEng were measured in samples from all study participants who satisfied the following inclusion criteria: primigravidae (first pregnancy) or multigravidae (≥3 pregnancies) with live singleton birth, minimum of three peripheral plasma samples from different gestation points available for testing, and PM status determined at delivery. Participants infected with *Plasmodium* species other than *P. falciparum* were not included in this study.

To further evaluate the association of sEng levels with PM, and with poor clinical outcomes of PM, a second study population was also tested. Between 2001 and 2006, pregnant women who attended the Queen Elizabeth Central Hospital (QECH) were invited to participate in the study following delivery of a live singleton newborn. QECH is a referral hospital in urban Blantyre where malaria transmission is seasonal with an average of 1 infective bite per person per year (Calis, Phiri et al. 2008). Cases were defined by the presence of *P. falciparum* asexual parasites present in the placental blood, as assessed by smear. For each case, two controls that were equal in age (±2 years) and gravidity, and were negative for malaria parasites by both peripheral and placental smear, were enrolled. All gravidities were included in this study population with multigravidae being defined as more than one prior pregnancy. Peripheral EDTA plasma samples were collected and stored at -80°C until testing.

Poor clinical outcomes include LBW (<2500 g), anemia (<11 g Hb/dL), growth restriction (defined as small-for-gestational-age: <10th centile for growth, after (Landis, Ananth et al. 2009)), and preterm birth (<37 weeks gestation).
4.3.3 Plasma sEng Measurements

Maternal peripheral plasma concentrations of sEng (sEng) were measured by ELISA with a commercially available human sEng kit (R&D Systems, Minneapolis, MN). All samples were tested with the investigators blinded to group and outcome.

4.3.4 Statistical Analysis

Continuous variables are reported as median [interquartile range (IQR)] and analyzed by Mann-Whitney U test. Categorical data were analyzed by Pearson’s chi-square test or Fisher’s exact test, as appropriate. A mixed linear multivariate model was applied to analyze the prospective study, with gestational age, gravidity, presence of peripheral parasites, and placental malaria infection as fixed variables, and study participant as a random variable. End of gestation (delivery), primigravidae, absence of peripheral parasitemia and absence of placental malaria infection were set as reference points for the fixed variables, respectively. Two-factor effects of log-transformed data (normalized) were analyzed by 2-way ANOVA; between-group effects were analyzed by Bonferroni post-tests. Correlations between continuous variables were analyzed by Spearman’s correlation or partial correlation on log-transformed data to control for the effect of additional variables. All P values are two-sided. Statistical analyses were performed using Prism 4.03 (GraphPad Software, La Jolla, CA) or SPSS (Chicago, IL).

4.4 RESULTS

4.4.1 Characteristics of Study Populations

Characteristics of the prospective study population are listed in Table 4.1, and illustrated in Figure 4.1. Of 52 women who satisfied the criteria for the Cameroon study, 22 were primigravid and 30 were multigravid. As expected, multigravidae were older and delivered larger infants than primigravidae (Table 4.1). No difference was observed between gravidities in the gestational age of participants at enrolment, the number of antenatal visits, or the gestational age at delivery. Malaria infections were detected at least once (median [IQR] = 2 [1-3] times) in gestation (median [IQR] = 6 [5.5-7] prenatal visits) or at delivery in 28 women, 15 primigravidae and 13 multigravidae. 40% of the malaria infections were detected after 30 weeks of gestation, when the fetal growth velocity is at its highest (Rogerson Am J Trop Med Hyg 2007). The other 24 participants were blood-smear negative at all prenatal visits (median [IQR] = 6 [6-7] visits) and
at delivery. Together, the primigravid women delivered infants with lower birth weight than multigravid women. Only 3 women (2 primigravid and 1 multigravid) delivered infants weighing <2500g, this low incidence of LBW being likely due to the effective provision of monthly prenatal care including malaria prophylaxis and iron supplementation (Leke 2010).

Characteristics of the cross-sectional study population are listed in Table 4.2, and illustrated in Figure 4.1. Since we enrolled two malaria-negative participants for every malaria-positive participant, one third of the women in each gravidity group were malaria positive. PM was defined as placental blood smear-positive for asexual stages of P. falciparum by microscopy. Parasites were detected in both the peripheral and placental compartments in 116 women (77 primigravidae and 39 multigravidae) and only placental parasites were detected in 24 women (15 primigravidae and 9 multigravidae). Malaria-infected primigravid women had on average higher levels of placental parasitemia (Table 2) and mononuclear cell infiltrate than infected multigravid women (primigravid, 10.0 [0-22.5] vs. multigravid, 5.0 [0-18.8] mononuclear cells per 500 cells; p=0.016). Multigravidae were older and delivered infants with higher birth weight than primigravidae.
Women who participated in the longitudinal study in Cameroon were followed throughout gestation, at up to 7 antenatal visits and at delivery. Peripheral *P. falciparum* malaria infection was assessed by microscopy of peripheral blood smear at each visit; MiP+, blood-smear positive. Women who participated in the cross-sectional study in Malawi were enrolled at delivery. Placental malaria (PM) was determined by microscopy of placental blood smear. Birth outcomes were defined as normal birth weight (NBW, ≥2500 g), low birth weight (LBW, <2500g), small for gestational age (SGA, less than 10th centile according to (Landis, Ananth et al. 2009)), preterm delivery (PTD, <37 weeks of gestation).
4.4.2 Soluble Endoglin Levels Change with Gestation and Gravidity

Since sEng is secreted by the placental syncytiotrophoblast and has anti-angiogenic effects on endothelial cells that might be important for physiologic placental vascular remodeling during gestation (Venkatesha, Toporsian et al. 2006), we first examined if its expression is altered with the stage of gestation in the prospective study population in Cameroon. Maternal peripheral sEng levels increased with gestational age (p<0.0001 by univariate analysis). In a multivariate analysis taking into account gestational age, gravidity and presence of peripheral malaria infection, sEng concentrations were significantly higher in early (<14 weeks) and late (>33 weeks) gestation than at delivery (p<0.004 and p<0.0001, respectively; Figure 4.2A).

4.4.3 Soluble Endoglin Levels Change with Gravidity

Gravidity is an important factor in the susceptibility to PM, with primigravidae being more susceptible to both infection and to sequelae resulting from infection (McGregor, Wilson et al. 1983). The same multivariate analysis on the Cameroonian study population results, accounting for gestational age and the presence of peripheral or placental parasites, showed that maternal sEng levels were higher in primigravid than in multigravid women (p<0.046; Figure 4.2B). This association was also observed at delivery in the Malawian population, with primigravidae having higher sEng levels (50.2 [39.5-64.3] ng/mL) than multigravidae (43.2 [31.9-52.8] ng/mL; p<0.001).
Figure 4.2 Maternal sEng levels are affected by gravidity, gestational age and malaria infection

sEng levels measured in peripheral plasma samples from study participants were peripheral blood-smear negative at all study visits, shown as (A) all gravidities together, and (B) split into primigravidae (PG) and multigravidae (MG). (C) Levels of sEng from all study participants split into those with *P. falciparum* infection detected at one or more antenatal visit or at delivery (Malaria) or blood-smear negative at all study visits (Uninfected). Multi-order polynomial non-linear regression curves with the best fits to the data are depicted to illustrate the effect trends in peripheral sEng levels throughout gestation.
4.4.4 Malaria in Pregnancy Increases Soluble Endoglin Levels

In the Cameroonian study population, the presence of peripheral parasitemia during pregnancy was associated higher maternal sEng levels when gestational age and gravidity were taken into account (p=0.006; Figure 4.2C). Likewise, maternal sEng was higher on average in women with PM infection than in those without PM in the Malawian population (p<0.05), after controlling for gestational age and gravidity (Figure 4.3).

![Graph showing sEng levels in primigravidae and multigravidae](image)

Figure 4.3 Maternal sEng at delivery is increased with placental malaria in primigravidae and not multigravidae

sEng was measured in peripheral plasma samples of Malawian women at delivery. Placental malaria (PM+) was defined as positive by placental blood smear microscopy. Bars represent medians. 2-way ANOVA on log-transformed data: gravidity, p<0.0001. *, p<0.05; **, p<0.01; ***, p<0.001 by Bonferroni post-test.
Since the Malawian cross-sectional study population had a larger number of women, it was possible to further explore the association of increased sEng and PM at delivery. A weak but significant correlation was observed in primigravidae between peripheral sEng levels and peripheral or placental parasitemia (peripheral: Spearman’s rho=0.198, p=0.001; placental: rho=0.179, p=0.002). The extent of placental monocyte infiltration was positively correlated with peripheral sEng levels, regardless of gravidity or malaria infection (rho=0.127, p=0.007). Among subjects with a malaria-associated inflammatory response in the placenta, the extent of monocyte infiltration with sEng levels was strongest in primigravidae (rho=0.303, p=0.004).

**4.4.5 Soluble Endoglin Levels are Highest Among Women with Poor Clinical Outcomes of Malaria in Pregnancy**

In the Malawian study population, 51 (17.5%) of the primigravid women and 9 (5.5%) of the multigravid women delivered LBW infants. Among primigravidae only, maternal sEng levels were higher in mothers of LBW infants than in mothers of normal-weight infants (Mann-Whitney, p=0.017; Figure 4.4A). These higher concentrations of sEng in primigravidae were associated with fetal growth restriction (small for gestational age (n=92) vs. normal for gestational age (n=197), p=0.003) but not with preterm birth (preterm (n=54) vs. term birth (n=235), p=0.286).

Stratification of primigravid participants by malaria infection and birth weight illustrates that both PM and LBW outcomes were associated with increased peripheral sEng levels at delivery (2-way ANOVA (log$_{10}$ [sEng]): PM, p=0.008; LBW, p=0.006; Figure 4.4B). Peripheral sEng levels in primigravidae with malaria were negatively correlated with birth weight when correcting for gestational age at delivery (rho=-0.237, p<0.001). This correlation was not observed in the multigravid group (rho=0.031, p=0.693).

We also examined the change in sEng with anemia, another poor clinical outcome associated with both PM and LBW (Steketee, Nahlen et al. 2001). Anemia was associated with an increase in sEng in primigravidae (p=0.019; Figure 4.4C). However, in a multivariable logistic regression
model with age and parasitemia as covariates, sEng was not an independent predictor of anemia (p=0.102).

Figure 4.4 Elevated maternal sEng at delivery is associated with poor clinical outcomes in primigravidae

sEng was measured in peripheral plasma samples of Malawian women at delivery. Results displayed by (A) gravidity and birth weight; (B) birth weight and placental malaria (PM) status for primigravidae only; (C) anemia and PM status for primigravidae only. PM was defined as *P. falciparum* positivity in placental blood smear by microscopy. Anemia was defined as <11 g Hb/dL. NBW, normal birth weight; LBW, low birth weight. Bars represent medians. *, p<0.05; **, p<0.01; ***, p<0.001 by Bonferroni post-test of 2-way ANOVA on log-transformed data.
4.5 DISCUSSION

Systemic sEng levels correlate with severity of disease in both preeclamptic women and *P. falciparum*-infected children (Venkatesha, Toporsian et al. 2006; Dietmann, Helbok et al. 2009). By measuring sEng in maternal plasma at various stages of pregnancy in geographically distinct study populations in malaria-endemic regions with different transmission rates, we were able to show that systemic sEng levels were associated with poor outcomes of malaria infection in pregnancy. sEng was increased in pregnant women with peripheral malaria parasitemia during pregnancy, and peripheral sEng levels at delivery were increased in the presence of PM. Maternal sEng levels at delivery also correlated with fetal growth restriction.

In this study, we measured sEng levels in a longitudinal study in Cameroon and in a large case-control cross-sectional study from Malawi. These two geographically distinct populations showed similar increases in sEng levels in primigravidae and malaria infected women, despite differences in malaria endemicity and likely diversity in host and parasite genetics. These results suggest that these findings may be generalizable to other populations.

The source of the systemic sEng we measured in maternal plasma samples is unknown. Cell types that have been implicated in PM, including endothelial cells, activated monocytes and macrophages, have been shown to express Eng (Gougos and Letarte 1988; Gougos, St Jacques et al. 1992; Lastres, Bellon et al. 1992). Syncytiotrophoblast is a source of sEng (Venkatesha, Toporsian et al. 2006); however sEng has been detected in non-pregnant individuals (e.g., children with severe malaria (Dietmann, Helbok et al. 2009)), and thus is likely also cleaved from the surface of other Eng-expressing cells by matrix metalloproteinases (Velasco-Loyden, Arribas et al. 2004).

The changes we observed in the levels of sEng with malaria were small, though consistent. This small range coupled with natural variation between individuals pre-empts the use of sEng as a stand-alone biomarker for either PM or for individuals at risk of poor outcomes. However, it may prove to be more informative when coupled with other biomarkers of PM, as is the case for preeclampsia, where the utility of sEng as a prognostic biomarker is strengthened when coupled
with measurements of soluble Flt-1 and placental growth factor (Levine, Lam et al. 2006; Romero, Nien et al. 2008).

Our findings, coupled with an understanding of the biological effects of sEng, lead us to suggest that sEng may play a role in mediating the pathogenesis of fetal growth restriction associated with PM. sEng binds to TGF-β, and thereby limits TGF-β bioavailability (Venkatesha, Toporsian et al. 2006). The increase of sEng we observed in maternal plasma of primigravid women with PM is consistent with the reports that PM is associated with a decrease in maternal and placental TGF-β levels (Moormann, Sullivan et al. 1999; Abrams, Brown et al. 2003; Abrams, Milner et al. 2004). The levels of TGF-β1 have also been shown to be inversely correlated with malaria disease severity (Perkins, Weinberg et al. 2000; Chaiyaroj, Rutta et al. 2004), and to be important in dictating severity of pathology in murine models of malaria (Omer and Riley 1998; Li, Sanni et al. 2003; Omer, de Souza et al. 2003). TGF-β has multiple biological functions, including immune regulation, vascular development, and hematopoiesis (Letterio and Roberts 1998; Cho, Bourdeau et al. 2001; Rossant and Howard 2002). sEng may be mediating LBW in the context of PM by interfering with any of these effects, and, owing to the pleiotropic nature of its ligand, it is likely that any effect sEng may have on fetal growth restriction occurs through several mechanisms.

Monocytic infiltration of the placenta is a strong correlate of LBW in PM (Menendez, Ordi et al. 2000; Rogerson, Pollina et al. 2003). TGF-β can have both pro- and anti-inflammatory effects on immune cells from the monocyte lineage depending on the activation state of the cells (Li, Wan et al. 2006). TGF-β is a potent chemoattractant for monocytes and has been shown to potentiate inflammation via IL-1 and IL-6 secretion (Wahl, Hunt et al. 1987; Wiseman, Polverini et al. 1988; Turner, Chantry et al. 1990). TGF-β also induces monocyte secretion of matrix metalloproteinases (Wahl, Hunt et al. 1987). In this way sEng may be cleaved from syncytiotrophoblast to control TGF-β–dependent monocyte infiltration and inflammation. This is supported by the correlation between mononuclear cell infiltrates and sEng levels in Malawian primigravidae with PM.

Once monocytes differentiate into macrophages, TGF-β may now induce an anti-inflammatory effect by inhibiting secretion of TNF, chemokines, and nitric oxide (Li, Wan et al. 2006). This
effect was observed in mice in whom recombinant TGF-β treatment induced IL-10, decreased TNF, and prolonged survival time to infection with the rodent parasite *Plasmodium berghei* (Omer and Riley 1998). Conversely, increases in sEng that result in reduction of bioactive TGF-β in PM may interfere with this anti-inflammatory role, and favor the establishment of a proinflammatory environment. A ratio of TNF:IL-10 that favors TNF has been associated with severe malaria in children (Perkins, Weinberg et al. 2000), and in LBW outcomes of PM (Fried, Muga et al. 1998).

Increased sEng may also mediate fetal growth restriction by acting directly on the placental vasculature. This contention is supported by our observations that systemic sEng is expressed at higher amounts at times in gestation that correspond to uterine invasion by trophoblast (early gestation) (Pijnenborg, Bland et al. 1981) and active vascular remodeling to support optimal fetal growth (last trimester; Figure 4.2A). TGF-β is known to both inhibit trophoblast proliferation, migration and uterine invasion (Graham and Lala 1991; Caniggia, Taylor et al. 1997), and to promote endothelial cell proliferation and migration (Goumans, Valdimarsdottir et al. 2002), depending on the signaling pathway and cell type targeted. sEng has been shown to reduce endothelial cell sprouting and capillary vasodilation by blocking TGF-β activity (Venkatesha, Toporsian et al. 2006). Increased levels of sEng have been correlated with increased impedance to uterine and umbilical arterial blood flow (Chaiworapongsa, Romero et al. 2010). The early increase in sEng level, which does not appear to change with gravidity (Figure 4.2A), is likely required for normal placentation while malaria-induced increases in sEng in later gestation (Figure 4.2C) may restrict TGF-β–mediated placental vessel growth and lead to functional placental insufficiency and impaired fetal growth.

A third way that sEng may mediate fetal growth restriction is by dysregulating erythropoeisis. We observed an increase in sEng levels with maternal anemia in primigravidae, and previous reports have associated anemia with LBW (Fried, Muga et al. 1998; Kidanto, Mogren et al. 2009). Erythrocyte proliferation and differentiation requires TGF-β signaling through membrane-bound Eng (Cho, Bourdeau et al. 2001; Moody, Singbrant et al. 2007), thus decreased bioavailable TGF-β as a result of increased sEng may reduce the number of erythrocytes in circulation and, therefore, the amount of oxygen that is delivered to the fetus. Given that sEng
was not an independent predictor of anemia, it is also possible that high parasitemia caused proportional but unrelated variations in both sEng levels and hemoglobin levels in primigravidae.

The mechanism(s) by which sEng may mediate growth restriction in PM will be more thoroughly informed by a prospective study that involves serial blood sampling together with ultrasound measurements of fetal growth to assess how changes in sEng at different points in gestation relate to fetal development. It would also be informative to measure TGF-β alongside sEng; however, circulating TGF-β must be measured in platelet poor plasma, which was not collected from the current study populations.

HIV infection status was unknown in our study participants, but data put the prevalence of HIV infection in pregnant women at 7% and 30% in Cameroon and Malawi, respectively, during the periods of recruitment (Kongnyuy, Soskolne et al. 2008; Kwiek, Mwapasa et al. 2008). While HIV, and other co-infections, may affect sEng levels, it is unlikely to be a significant confounder of the results presented in this study, as the two populations with different levels of HIV prevalence showed the same results.

To our knowledge, this is also the first study to describe elevated sEng levels in first pregnancies. Primigravidae are known to be more susceptible to malaria infection and sequelae associated with malaria (Duffy 2007). The increased susceptibility in primigravidae is thought to be primarily due to a lack of antibodies specific for CSA-binding parasites that can prevent sequestration and promote opsonic clearance of infected erythrocytes in the placenta (Fried and Duffy 1998; Duffy and Fried 2003; Keen, Serghides et al. 2007). Primigravidae are also at higher risk of developing preeclampsia. Immune maladaptation to the semi-allogenic fetus has been suggested as an explanation for this susceptibility (Dekker, Robillard et al. 1998). Higher baseline systemic levels of sFlt-1, an anti-inflammatory and anti-angiogenic protein, have also been noted in first time pregnancies (Wolf, Shah et al. 2005). Increased constitutive sFlt-1 and sEng expression may keep heightened basal immune system activation in check, but also predispose primigravidae to poor clinical outcomes such as hypertension and fetal growth restriction. The absence of increased sEng in malaria-infected multigravidae (Figure 4.2 and Figure 4.4A), i.e., those less susceptible to PM sequelae, is also consistent with this hypothesis.
4.6 CONCLUSIONS

Maternal circulating sEng is increased with *P. falciparum* infection in pregnancy and with fetal growth restriction in primigravidae with PM. These changes are not sufficient for sEng on its own to be a useful biomarker of these conditions, but its potential contribution to compound biomarkers deserves further study. Our findings are compatible with the hypothesis that sEng may play a role in the pathogenesis of PM-related fetal growth restriction.
### Table 4.1 - Characteristics of prospective study population (Cameroon)

<table>
<thead>
<tr>
<th></th>
<th>Primigravid (n=22)</th>
<th>Multigravid (n=30)</th>
<th>P value</th>
</tr>
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<td>20 (18-24)</td>
<td>28 (24-32)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Number (%) who were blood-smear positive ≥ 1 times during pregnancy</td>
<td>15 (68%)</td>
<td>13 (43%)</td>
<td>0.096 (a)</td>
</tr>
<tr>
<td>Number (%) with PM at delivery</td>
<td>7 (32%)</td>
<td>5 (17%)</td>
<td>0.320 (a)</td>
</tr>
<tr>
<td>Gestational age at enrolment, weeks</td>
<td>14 (13-15.5)</td>
<td>13 (12-15.5)</td>
<td>0.201</td>
</tr>
<tr>
<td>Number of antenatal visits</td>
<td>6 (6-7)</td>
<td>6 (6-7)</td>
<td>0.289</td>
</tr>
<tr>
<td>Gestational age at delivery, weeks</td>
<td>39.5 (39-41)</td>
<td>40 (39-41)</td>
<td>0.831</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>2815 (2600-3200)</td>
<td>3400 (2975-3710)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Data shown as median (interquartile range), except where noted.

\(a\) Fisher’s exact test

### Table 4.2 - Characteristics of cross-sectional study population (Malawi)

<table>
<thead>
<tr>
<th></th>
<th>Primigravid (n=292)</th>
<th>Multigravid (n=166)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>18.0 (17.0-20.0)</td>
<td>25.0 (22.0-28.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin concentration, g/dL</td>
<td>12.0 (10.5-13.3)</td>
<td>11.8 (10.6-12.9)</td>
<td>0.388</td>
</tr>
<tr>
<td>Number (%) with PM at delivery (^a)</td>
<td>92 (31.5)</td>
<td>48 (28.9)</td>
<td>0.563</td>
</tr>
<tr>
<td>Placental parasites in infected mothers, per µL</td>
<td>70.0 (21.0-446.0)</td>
<td>40.0 (9.3-111.5)</td>
<td>0.013</td>
</tr>
<tr>
<td>Gestational age at delivery, weeks</td>
<td>38 (38-40)</td>
<td>40 (38-40)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>2800 (2563-3138)</td>
<td>3150 (2900-3450)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data shown as median (interquartile range), except where noted.

\(^a\) Product of study design with two malaria-negative control enrolled for each malaria-positive case enrolled.
Chapter 5
Performance Characteristics of Combinations of Host Biomarkers Identify Women with Occult Placental Malaria: A Case-Control Study from Malawi

5.1 ABSTRACT

5.1.1 Background
Because of its propensity to sequester in the placental intervillous space, *Plasmodium falciparum* can evade detection by peripheral smear in women with placental malaria (PM). We evaluated host biomarkers as potential indicators of occult PM infections.

5.1.2 Methods and Findings
Using a case-control design, we evaluated the ability of biomarkers to identify PM in the absence of circulating peripheral parasites (n=24) compared to placental smear-negative controls (n=326). We measured levels of biomarkers (C3a, C5a, CRP, angiopoietin-1, angiopoietin-2, sTie-2, sEndoglin, VEGF, sFlt-1, tissue factor, and leptin) in maternal peripheral plasma at delivery. Using ROC curve analysis, we assessed the ability of clinical parameters and biomarkers to accurately detect PM infections identified by placental smear. We show that decreases in sFlt-1 and leptin and increases in CRP were associated with occult PM infections (p<0.01) and correlated with placental parasitemia (p<0.01). Individually, all markers had moderate ability to diagnose occult PM infections with areas under the ROC between 0.62 and 0.72. In order to improve diagnostic performance, we generated simple scoring systems to identify PM infections using either a clinical score (0-2), a biomarker score (0-3) or a clinical plus biomarker score (0-5). The combinatorial model that incorporated both clinical parameters and biomarkers had an area under curve (AUC) of 0.85 (95% CI, 0.81-0.89), which was significantly better at identifying occult PM infections than the clinical score alone (p=0.001).
5.1.3 Conclusion

These data suggest that host biomarkers in the maternal peripheral blood may improve the detection of PM in the absence of peripheral parasitemia.
5.2 INTRODUCTION

Every year 85 million pregnant women are at risk of infection by the malaria parasite *Plasmodium falciparum* (Dellicour, Tatem et al. 2010). Malaria in pregnancy (MiP) may lead to adverse consequences for both the mother and the fetus, including severe maternal anemia, spontaneous abortion, stillbirth and low birth weight (LBW). In areas of unstable transmission of *P. falciparum*, mothers are at increased risk of severe malarial disease, including cerebral malaria and hypoglycemia (Desai, ter Kuile et al. 2007). As the level of endemicity and prior clinical immunity to malaria increases, MiP is more likely to be asymptomatic or paucisymptomatic (Desai, ter Kuile et al. 2007).

The proclivity of *P. falciparum*- PEs to bind to chondroitin-sulfate A (CSA) in the placental intervillus space (Fried and Duffy 1998) can make it difficult to detect placental malaria (PM) by either microscopic examination of Giemsa-stained peripheral blood smears or point-of-care rapid diagnostic testing (RDT) alone (Desowitz and Alpers 1992; Leke, Djokam et al. 1999; Mockenhaupt, Bedu-Addo et al. 2006). Occult placental malaria may still lead to adverse pregnancy outcomes. One strategy to decrease pregnancy-related malaria complications is intermittent preventive treatment in pregnancy (IPTp), the widespread administration of antimalarials (typically sulfadoxine-pyrimethamine) to pregnant women irrespective of their infection status at two or more scheduled antenatal visits during pregnancy (WHO 2004). Although IPTp has reduced infant low birth weight by 43% (Desai, ter Kuile et al. 2007), declining malaria transmission and increasing resistance to sulfadoxine-pyrimethamine will change the cost-benefit ratio for IPTp, potentially favoring intermittent screening and treatment (IST) involving newer, more costly and potentially less safe therapeutic agents (Tagbor, Bruce et al. 2010). Given the limitations of traditional and field diagnostics to detect placental malaria, the identification of biomarkers present in the maternal peripheral blood that could identify PM in the absence of patent peripheral parasitemia, could minimize unnecessary drug treatment during pregnancy, improve the sensitivity of IST to detect placental malaria, reduce drug pressure and the selection of resistant parasites, and improve maternal and fetal outcomes.

Because occult PM induces a local host response in the placenta, and soluble components of the placental compartment may circulate in the peripheral blood, we evaluated a number of host
proteins as potential candidate biomarkers for the detection of placental malaria, in the absence of detectable circulating parasites. We measured markers of inflammation (C-reactive protein), complement activation (C3a, C5a), angiogenesis (angiopoietin-1, angiopoietin-2, soluble Tie-2, vascular endothelial growth factor (VEGF), soluble fms-like tyrosine kinase-1 (sFlt-1), and soluble Endoglin), coagulation (tissue factor), and nutrient availability (leptin). Our results indicate that decreases in sFlt-1 and leptin and an increase in CRP in peripheral plasma were associated with the presence of placental parasites.

5.3 METHODS

5.3.1 Ethics Statement

Ethical approval for this study was granted from The College of Medicine Research Ethics Committee in Blantyre, Malawi (COMREC) and all women gave written informed consent for enrolment into the study.

5.3.2 Study Population

From 2001-2006, a cross-sectional study was carried out in Blantyre, Malawi, in which pregnant women were prospectively recruited at the Gogo Chatinkha Banda Maternity Unit of Queen Elizabeth Central Hospital. Upon delivery, a thick blood film was prepared from the cut surface of the placenta, and women were recruited as cases if they were positive for placental malaria and had delivered a live singleton newborn and had a peripheral blood thick film that was negative for malaria parasites. Twenty-one women were excluded from the study because they had malaria parasites detected by peripheral smear, but not placental smear. Age and gravidity-matched controls (peripheral and placental smear negative for malaria) were chosen in a 2:1 ratio. Controls enrolled based on a negative placental smear were not reclassified as cases based on the identification of parasites by subsequent histology, as these sub-microscopic infections were not associated with significantly different clinical outcomes than histology negative controls in this cohort. Placental and peripheral EDTA plasma samples were collected after women had given their informed consent, and samples were stored at -80ºC until testing.
5.3.3 Biomarker ELISAs

Plasma concentrations of biomarkers C5a, angiopoietin (Ang)-1, Ang-2, sTie-2, sEng, VEGF, sFlt-1, leptin, and CRP (DuoSets, R&D Systems, Minneapolis, MN) were measured in peripheral plasma by ELISA, as described (Conroy, Phiri et al. 2010). C3a (capture: clone 354113, detection: purified PAb, Goat IgG) and tissue factor (capture: clone 323514, detection: PAb, goat IgG) were measured using antibody pairs (R&D Systems, Minneapolis, MN) with biotin swine anti-goat IgG (human and mouse adsorbed, clone CLCC50015) as a secondary antibody as described previously (Conroy, Phiri et al. 2010). ELISAs were performed in 2009 by a single experienced technician who was blinded to the microscopy results during sample testing.

5.3.4 Statistical Analysis

GraphPad Prism v5, SPSS v16, and MedCalc software were used for analysis. Comparisons of continuous variables were performed using the Mann-Whitney U test and Spearman rank correlation coefficient. Comparisons of proportions were performed using Pearson chi-square test or Fisher’s exact test, as appropriate. Odds ratios (OR) were calculated using Pearson’s Chi-square. The diagnostic accuracy of biomarkers and laboratory findings were assessed using non-parametric receiver operating characteristic (ROC) curves, and the areas under the ROC (AUC) were compared using the method of Delong et al., (DeLong, DeLong et al. 1988). Biomarker cut-points were determined using the Youden index (J = max[sensitivity + specificity – 1]).

5.4 RESULTS

5.4.1 Description of Study Population

A total of 465 women with live singleton deliveries were included in the study. Of these women, 326 were peripheral and placental smear-negative for malaria and 139 were PM-positive based on placental smears. Of the 139 placental smear-positive women, 24 had negative peripheral smears. Using histology as the gold-standard for diagnosing malaria infections, over half (53.4%) of the placental smear-negative women had evidence of a malaria infection (parasites ± pigmented fibrin/mononuclear cells) (Rogerson, Pollina et al. 2003). However, there were no differences in peripheral blood biomarkers, maternal hemoglobin, or fetal outcome (gestational age or birth weight) in placental smear-negative women with histologically defined PM infections compared to those with no detectable placental parasites (p>0.05 for all comparisons,
data not shown). Since histology-only infections were not associated with signs of disease or clinical outcome, the women were included in the study as smear-negative controls according to the original design. In order to evaluate biomarkers in maternal peripheral plasma that were the most sensitive for placental-specific infections, the 326 peripheral and placental smear-negative women were compared to women who were PM positive without detectable infections by peripheral smear. The study population is described in Table 5.1.

Women with placental smear-positive PM had a significantly lower median peripheral-blood hemoglobin concentration ([Hb]) than placental smear-negative women (10.9g/dL vs. 12.3g/dL, p=0.003, Mann-Whitney U test) (Table 5.1). Anemia (peripheral blood [Hb] <11g/dL) in the mother was associated with a 3.3-fold (95% CI, 1.4-7.6) increased odds of PM. Women with PM more commonly reported non-specific symptoms (fever, chills, headache) within one week prior to delivery (p<0.0001) (Table 5.1). The presence of one or more of the above symptoms was associated with a 7.0-fold (2.9-16.6) increased odds of placental infection (p<0.0001).

5.4.2 sFlt-1, Leptin and CRP are Clinically Informative Biomarkers of Placental Malaria Infection

We included a number of biomarkers in our analysis that have previously been shown to be altered in the peripheral blood in women with placental malaria, including CRP (Mockenhaupt, Rong et al. 2000; Mockenhaupt, Bedu-Addo et al. 2006), complement C5a (Conroy, Serghides et al. 2009), the angiopoietin (Ang) family of Ang-1, Ang-2 and a soluble form of their cognate receptor, Tie-2 (sTie-2) (Silver, Zhong et al. 2010), anti-angiogenic proteins sFlt-1 and sEng(Muehlenbachs, Mutabingwa et al. 2006), and leptin (Kabyemela, Muehlenbachs et al. 2008). Median Ang-1, sFlt-1, and leptin levels were significantly lower, and median CRP levels were significantly higher in women with placental malaria than in uninfected controls (Figure 5.1, Table 5.2). These markers were significantly correlated with quantitative placental parasite density on placental blood smear (Spearman’s rho, p-value): Ang-1, -0.110, p=0.044; sFlt-1, -0.174, p=0.001; leptin, -0.203, p<0.001; and CRP, 0.188, p<0.001. Ang-2 was the only marker significantly associated with placental mononuclear cell counts, as assessed by histology (0.141, p=0.009).
We assessed the diagnostic accuracy of our biomarkers using receiver operating characteristic (ROC) curve analysis (Figure 5.1). sFlt-1, leptin and CRP all had AUC ≥ 0.70 for discrimination between cases and controls, and provided significant discrimination in univariable analysis after applying a correction factor for multiple comparisons (Figure 5.1, Table 5.2). We used the Youden index to create a cut-point for each biomarker, and low sFlt-1 (<16.9ng/mL), low leptin (<8.8ng/mL) and high CRP (>30.5mg/mL) were associated with increased odds ratios (95% CI) of placental malaria of 4.7 (2.0-11.2), 4.6 (1.8-11.5), and 6.1 (2.3-16.0) respectively. The sensitivity and specificity for the biomarkers at their respective cut-points are shown in Table 5.2.
Figure 5.1 Biomarkers of occult placental malaria infection

(A-C) Box plots showing the median (IQR) of peripheral blood biomarkers (whiskers denote the 5-95% percentiles and outliers are plotted as dots), with the associated (D-F) receiver operating characteristic (ROC) curves and (G-I) decision plots of sensitivity and specificity generated from the ROC curves. Diagnostic accuracy was assessed using receiver operating characteristic (ROC) curves and determining the area under the ROC curve (AUC). The AUC were compared and all three biomarkers had comparable diagnostic performance (method of Delong et al.). The dotted lines represent the cut-point for dichotomizing the biomarkers, as defined using the Youden index.
5.4.3 Predicting Placental Malaria using Combinations of Biomarkers

We hypothesized that combinations of biomarkers would improve diagnostic accuracy, as each protein could contribute unique information to a predictive model. Therefore, we generated a biomarker score based on dichotomization and summation of the individual biomarkers with the best performance: sFlt-1, leptin, and CRP. For each marker, one point was assigned if the value was lower (sFlt-1, leptin) or higher (CRP) than the corresponding cut-point, and zero points were assigned otherwise. None of the dichotomized biomarkers were significantly correlated (p<0.05, Spearman correlation).

ROC curve analysis of the 3 biomarker score yielded an AUC of 0.83 (0.78-0.86). A score of 1 or more was able to identify 95.7% of all infections (sensitivity), and a score of 3 had a specificity of 100% (Figure 5.2). A score of 2 had a sensitivity and specificity of 73.9% and 80.9%, respectively. Evaluation of more parsimonious biomarker scoring systems showed that 2-biomarker scores incorporating leptin and CRP (AUC=0.78 [0.73-0.82]), as well as sFlt-1 and CRP (AUC=0.78 [0.74-0.83]), provided similar discriminative ability to the 3-biomarker score.

Next, we sought to integrate our biomarkers into models that included established clinical parameters associated with placental infection, including maternal anemia and febrile symptoms. To do this, we first generated a clinical score (0-2) based on the presence of maternal anemia and non-specific febrile symptoms. The clinical score had an AUC of 0.72 (0.67-0.77) (Figure 5.2). Then by combining the 3-marker biomarker score with the clinical score (into a five-point score), we were able to achieve an AUC of 0.85 (0.81-0.89), which was significantly better at identifying PM infections than the clinical score alone (p=0.001) (Figure 5.2), but not better than the 3-marker biomarker score (p=0.39).
Figure 5.2 Combinations of biomarkers and clinical parameters improve diagnostic accuracy

(A-C) We generated a simple scoring system based on the summation of dichotomous variables that were significantly associated with the presence of smear positive parasites in the placenta, but not the periphery. (A) The clinical score (0-2) consisted of maternal anemia (1) and non-specific febrile symptoms (any one of: fever, chills, headache=1). (B) The biomarker score (0-3) consisted of high CRP levels (>30.5mg/mL=1), low leptin levels (<8.8ng/mL=1), and low sFlt-1 levels (<16.9ng/mL=1). (C) The clinical score and biomarker score were integrated to generate the final score (0-5). (D) The diagnostic ability of the different scoring systems was assessed using ROC curve analysis. The clinical + biomarker score had an AUC of 0.85 (95% CI, 0.81-0.89) and was significantly better than the clinical score at discriminating between women with occult PM infections compared to smear-negative controls, p=0.001 (method of Delong, et al.).
5.5 DISCUSSION

It is well-established that malaria in pregnancy increases the risk of low birth-weight delivery. In some women with placental malaria, parasites cannot be detected in the peripheral blood. Such infections are commonly asymptomatic or paucisymptomatic, yet they are still associated with adverse effects on pregnancy outcome. The existence of such ‘hidden’ *P. falciparum* infections makes it difficult to assess the true impact of malaria in pregnancy and poses challenges to the implementation of proposed programs of intermittent screening and treatment (Smith, Jones et al. 2010).

In this study we assessed the utility of host biomarkers to identify occult PM infections in a case-control study of Malawian women. We compared peripheral plasma levels of these biomarkers between women with placental smear-positive *P. falciparum* infections (peripheral blood smear negative) and placental smear-negative controls. We identified three biomarkers that were significantly associated with the presence of placental parasites (sFlt-1, leptin, CRP) and evaluated the diagnostic performance of these biomarkers alone and in combination using a simple scoring system. The biomarker score was associated with occult PM infections, had good diagnostic performance, and allowed more accurate prediction of placental malaria than a score based on clinical criteria alone. Our data suggest that a combinatorial biomarker score could be used to identify women likely to benefit from targeted antimalarials during pregnancy.

sFlt-1 is an anti-angiogenic protein that is an alternatively spliced variant of Flt-1 (VEGFR-1). Elevated sFlt-1 has been implicated in the pathophysiology of placental malaria (Muehlenbachs, Mutabingwa et al. 2006; Muehlenbachs, Fried et al. 2008; Conroy, Serghides et al. 2009) and has also been identified as a biomarker that is elevated in pregnancy-associated hypertension and preeclampsia (Maynard, Min et al. 2003; Levine, Qian et al. 2006; Muehlenbachs, Mutabingwa et al. 2006; Hirokoshi, Maeshima et al. 2007). We observed a substantial decrease in peripheral sFlt-1 levels in PM infections that were peripheral smear negative compared to controls. The identification of decreased sFlt-1 in maternal peripheral blood in malaria is a novel finding, and these data suggest that sFlt-1 may be particularly useful as a blood test in pregnancy in malaria-endemic areas, with decreases in sFlt-1 being associated with occult PM infections and elevations in sFlt-1 predicting the onset of preeclampsia. Further studies will be required to
assess the utility of this marker in populations where PM and preeclampsia may interact to drive adverse pregnancy outcomes (Muehlenbachs, Mutabingwa et al. 2006).

Leptin is a peptide hormone that influences energy homeostasis and regulates neuroendocrine function, in addition to its role as an inflammatory molecule in immune function. The syncytiotrophoblast produces an abundance of leptin in later stages of pregnancy and decreased leptin levels in pregnancy have been associated with fetal growth restriction (Yildiz, Avci et al. 2002; Pighetti, Tommaselli et al. 2003). We observed a significant reduction in maternal leptin levels in occult PM compared to controls. As most placentally-derived leptin is released into the maternal circulation (Linnemann, Malek et al. 2000), we postulate that PM infection may reduce the release of leptin into the maternal circulation.

CRP is an acute phase reactant and a non-specific marker of inflammation. Our results indicate that CRP is elevated in occult PM infections, which is consistent with a previous report from Gabon that described elevated CRP in PM, but not sub-microscopic infections (assessed by PCR) (Adegnika, Verweij et al. 2006). In this study, CRP was the best single predictor with the highest AUC. It is an attractive candidate biomarker given its commercial availability and widespread use in other conditions. However, owing to its lack of specificity, the utility of CRP as a biomarker of occult PM requires further investigation in studies that include other common infections in pregnancy such as chorioamnionitis (van de Laar, van der Ham et al. 2009).

Combinations of biomarkers have sometimes been shown to provide better diagnostic or prognostic accuracy than single markers, particularly if drawn from distinct pathobiological pathways (Morrow and Braunwald 2003). In this study, we combined markers of angiogenesis (sFlt-1), metabolism (leptin), and inflammation (CRP) to improve identification of occult PM infections. By combining the three biomarkers with the highest individual AUC, we were able to achieve better predictive ability than with any of the biomarkers used on their own (comparison of AUC between 3 biomarker model and individual models by method of Delong et al., p<0.05). Assays for both CRP and sFlt-1 have been commercialized and are available for immediate use. We were able to integrate the biomarker data with easily obtained clinical data (self-reported febrile symptoms, anemia) to generate a simple scoring system with an AUC of 0.85.
While the results of this study are promising, they require validation in other populations with differing levels of malaria endemicity. In this population, we did not find the presence of placental parasites detectable by histology only associated with impaired fetal growth, but this may not be the case in all populations. The biomarkers that we have used should also be compared to other inflammatory biomarkers that have recently been associated with placental malaria (Thevenon, Zhou et al. 2010). The biomarkers discussed in this study will need to be evaluated at earlier stages in pregnancy, at times when presumptive therapy might usefully be applied, as well as in the context of other clinically important infections, in particular HIV.

The case-control design of this study enabled us to look at biomarkers in a broader context by removing the effect of age and gravidity on susceptibility to PM. Future studies should validate these biomarkers in a prospective study.

### 5.6 CONCLUSIONS

Biomarkers circulating in the peripheral blood of pregnant women may provide more accurate prediction of placental malaria than clinical indicators alone, in women without peripheral parasitemia. Analysis of biomarkers in peripheral blood could contribute to the accurate identification of women most in need of antimalarial therapy in pregnancy, and thereby facilitate programs of intermittent screening and therapy aimed at reducing the impact of malaria on the pregnant woman and unborn child.
Table 5.1: Description of Study Population

<table>
<thead>
<tr>
<th></th>
<th>Placental smear positive PM</th>
<th>Placental and peripheral smear negative</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>20.5 (18.3-25.3)</td>
<td>20.0 (18.0-23.0)</td>
<td>0.603</td>
</tr>
<tr>
<td>Gravidity</td>
<td>1 (1-3.75)</td>
<td>1 (1-2)</td>
<td>0.580</td>
</tr>
<tr>
<td>% malaria-positive by histology</td>
<td>100</td>
<td>53.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total # antimalarial doses†</td>
<td>2.0 (2.0-2.25)</td>
<td>2 (1-3)</td>
<td>0.575</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>56.0 (50.25-60.25)</td>
<td>56.0 (51.0-60.0)</td>
<td>0.754</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>155 (150-159)</td>
<td>154 (150-158)</td>
<td>0.743</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.5 (36.0-37.0)</td>
<td>36.1 (36.0-36.6)</td>
<td>0.076</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>10.9 (10.1-12.2)</td>
<td>12.3 (11.1-13.5)</td>
<td>0.003</td>
</tr>
<tr>
<td>Febrile symptoms in last 7 d, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>15 (62.5)</td>
<td>63 (19.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Chills</td>
<td>9 (37.5)</td>
<td>15 (4.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Headache</td>
<td>9 (37.5)</td>
<td>22 (6.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>15 (62.5)</td>
<td>56 (17.2)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Weight of baby (kg)</td>
<td>2.9 (2.5-3.1)</td>
<td>3.0 (2.7-3.3)</td>
<td>0.036</td>
</tr>
<tr>
<td>Weeks gestation at delivery</td>
<td>38 (36-40)</td>
<td>39 (38-40)</td>
<td>0.030</td>
</tr>
<tr>
<td>Mononuclear cell count*</td>
<td>18.5 (13.25-30.75)</td>
<td>3 (0-13)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mononuclear cell pigment score (0-4)*</td>
<td>1.0 (0.25-2.0)</td>
<td>0 (0-0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fibrin pigment score (0-4)*</td>
<td>2.0 (0-3.0)</td>
<td>0 (0-1)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range) unless otherwise indicated.
Groups were compared using the Mann-Whitney U test (for continuous variables) or Pearson Chi-square (for nominal variables).
†Number of doses of SP recorded on antenatal clinic card
*Assessed using a semi-quantitative scale by a single observer
### Table 5.2: Peripheral plasma biomarkers in occult placental malaria infections

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Infected</th>
<th>Uninfected</th>
<th>P-value</th>
<th>AUC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cut-point</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.2 (16.7-72.7)</td>
<td>18.5 (8.2-39.7)</td>
<td>0.002*</td>
<td>0.71 (0.66-0.76)</td>
<td>30.5</td>
<td>73.9 (51.6-89.8)</td>
<td>68.3 (62.9-73.4)</td>
</tr>
<tr>
<td>C3a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0 (1.6-4.4)</td>
<td>2.4 (1.6-4.5)</td>
<td>0.704</td>
<td>0.56 (0.50-0.60)</td>
<td>6.4</td>
<td>50.0 (29.1-70.9)</td>
<td>66.0 (60.5-71.1)</td>
</tr>
<tr>
<td>C5a</td>
<td>63.0 (32.2-76.1)</td>
<td>58.1 (39.9-78.4)</td>
<td>0.307</td>
<td>0.53 (0.47-0.58)</td>
<td>35.4</td>
<td>34.8 (16.4-57.3)</td>
<td>81.5 (76.8-85.6)</td>
</tr>
<tr>
<td>Ang-1</td>
<td>13.7 (6.7-20.6)</td>
<td>17.9 (9.4-32.2)</td>
<td>0.037</td>
<td>0.62 (0.57-0.67)</td>
<td>8.5</td>
<td>43.5 (23.2-65.5)</td>
<td>79.9 (75.1-84.2)</td>
</tr>
<tr>
<td>Ang-2</td>
<td>4.4 (2.8-7.7)</td>
<td>5.1 (3.2-7.9)</td>
<td>0.555</td>
<td>0.54 (0.49-0.59)</td>
<td>2.9</td>
<td>34.8 (16.4-57.3)</td>
<td>84.3 (79.9-88.1)</td>
</tr>
<tr>
<td>sTie-2</td>
<td>28.9 (22.1-32.1)</td>
<td>24.6 (20.0-30.4)</td>
<td>0.311</td>
<td>0.60 (0.55-0.65)</td>
<td>27.8</td>
<td>60.9 (38.5-80.3)</td>
<td>64.6 (59.1-69.8)</td>
</tr>
<tr>
<td>sEng</td>
<td>43.0 (29.0-72.6)</td>
<td>46.7 (36.5-57.5)</td>
<td>0.819</td>
<td>0.49 (0.43-0.54)</td>
<td>69.95</td>
<td>30.4 (13.2-52.9)</td>
<td>90.6 (86.8-93.6)</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>16.9 (11.4-34.7)</td>
<td>27.3 (19.3-39.7)</td>
<td>0.003*</td>
<td>0.70 (0.64-0.74)</td>
<td>16.9</td>
<td>52.2 (30.6-73.2)</td>
<td>83.7 (7912-87.6)</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.10 (0.04-0.23)</td>
<td>0.14 (0.06-0.41)</td>
<td>0.242</td>
<td>0.57 (0.52-0.63)</td>
<td>0.075</td>
<td>47.8 (26.8-69.4)</td>
<td>70.2 (64.9-75.2)</td>
</tr>
<tr>
<td>Tissue Factor</td>
<td>0.10 (0.06-0.14)</td>
<td>0.08 (0.05-0.14)</td>
<td>0.370</td>
<td>0.56 (0.50-0.61)</td>
<td>0.043</td>
<td>95.5 (77.2-99.9)</td>
<td>23.8 (19.1-29.4)</td>
</tr>
<tr>
<td>Leptin</td>
<td>5.6 (4.7-9.8)</td>
<td>11.2 (7.4-18.9)</td>
<td>&lt;0.001*</td>
<td>0.73 (0.67-0.77)</td>
<td>8.8</td>
<td>73.9 (51.6-89.8)</td>
<td>66.5 (61.0-71.6)</td>
</tr>
</tbody>
</table>

Groups (infected vs. uninfected) were compared using the Mann-Whitney U test. * Significant (p<0.05) after Holm’s correction for 11 pair-wise comparisons. <sup>a</sup>Non-parametric estimation (DeLong et al., 1988 (AUC), 95% CI estimated using binomial exact method (MedCalc)). Biomarkers are ng/mL unless otherwise indicated.<sup>b</sup>mg/mL
Chapter 6
Whole Blood Angiopoietin-1 and -2 Levels Discriminate Cerebral and Severe (Non-Cerebral) Malaria from Uncomplicated Malaria

6.1 ABSTRACT

6.1.1 Background

Severe and cerebral malaria are associated with endothelial activation. Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are major regulators of endothelial activation and integrity. The aim of this study was to investigate the clinical utility of whole blood angiopoietin (Ang) levels as biomarkers of disease severity in Plasmodium falciparum malaria.

6.1.2 Methods

The utility of whole blood Ang levels was examined in Thai patients to distinguish cerebral (CM; n=87) and severe (non-cerebral) malaria (SM; n=36) from uncomplicated malaria (UM; n=70). Comparative statistics are reported using a non-parametric univariate analysis (Kruskal-Wallis test or Chi-squared test, as appropriate). Multivariate binary logistic regression was used to examine differences in whole blood protein levels between groups (UM, SM, CM), adjusting for differences due to ethnicity, age, parasitemia and sex. Receiver operating characteristic curve analysis was used to assess the diagnostic accuracy of the Angiopoietins in their ability to distinguish between UM, SM and CM. Cumulative organ injury scores were obtained for patients with severe disease based on the presence of acute renal failure, jaundice, severe anemia, circulatory collapse or coma.

6.1.3 Results

Ang-1 and Ang-2 were readily detectable in whole blood. Compared to UM there were significant decreases in Ang-1 (p<0.001) and significant increases in Ang-2 (p<0.001) levels and the ratio of Ang-2: Ang-1 (p<0.001) observed in patients with SM and CM. This effect was independent of covariates (ethnicity, age, parasitemia, sex). Further, there was a significant
decrease in Ang-1 levels in patients with SM (non-cerebral) versus CM (p<0.001). In participants with severe disease, Ang-2, but not Ang-1, levels correlated with cumulative organ injury scores; however, Ang-1 correlated with the presence of renal dysfunction and coma. Receiver operating characteristic curve analysis demonstrated that the level of Ang-1, the level of Ang-2 or the ratio of Ang-2: Ang-1 discriminated between individuals with UM and SM (area under the curve, p-value: Ang-2, 0.763, p<0.001; Ang-1, 0.884, p<0.001; Ratio, 0.857, p<0.001) or UM and CM (area under the curve, p-value: Ang-2, 0.772, p<0.001; Ang-1, 0.778, p<0.001; Ratio, 0.820, p<0.001).

6.1.4 Conclusions

These results suggest that whole blood Ang-1/2 levels are promising clinically informative biomarkers of disease severity in malarial syndromes.
6.2 INTRODUCTION

Only a small proportion of individuals with *Plasmodium falciparum* malaria progress to severe and potentially fatal forms of infection (World Health Organization 2000; Snow, Guerra et al. 2005). The definitive diagnosis of severe and cerebral malaria is challenging due to the non-specific nature of the clinical presentation and the confounder of incidental parasitemia in malaria-endemic areas (Taylor, Fu et al. 2004). These factors may result in misdiagnosis and adverse outcomes due to the failure to treat other life-threatening infections (World Health Organization 2000; Beare, Southern et al. 2004). In studies of African children diagnosed with cerebral malaria, over 20% were shown to have an alternative cause for their neurological syndrome at post-mortem examination (Taylor, Fu et al. 2004). A rapid point-of-care test that accurately identifies patients with severe or cerebral malaria, or those at risk of progressing to these syndromes, would be of clinical and public health utility. However, limited prognostic or diagnostic laboratory tools for severe malaria are currently available.

Endothelial activation and dysfunction have been implicated in the pathogenesis of severe and cerebral malaria (Brown, Hien et al. 1999; Yeo, Lampah et al. 2008). The angiogenic factors, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) have recently been shown to function as essential regulators of endothelial activation and integrity (Fiedler, Reiss et al. 2006). Ang-1 is constitutively expressed and maintains vascular quiescence by signaling through the Tie-2 receptor (Fiedler, Reiss et al. 2006). Ang-2 is released from Weibel-Palade (WP) bodies in association with endothelial activation and displaces Ang-1; sensitizing the endothelium to become responsive to sub-threshold concentrations of cytokines such as TNF (Fiedler, Reiss et al. 2006). Elevations in plasma or serum Ang-2 levels have been reported in patients with sepsis, acute lung injury/acute respiratory distress syndrome (Parikh, Mammoto et al. 2006; Orfanos, Kotanidou et al. 2007), and severe malaria (Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009).

Based on the hypothesis that dysregulation of angiopoietins is associated with severe malaria syndromes, Ang-1 and Ang-2 levels were examined in malaria-infected patients to determine if they would distinguish between uncomplicated, severe and cerebral malaria. This study focused on the predictive value of Ang-1 and Ang-2 levels in whole blood, since unprocessed whole
blood obtained by finger prick is a preferred clinical specimen for point-of-care testing (Bell and Peeling).

6.3 METHODS

6.3.1 Study Population and Specimen Collection

Individuals (≥ 13 years of age) presenting with falciparum malaria to Hospital for Tropical Disease clinics (Mahidol University, Thailand) were eligible for enrolment into this study. Patients were classified into uncomplicated (UM), cerebral (CM) or severe (non-cerebral) malaria (SM) according to WHO criteria (World Health Organization 2000). Whole blood samples (pre-treatment) were collected from all patients for thick and thin blood film preparation, PCR, and complete blood counts. A heparinized aliquot was frozen at –80°C for determination of subsequent angiopoietin levels. An expert microscopist, who was blinded to the results of additional diagnostic testing, examined the blood films. Smears were considered negative if no parasites were seen in two consecutive thick blood films. Parasite density was calculated by thick or thin film determining the number of parasites per 200 white blood cells for thick blood films or per 1,000 red blood cells (RBC) for thin blood films. Baseline white blood cell counts or RBC counts were used to calculate parasitemia (parasites per μL). Plasmodium falciparum diagnosis was confirmed by PCR as previously described (Perandin, Manca et al. 2004). The institutional review board of the Faculty of Tropical Medicine, Mahidol University approved this study and informed consent was obtained from all patients or their legal guardians prior to specimen collection.

6.3.2 Quantification of Ang-1 and Ang-2 in Whole Blood

Whole blood concentrations of Ang-1 and Ang-2 were measured by ELISA (R&D Systems, Minneapolis MN) according to the manufacturers’ instructions. Concentrations were interpolated from 4-parameter-fit standard curves generated using a standard curve of recombinant human angiopoietin proteins. The lower and upper limits of detection for each assay were as follows: Ang-1 (156.25 - 10,000 pg/mL) and Ang-2 (54.69 - 3,500 pg/mL).
6.3.3 Statistical Analysis

Statistical analysis was performed using SPSS v17.0. Comparative statistics are reported from a non-parametric univariate analysis (Kruskal-Wallis test, followed by Mann-Whitney U test with adjustment for multiple comparisons or Chi-squared test, as appropriate). Multivariate binary logistic regression was used to examine differences in whole blood protein levels between groups (UM, SM, CM), adjusting for differences due to ethnicity, age, parasitemia and sex. Spearman’s correlation was used to examine correlations between angiopoietin levels and parasitemia or cumulative organ injury scores. Receiver operating characteristic (ROC) curves and area under the ROC curves were generated using SPSS v.17.0. Optimal test thresholds were derived mathematically from the ROC curves. To adjust for multiple comparisons at a family-wise simultaneous error rate of $\alpha=0.05$, Bonferroni corrections were applied as appropriate.
6.4 RESULTS

6.4.1 Study Population

193 patients with *P. falciparum* malaria were enrolled during the study. Patients were classified as having UM, (n=70), SM (n=36) or CM (n=87) (World Health Organization 2000). In patients with complicated disease (SM and CM), 22 participants (17.9%) had evidence of acute renal failure, 71 had evidence of liver dysfunction (57.7%), three had algid malaria with a systolic blood pressure less than 80 mmHg (2.4%), 34 were anemic and required a blood transfusion (27.6%) and 87 were in a coma (70.7%). Of study participants with severe malaria, 46 had a single organ system involved, 41 had two organ systems affected, 22 had three and five patients had involvement of four organ systems. There was a single fatality in the study with the study participant having cerebral malaria and algid malaria. Demographic and baseline clinical characteristics of the study participants are shown in Table 6.1.

6.4.2 Whole Blood Ang-2 Levels are Increased and Ang-1 Levels are Decreased in SM or CM Compared to UM

Whole blood Ang-2 levels were significantly higher in individuals with SM or CM compared to individuals with UM (*Figure 6.1A*; p<0.001). Furthermore, Ang-1 levels were significantly lower in those with SM compared to individuals with either UM or CM, and in individuals with CM compared to UM (*Figure 6.1B*; p<0.001). Since Ang-2 and Ang-1 have divergent roles in vascular activation, the ratio of Ang-2:Ang-1 was used as an additional measure for each patient. This ratio was significantly higher in patients with SM or CM compared to those with UM (*Figure 6.1C*; p<0.001).
Figure 6.1: Comparison of angiopoietin-1 and -2 levels in whole blood samples from Thai adults with malaria

A-C. Whole blood concentrations of (A) angiopoietin-2 (Ang-2), (B) angiopoietin-1 (Ang-1), and (C) the ratio of Ang-2:Ang-1 (Ratio, expressed as log base 10) were measured in 70 uncomplicated malaria (UM) patients, 36 severe malaria (SM) patients, and 87 cerebral malaria (CM) patients. ***p<0.0001 by Kruskal-Wallis test with posthoc comparisons using Mann-Whitney U test and Bonferroni correction. D-F. Receiver operating characteristic (ROC) curves were generated for each test to compare (D) UM with SM patients, (E) UM with CM patients, and (F) SM with CM patients, with the null hypothesis (diagonal line) that the area under the curve equals 0.5.
6.4.3 Receiver Operating Characteristic (ROC) Curves Indicate that Angiopoietin Levels Discriminate Between UM and SM or CM

ROC curves were plotted for Ang-2, Ang-1, and the ratio of Ang-2:Ang-1 to assess the ability of each biomarker to discriminate between UM, SM and CM. Comparing individuals with UM vs. individuals with SM, all markers have an area under the curve (AUC) that differs significantly from that of a chance result (AUC: 0.5) (**Figure 5.1D**: AUC, p-value: Ang-2, 0.763, p<0.001; Ang-1, 0.884, p<0.001; Ratio, 0.857, p<0.001). Similarly, all markers discriminated between UM and CM (**Figure 5.1E**: AUC, p-value: Ang-2, 0.772, p<0.001; Ang-1, 0.778, p<0.001; Ratio, 0.820, p<0.001). Finally, Ang-1 but not Ang-2 or the Ang-2:Ang-1 ratio, was able to discriminate between SM and CM (**Figure 6.1F**: AUC, p-value: Ang-1; 0.735, p<0.001: Ang-2; 0.527, p=0.663: Ratio; 0.599, p=0.084) with Ang-1 levels being lower in SM than CM. In this population, Ang-1 discriminated between UM and severe malarial syndromes better than either Ang-2 or the ratio of Ang-2:Ang-1, and was able to distinguish SM from CM.

6.4.4 The Ratio of Ang-2:Ang-1 Displays High Sensitivity and Specificity as a Biomarker for Disease Severity

The sensitivity, specificity, and positive and negative likelihood ratios (LR(±)) were determined for Ang-1, Ang-2, and the ratio of Ang-2:Ang-1. Based on sensitivity and specificity, Ang-1 was best able to discriminate between UM and SM (cut-off (12.38 ng/mL): sensitivity: 0.861, specificity: 0.857, LR(+): 6.028, LR(-): 0.162)); whereas Ang-2 was better at discriminating between UM and CM (cut-off (1.33 ng/mL): sensitivity: 0.759, specificity: 0.771, LR(+): 3.319, LR(-): 0.313)). The ratio of Ang-2:Ang-1 was best at discriminating between complicated disease of mixed phenotype (SM+CM) (cut-off 0.08): sensitivity: 0.789, specificity: 0.829, LR(+): 4.60, LR(-): 0.264)) (Table 5.2). Collectively, our data suggest that the ratio of Ang-2:Ang-1 may be the best predictor of patients with uncomplicated disease versus patients with complicated (severe or cerebral) disease, whereas Ang-1 may have utility in differentiating between cerebral malaria vs. severe (non-cerebral) disease.

6.4.5 Angiopoietins Predict Severe Malaria Independently of Covariates

In order to examine whether endothelial activation was independent of parasite burden, levels of Ang-1 and Ang-2 were correlated with parasitemia. Overall, there was a positive correlation
between Ang-2 and parasitemia (Spearman’s rho 0.339, p<0.0001) and a negative correlation between Ang-1 and parasitemia (Spearman’s rho -0.446, p<0.0001). Furthermore, Ang-2 and Ang-1 were negatively correlated with one another (Spearman’s rho -.320, p<0.0001).

Multivariate logistic regression analysis revealed that the observed differences in Ang levels between groups remained even after adjusting for differences due to ethnicity, age, gender, and parasitemia. There remained a significant increase in Ang-2 (and the ratio of Ang-2:Ang-1) in SM vs. UM, and CM vs. UM, with no significant difference between SM and CM. For Ang-1, there was a significant decrease in SM vs. UM, CM vs. UM, and SM vs. CM (p<0.0001).

6.4.6 Angiopoietin-2 is Associated with Cumulative Organ Injury

To address whether Ang levels are a measure of disease severity, an organ injury score ranging from 0-5 was assigned to each participant based on the number of organ systems involved. A value of 1 was added to the organ injury score for each participant based on the presence of each of the following: acute renal failure requiring hemodialysis, clinically apparent jaundice, severe anemia requiring a blood transfusion, circulatory collapse with a systolic pressure <80mmHg, and coma. Ang-2 and the ratio of Ang-2: Ang-1 were positively correlated with cumulative organ injury (Spearman’s rho, p-value: Ang-2; 0.378, p<0.0001: Ang-2:Ang-1 ratio; 0.288, p=0.001). Interestingly, Ang-1 also trended towards a positive correlation (Spearman’s rho 0.166, p=0.071) despite its ability to discriminate between individuals with cerebral vs. severe non-cerebral malaria. To investigate this further, a post-hoc analysis was performed to examine how Ang-1 correlated with the organ systems included in the analysis. There were no associations observed between Ang-1 levels in participants with hepatic dysfunction, severe anemia or circulatory collapse, although the latter was underpowered. However, Ang-1 was positively associated with renal dysfunction (Spearman’s rho, p-value: 0.243, p=0.007) and coma (Spearman’s rho, p-value: 0.370, p<0.0001).

6.5 DISCUSSION

These results demonstrate that whole blood Ang-1 and Ang-2 levels are robust biomarkers of severe and cerebral malaria. Ang-2, Ang-1 and the ratio of Ang-2: Ang-1 were significantly different between UM and both SM and CM (Figure 6.1A-C). Of particular interest, Ang-1 levels further distinguished patients with severe (non-cerebral) malaria from those with cerebral involvement (Figure 6.1B). Finally, ROC curve analysis indicated good diagnostic accuracy for
these biomarkers in discriminating uncomplicated malaria from severe malarial syndromes (Figure 6.1D-F; Table 6.2). Significant differences between groups remained after correcting for potential confounding factors, and were independent of parasitemia, ethnicity, age and gender. Taken together with recent studies, these data implicate dysregulation of angiopoietins in the pathogenesis of complicated malaria and suggest they may be clinically informative biomarkers of disease severity (Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009).

The ability of angiopoietins to discriminate between uncomplicated and complicated malaria has now been confirmed in three distinct geographic regions and populations (children in Uganda, adults in Papua and Thailand). These proteins have previously been measured in serum (Ang-1, Ang-2) (Lovegrove, Tangpukdee et al. 2009), plasma (Ang-2) (Yeo, Lampah et al. 2008), and here we show that they can be measured directly in whole blood samples acquired from an independent population of malaria-infected individuals. Only a small number of patients progress to severe and fatal malaria (World Health Organization 2000; Snow, Guerra et al. 2005) and a rapid point-of-care test to identify malaria-infected individuals with or at risk of progressing to severe disease could be of clinical utility. Ang-1 and Ang-2 are attractive candidates for incorporation into rapid lateral flow immunochromatographic tests combined with malaria antigen detection due to their detection in whole blood and ability to discriminate between patients with and without severe disease. However, before biomarkers like Ang-1 or Ang-2 can be incorporated into rapid malaria tests and clinical practice, additional prospective studies will be needed to confirm and validate their usefulness in different populations. Optimal cut-offs need to be determined using standardized methods of sample collection, processing, and laboratory protocols for biomarker testing.

Ang-1 and Ang-2 are stable proteins able to withstand repeated freeze thaws without significant changes in protein levels, and are readily detectable, as this study demonstrates, in whole blood. Furthermore, the angiopoietins, which are linked with inflammation, angiogenesis, and endothelial function and integrity, play a central role in many of the disease processes implicated in the pathogenesis of severe malarial syndromes. Ang-2 exists pre-stored within WP bodies and is rapidly released upon, and may further contribute to, endothelial activation (Fiedler, Reiss et al. 2006). As a result, changes in Ang-2 levels may represent early changes in the endothelial beds, and future studies should examine its utility as a predictive biomarker. Furthermore, different endothelial beds may be differentially responsive to exogenous stimuli, providing a
putative mechanism to explain why certain syndromes of organ dysfunction are more common in severe malaria, particularly acute renal injury, respiratory distress, or cerebral malaria. This may be particularly relevant with Ang-1, which differentiated between severe (non-cerebral) malaria and cerebral malaria in this study.

In the current study, Ang-2 levels were elevated in severe malarial syndromes compared to uncomplicated malaria but were also correlated with scores of cumulative organ injury in the cohort of severe malaria patients. This confirms other studies suggesting that Ang-2 is a marker of disease severity in conditions associated with endothelial activation (Parikh, Mammoto et al. 2006; Orfanos, Kotanidou et al. 2007; Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009). However, this is the first study to show that Ang-1 differentiates between severe (non-cerebral) and cerebral malaria. Interestingly, Ang-1 levels did not correlate significantly with the cumulative organ injury score but were specifically correlated with renal dysfunction and coma. It is unclear whether the association between Ang-1 levels and the presence of renal impairment and coma are due to the nature of microvascular environment in those organ systems or other physiological derangements. In order to address those questions, these observations will need to be confirmed in a larger population of individuals with well-characterized clinical phenotypes.

The use of biomarkers is inherently limited by their specificity for the disease in question. Dysregulation of angiopoietins may occur in a number of severe infectious syndromes associated with endothelial activation and dysfunction. As a result, it will be important to consider the implications of co-infections, including bacterial and viral (e.g. HIV) infections if these proteins are to be used as prognostic indicators for malaria.

Finally, based on our study design it was not possible to examine the predictive value of angiopoietins and future prospective studies are needed to examine the kinetics of angiopoietin levels and their relation to symptom onset, clinical history of malaria, and response to treatment. However, should angiopoietins fail to distinguish between malaria and other infectious diseases, these proteins may still be informative biomarkers to facilitate clinical decision-making (e.g. patient triage, referral, admission) and optimal allocation of health resources for the treatment of severe infections associated with endothelial dysfunction.
6.6 CONCLUSIONS

In summary, these data suggest that Ang-1 and Ang-2 are promising biomarkers of severe malaria. Furthermore, the ability to robustly detect angiopoietin levels in whole blood makes them attractive candidates for potential integration into point-of-care diagnostic/theranostic devices of disease severity.
Table 6.1: Demographic characteristics of Thai adults with malaria

<table>
<thead>
<tr>
<th></th>
<th>Uncomplicated Malaria</th>
<th>Severe Malaria (Non-cerebral)</th>
<th>Severe Malaria (Cerebral)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>70</td>
<td>36</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Sex, Number (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>56 (80)</td>
<td>27 (75)</td>
<td>73 (84)</td>
<td>0.508^a</td>
</tr>
<tr>
<td>Female</td>
<td>14 (20)</td>
<td>9 (25)</td>
<td>14 (16)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>26 (13-50)</td>
<td>24 (14-59)</td>
<td>26 (14-61)</td>
<td>0.184^b</td>
</tr>
<tr>
<td>Parasitemia, Parasites/uL</td>
<td>5360 (422385)</td>
<td>119850 (943890)</td>
<td>84840 (1188626)</td>
<td>&lt;0.0001^b</td>
</tr>
</tbody>
</table>

Data are median (range) unless otherwise indicated.

^a Chi-squared test.
^b Kruskal-Wallis U test.
Table 6.2: Optimal biomarker cut-off values for receiver operating characteristic curve sensitivity, specificity, positive likelihood ratio (LR(+)) and negative likelihood ratio (LR(-)) at the chosen cutoffs.

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Sensitivity # (95% CI)</th>
<th>Specificity # (95% CI)</th>
<th>LR(+) # (95% CI)</th>
<th>LR(-) # (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiopoietin-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM vs. SM</td>
<td>1.43</td>
<td>0.69 (0.53-0.82)</td>
<td>0.79 (0.68-0.87)</td>
<td>3.2 (1.97-5.33)</td>
</tr>
<tr>
<td>UM vs. CM</td>
<td>1.33</td>
<td>0.76 (0.66-0.84)</td>
<td>0.77 (0.66-0.85)</td>
<td>3.32 (2.12-5.19)</td>
</tr>
<tr>
<td>UM vs. (CM+SM)</td>
<td>1.33</td>
<td>0.74 (0.66-0.81)</td>
<td>0.77 (0.66-0.85)</td>
<td>3.24 (2.08-5.04)</td>
</tr>
<tr>
<td>SM vs. CM</td>
<td>3.14</td>
<td>0.47 (0.32-0.63)</td>
<td>0.64 (0.54-0.74)</td>
<td>1.33 (0.85-2.07)</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM vs. SM</td>
<td>12.38</td>
<td>0.86(0.71-0.94)</td>
<td>0.85(0.76-0.92)</td>
<td>6.03(3.35-10.86)</td>
</tr>
<tr>
<td>UM vs. CM</td>
<td>15.86</td>
<td>0.71(0.61-0.80)</td>
<td>0.74(0.63-0.83)</td>
<td>2.77 (1.82-4.22)</td>
</tr>
<tr>
<td>UM vs. (CM+SM)</td>
<td>15.16</td>
<td>0.71(0.62-0.78)</td>
<td>0.77(0.66-0.85)</td>
<td>3.09(1.98-4.83)</td>
</tr>
<tr>
<td>SM vs. CM</td>
<td>11.21</td>
<td>0.72 (0.56-0.84)</td>
<td>0.66(0.55-0.75)</td>
<td>2.09(1.47-2.98)</td>
</tr>
<tr>
<td>Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM vs. SM</td>
<td>0.082</td>
<td>0.81(0.65-0.90)</td>
<td>0.83(0.72-0.90)</td>
<td>4.70(2.74-8.06)</td>
</tr>
<tr>
<td>UM vs. CM</td>
<td>0.080</td>
<td>0.78(0.68-0.86)</td>
<td>0.83(0.72-0.90)</td>
<td>4.56(2.69-7.72)</td>
</tr>
<tr>
<td>UM vs. (CM+SM)</td>
<td>0.080</td>
<td>0.79(0.71-0.85)</td>
<td>0.83(0.72-0.90)</td>
<td>4.60(2.73-7.76)</td>
</tr>
<tr>
<td>SM vs. CM</td>
<td>0.448</td>
<td>0.44(0.30-0.60)</td>
<td>0.83(0.74-0.89)</td>
<td>2.58(1.43-4.64)</td>
</tr>
</tbody>
</table>
Chapter 7

Endothelium-Based Biomarkers are Associated with Cerebral Malaria in Malawian children: a Retrospective Case-Control Study

7.1 ABSTRACT

7.1.1 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 parasitemia. CM is associated with endothelial activation. 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.

7.1.2 Methods and Findings

Plasma samples were tested from children recruited with uncomplicated malaria (UM; n=32), cerebral malaria with retinopathy (CM-R; n=38), clinically defined CM without retinopathy (CM-N; n=29), or non-malaria febrile illness with decreased consciousness (CNS; n=24). Admission levels of angiopoietin-2 (Ang-2), Ang-1, soluble Tie-2 (sTie-2), von Willebrand factor (VWF), its propeptide (VWFpp), vascular endothelial growth factor (VEGF), soluble ICAM-1 (sICAM-1) and interferon gamma-induced protein 10 (IP-10) were measured by ELISA. 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 CM-N. Children with CM-R had significantly lower median levels of Ang-1 and higher median concentrations of Ang-2:Ang-1, sTie-2, VWF, VWFpp, VEGF and sICAM-1 compared to UM, and significantly lower median levels of Ang-1 and higher median levels of Ang-2, Ang-2:Ang-1, VWF and VWFpp compared to children with fever and altered consciousness due to other causes. Ang-1 was the best discriminator between UM and CM-R and between CNS and CM-R (areas under the ROC curve of 0.96 and 0.93, respectively). A comparison of biomarker levels in CM-R between admission
and recovery showed uniform increases in Ang-1 levels, suggesting this biomarker may have utility in monitoring clinical response.

### 7.1.3 Conclusions

These results suggest that endothelial proteins are informative biomarkers of malarial disease severity. These results require validation in prospective studies to confirm that this group of biomarkers improves the diagnostic accuracy of CM from similar conditions causing fever and altered consciousness.
7.2 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 parasitemia in malaria-endemic areas (Taylor, Fu et al. 2004) (Wright, Avery et al. 1993; Berkley, Mwangi et al. 1999; Okubadejo and Danesi 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 hemorrhages, 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 (Hollestelle, 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 the 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 tumor 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). Tie-2 is the cognate receptor for Ang-1 and Ang-2 and the soluble form of the receptor, sTie-2, can bind the angiopoietins and regulate their function. WPBs are also an important source of VWF, particularly ultra large 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 between cerebral 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 parasitemia.
7.3 METHODS

7.3.1 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.

7.3.2 Study Population

This study was nested within prospective studies examining 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). Convenience samples were selected for children between 1 month and 14 years of age presenting with fever to the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi between 1997 and 2009, who met the eligibility criteria for the clinical syndromes and had sufficient plasma samples available. Admission lithium heparin plasma samples were obtained from children after their parents or guardians had given their informed consent. 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. Efforts were made to minimize bias by selecting subjects from a single population (children presenting to QECH), and by using two types of controls, uncomplicated malaria illness (outpatient) and non-malarial febrile illness with decreased consciousness (inpatient). All participants received standard treatment, including antimalarial and/or antibacterial therapy as indicated, according to Malawian National guidelines.

7.3.3 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-centered hemorrhages, 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.
7.3.4 Definitions of clinical syndromes

7.3.4.1 Cerebral Malaria (CM)

Children meeting the case definition for CM (World Health Organization 2000; Taylor, Fu et al. 2004) including *P. falciparum* asexual parasitemia, a Blantyre coma score ≤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 (representing clinical recovery) were collected for each surviving child available for follow-up.

7.3.4.2 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.

7.3.4.3 CNS Controls

Children 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.

7.3.5 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 healthy adult Caucasian 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 plated in duplicate and 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 H₂SO₄. 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.

7.3.6 Statistical Analysis

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 operating 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.
7.4 RESULTS

7.4.1 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). Children with CM-R were significantly younger than children with clinically defined CM without retinopathy. Demographic and clinical data for these children are shown in Table 7.1.

7.4.2 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 7.1, Table 7.2). For each analyte tested, a receiver operating 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 (AUC) 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 AUCs of 0.83 (95% CI: 0.73-0.93) and 0.77 (95% CI: 0.65-0.89) respectively.
Figure 7.1 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). ns (not significant, p>0.05), *p<0.05 after Holms correction for multiple comparisons (9 pair-wise comparisons). The limit of detection is represented by a dotted line (VEGF).
7.4.3 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 7.2, Table 7.3).

ROC curves were generated to assess the diagnostic accuracy of the biomarkers to discriminate between UM and CM-R. The AUC curve was computed and the sensitivity, specificity and positive and negative likelihood ratios were calculated at the optimal biomarker cut-off (Table 7.4). 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 AUC as good as Ang-1 and resulted in an improved positive likelihood ratio (LR(+)) versus Ang-1 alone [Ang-1 LR(+)=7.1 compared to Ang-2:Ang-1 LR(+)=18].
Figure 7.2 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). ns (not significant, p>0.05), *p<0.05 after Holms correction for multiple comparisons (9 pair-wise comparisons). The limit of detection is represented by a dotted line (VEGF).
7.4.4 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 7.2, Table 7.3).

Using ROC curve analysis, three biomarkers, Ang-1, VWFpp, and VWF, discriminated children with CM-R from children with other suspected CNS infections (Table 7.5). Median Ang-2 was elevated in CM-R compared to the CNS controls, and the Ang-2:Ang-1 ratio had an AUC equal to that of Ang-1 alone but with a two-fold increase in the positive likelihood ratio (Table 7.5). sICAM-1, sTie-2, and VEGF, while useful biomarkers for UM vs. CM-R, did not discriminate between CM-R vs. CNS (Table 7.5).

7.4.5 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, Ang-2, VWF, VWFpp, sICAM-1 and IP-10 (Table 7.6). Most of the endothelial biomarkers were significantly associated with each other, except for VEGF, which was only associated with IP-10.

7.4.6 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 7.3). Overall, VEGF showed a significant increase in levels at convalescence. 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.
Figure 7.3 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).
7.5 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) (Burton, Nyong'o et al. 2004; Beare, Taylor et al. 2006; Lewallen, Bronzan et al. 2008), 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 in children with clinical defined CM (Figure 7.1, Table 7.2). 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 7.2, Tables 7.3 & 7.4). 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). Further, we show a marked and uniform decrease in Ang-2:Ang-1 at follow-up (Figure 7.3), suggesting that the ratio between these two proteins may offer an approach to monitor clinical response. This study represents a significant extension to previous studies of endothelial biomarkers in malaria as it includes a retinopathy-confirmed group of children with cerebral malaria and also includes children with decreased consciousness due to other febrile illnesses. Finally, we are able to go beyond description to examine different putative causal biomarkers of endothelial activation and postulate on their role in disease pathogenesis and clinical recovery.
The endothelium is a dynamic organ system representing the interface between the vascular space and vital organs. The regulation of 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 pediatric 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 pediatric 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 signaling 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 pediatric 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). These studies postulate that VEGF may be neuroprotective and associated with wound healing (Jain, Armah et al. 2008), or may reflect the accumulation of VEGF within the parasitophorous vacuole (Sachanonta, Medana et al. 2008; 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.). Together, our VEGF data supports the idea that VEGF may be associated with disease severity and neurologic complications in CM; however, due to its local action, future studies are warranted to further elucidate the role of VEGF in disease severity.

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. In this population, IP-10 was non-significantly elevated in retinopathy-confirmed CM and displayed poor performance as a biomarker. Previous reports have identified IP-10 as a good prognostic biomarker for malaria mortality (Jain, Armah et al. 2008); however, this could not be assessed in the current study where fatal cases were excluded. 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 may 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.

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 pediatric 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) may 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.
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<td>Age, Months</td>
<td>33 (13-82)**</td>
<td>51 (14-159)</td>
<td>26 (1-109) &lt;</td>
<td>39 (8-96)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>17 (44.7)</td>
<td>11 (37.9)</td>
<td>9 (37.5)</td>
<td>17 (53.2)</td>
</tr>
<tr>
<td>Presenting BCS</td>
<td>1 (0-2)</td>
<td>1 (0-2)</td>
<td>4 (3-5) +</td>
<td>5</td>
</tr>
<tr>
<td>Severe Anemia, n (%)</td>
<td>28 (73.7%)***</td>
<td>2 (6.9)</td>
<td>1 (4.5) &lt;</td>
<td>0</td>
</tr>
<tr>
<td>Admission Parasitemia</td>
<td>62,956 (86-1,729,160)</td>
<td>43,631 (36-1,626,240)</td>
<td>0 (0-0) +</td>
<td>#</td>
</tr>
<tr>
<td>CSF White Cell Count</td>
<td>0 (0-17)</td>
<td>0 (0-15)</td>
<td>2 (0-6) &lt;</td>
<td>‡</td>
</tr>
<tr>
<td>History of Convulsions, n (%)</td>
<td>30 (78.9)</td>
<td>27 (93.1)</td>
<td>8 (44.4)†, &lt;</td>
<td>‡</td>
</tr>
<tr>
<td>White Blood Cell Count</td>
<td>9900 (2,800-25,700)</td>
<td>8,800 (1,400-28,000)</td>
<td>‡</td>
<td>‡</td>
</tr>
<tr>
<td>Platelet count</td>
<td>45,000 (5,000-262,000)</td>
<td>116,000 (6,000-545,000)</td>
<td>‡</td>
<td>‡</td>
</tr>
<tr>
<td>Venous Lactate</td>
<td>6.35 (2.4-16.4)</td>
<td>5.0 (1.6-21.4)</td>
<td>‡</td>
<td>‡</td>
</tr>
</tbody>
</table>

CM: retinopathy positive (CM-R), retinopathy negative (CM-N). Median (range) unless otherwise indicated, # Clinical information unavailable as children seen on an outpatient basis. **p<0.01, ***p<0.001 (CM-R vs. CM-N), †N=18, ‡ Unavailable, < p<0.01, + p<0.0001 (CM vs. CNS), BCS : Blantyre Coma Score.
Table 7.2: Endothelial-biomarkers are associated with retinopathy in a cohort of children with cerebral malaria (CM).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>AUC 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>0.046</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 (0.65-0.89)</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 (0.60-0.87)</td>
<td>0.001*</td>
<td>2.0</td>
<td>74 (58-85)</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>0.36 (0.20-0.65)</td>
</tr>
<tr>
<td>VWF propeptide</td>
<td>0.71 (0.58-0.85)</td>
<td>0.005*</td>
<td>51</td>
<td>76 (61-87)</td>
<td>62 (44-77)</td>
<td>2.0 (1.2-3.3)</td>
<td>0.38 (0.20-0.72)</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>0.006*</td>
<td>1000</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 Holms correction for 9 pair-wise comparisons. CM-R: Cerebral malaria, retinopathy positive, CM-N: Cerebral malaria, retinopathy negative, AUC: Area under the receiver operating characteristic curve.
Table 7.3. 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)
Table 7.4. 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>AUC UM vs. CM-R (95% CI)</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>0.96 (0.93-1.0)</td>
<td>&lt;0.0001*</td>
<td>5.3</td>
<td>94 (80-98)</td>
<td>87 (73-94)</td>
<td>7.1 (3.1-16)</td>
<td>0.072 (0.019-0.28)</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.65 (0.51-0.79)</td>
<td>0.0359</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 (0.32-0.86)</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 (80-98)</td>
<td>95 (83-99)</td>
<td>18 (4.6-69)</td>
<td>0.066 (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 (61-89)</td>
<td>74 (58-85)</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 (76-97)</td>
<td>84 (70-93)</td>
<td>5.7 (2.7-12)</td>
<td>0.11 (0.038-0.33)</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>870</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>VEGF</td>
<td>0.71 (0.58-0.84)</td>
<td>0.0025*</td>
<td>0.25</td>
<td>72 (55-84)</td>
<td>74 (58-85)</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>0.0504</td>
<td>0.72</td>
<td>69 (51-82)</td>
<td>66 (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 Holms correction for 9 pair-wise comparisons. CM-R: Cerebral malaria, retinopathy positive
UM: Uncomplicated malaria, AUC: Area under the receiver operating characteristic curve.
Table 7.5. 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></th>
<th>AUC 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 (2.9-15)</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 (0.26-0.86)</td>
</tr>
<tr>
<td>Ang-2: Ang-1</td>
<td>0.93 (0.87-0.99)</td>
<td>&lt;0.0001*</td>
<td>1.4</td>
<td>71 (51-85)</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>0.1087</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 propeptide</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>0.59 (0.41-0.74)</td>
<td>0.254</td>
<td>1000</td>
<td>38 (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 (0.57-1.2)</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.64 (0.50-0.78)</td>
<td>0.0673</td>
<td>0.91</td>
<td>52 (33-71)</td>
<td>66 (50-79)</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 Holms correction for 9 pair-wise comparisons. CM-R: Cerebral malaria, retinopathy positive. CNS: children with non-malarial fever with decreased consciousness, AUC: Area under the receiver operating characteristic curve.
Table 7.6. 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>0.33**</td>
<td>0.55**</td>
<td>0.42**</td>
<td>0.36**</td>
<td>0.12</td>
<td>0.54**</td>
<td></td>
</tr>
<tr>
<td>sTie-2</td>
<td>0.251**</td>
<td>0.25**</td>
<td>0.44**</td>
<td>0.17</td>
<td>0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWFpp</td>
<td></td>
<td>0.80**</td>
<td>0.58**</td>
<td>0.055</td>
<td>0.57**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWF</td>
<td></td>
<td></td>
<td>0.60**</td>
<td>0.14</td>
<td>0.37**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sICAM-1</td>
<td></td>
<td></td>
<td></td>
<td>0.16</td>
<td>0.43**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Spearman’s rho, *p<0.05; **p<0.01, n=123.
Chapter 8

Angiopoietin-2 Levels Predict Mortality in Malawian Children with Cerebral Malaria: A Retrospective Case-Control Study

8.1 ABSTRACT

8.1.1 Background

Vascular endothelium is the site of cytoadherence, sequestration, and microhemorrhage in cerebral malaria (CM). Based on the hypothesis that the angiopoietin-Tie-2 axis is a critical regulator of endothelial activation during malaria infection we investigated the relationship between this system, retinopathy and mortality in CM.

8.1.2 Methods

In a case-control study of children presenting with clinical CM with and without malarial retinopathy (n=155), we determined admission levels of angiopoietin-1, angiopoietin-2 and a soluble version of their cognate receptor (sTie-2).

8.1.3 Results

We show that angiopoietin-1 levels are decreased and angiopoietin-2 and sTie-2 levels are increased in children with CM who had retinopathy compared to those who did not. Angiopoietin-2 and sTie-2 were independent predictors of retinopathy (adjusted Odds ratio (95% CI): angiopoietin-2, 4.3 (1.3-14.6), p=0.019; sTie-2, 9.7 (2.1-45.8), p=0.004). Angiopoietin-2 and sTie-2 levels were elevated in children with CM who subsequently died and Angiopoietin-2 was an independent predictor of death (adjusted Odds ratio: 3.9 (1.2-12.7), p=0.024). When combined with clinical parameters, Angiopoietin-2 improved classification of patients using logistic regression and CRT.

8.1.4 Conclusions

These results provide insights into mechanisms of endothelial activation in CM and indicate that the angiopoietin-Tie-2 axis is associated with retinopathy and mortality in pediatric CM.
8.2 INTRODUCTION

*Plasmodium falciparum* causes approximately 250 million infections and 1 million deaths annually, with 85% of the fatalities occurring in children under the age of five (WHO 2009). The case fatality rate in CM remains high (>15%) despite the initiation of appropriate anti-malarial therapy and supportive care (Murphy and Breman 2001; Dondorp, Fanello et al. 2010). There are currently no effective adjunctive therapies for CM, in part owing to an incomplete understanding of underlying pathogenetic mechanisms. Sequestration of parasites in major organs is thought to be central to the pathogenesis of many severe malaria syndromes, including CM. Mature stage parasitized erythrocytes bind to cell adhesion molecules expressed on the endothelium (e.g. ICAM-1), where they are believed to contribute to endothelial activation, microvascular obstruction, localized hypoxia and associated tissue injury. In a post-mortem study of fatal CM cases, the highest density of sequestration was found in the brain, consistent with the neurological disturbances that define this syndrome (Seydel, Milner et al. 2006).

Clinically, it is challenging to differentiate CM from other febrile conditions associated with altered consciousness, owing to the high prevalence of incidental parasitemia in malaria endemic areas and the non-specific presentation of CM (fever, convulsions, coma) (Taylor, Fu et al. 2004). Post-mortem examinations have revealed that approximately one-quarter of children meeting the World Health Organization (WHO) case definition for CM had an alternative cause of death (Taylor, Fu et al. 2004). Malarial retinopathy has emerged as a powerful tool to identify children with CM and to better define disease pathobiology. Specific retinal changes – hemorrhages, retinal whitening, or vessel abnormalities – have been found to be associated with CM. Malarial retinopathy had 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), and can be used to accurately define pediatric patient groups for research studies. As the retina and the brain are formed from the same embryonic tissue and have analogous blood-tissue-barriers, analysis of retinopathy by direct and indirect ophthalmoscopy can provide insights into microvascular and tissue disturbances in the brain that are otherwise difficult to study. Ophthalmoscopy combined with retinal angiography has demonstrated that retinal whitening is associated with areas of non-perfusion, vessel abnormalities are indicative of non-perfusion of vessels (white colouration) or areas of reduced vascular lumen (orange colouration) (Beare,
Harding et al. 2009), and hemorrhage corresponds to focal loss of blood-retinal-barrier integrity. Consistent with these observations and their associated brain pathology, retinopathy is statistically (Lewallen, Bakker et al. 1996; Olumese, Adeyemo et al. 1997; Beare, Southern et al. 2004) associated with worse outcome in cerebral malaria.

The vascular pathology observed on retinal examination and at autopsy point to a key role for cerebral endothelial activation and dysfunction during the development of CM. The endothelium represents the critical interface between the brain parenchyma and the intravascular compartment containing sequestered parasites. In 75% of fatal CM cases, sequestration was associated with intravascular and perivascular pathology including microthrombus formation, perivascular edema, and petechial hemorrhages in the brain parenchyma surrounding ruptured vessels (Taylor, Fu et al. 2004). Markers of endothelial activation, such as endothelial microparticles, von Willebrand factor and its propeptide, and soluble cell-adhesion molecules (sCAMs), are increased in the plasma of malaria patients 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; Larkin, de Laat et al. 2009). Collectively these observations suggest a role for endothelial activation and loss of barrier integrity in the pathophysiology of CM.

The angiopoietin-Tie-2 system has been shown to regulate endothelial cell function and barrier integrity (Brindle, Saharinen et al. 2006; Fiedler and Augustin 2006). Angiopoietin-1 (Ang-1) engages endothelial receptor Tie-2, promoting endothelial cell quiescence and survival, whereas these effects are generally inhibited by Angiopoietin-2 (Ang-2) (Yuan, Khankin et al. 2009). Previous reports have demonstrated low Ang-1 and elevated Ang-2 levels in severe compared to uncomplicated malaria (Yeo, Lampah et al. 2008; Conroy, Lafferty et al. 2009; Lovegrove, Tangpukdee et al. 2009). In this study, we investigated markers of the angiopoietin-Tie-2 system in CM in relation to data derived from retinal findings and clinical outcome. We hypothesized that altered levels of Ang-1, Ang-2, and the soluble form of Tie-2 (sTie-2) would be associated with retinopathy in children with apparent CM, and that plasma concentrations of these markers would correlate with the severity of retinopathy. Since endothelium may have a crucial role in mediating malaria pathogenesis, we further postulated that these markers would have prognostic value in CM patients. 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. We also show
that Ang-2 is a clinically informative prognostic biomarker on its own or in combination with clinical findings.
8.3 METHODS

8.3.1 Ethics

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

8.3.2 Study Population

Children between 6 months and 14 years of age presenting with fever to the Queen Elizabeth Central hospital in Blantyre, Malawi were recruited between 1997 and 2009. Admission EDTA plasma samples were obtained from children after their parents or guardians had given their informed consent. Samples collected as part of ongoing prospective studies of the pathogenesis and management of CM were used to assess the relationship between endothelial regulatory proteins (Ang-1, Ang-2, sTie-2) and retinopathy and mortality. This study was a retrospective case control design of children with clinically defined CM, with retinopathy and mortality as outcome measures. Cases were selectively chosen to include a suitable number with a fatal outcome and/or retinopathy, but without knowledge of other clinical details. All participants received standard treatment, including antimalarial and/or antibacterial therapy as indicated, according to Malawian national guidelines. The clinical case definition for CM was *P. falciparum* asexual parasitemia, a Blantyre coma score ≤2 with no improvement following correction of hypoglycemia, and no evidence of an alternative cause for coma including meningitis on examination of cerebrospinal fluid (World Health Organization 2000; Taylor, Fu et al. 2004).

8.3.3 Ophthalmological Examination

After admission of a child with altered consciousness, the patient’s pupils were dilated by application of mydriatic eye 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 of the following retinal findings: hemorrhages, retinal whitening, or vessel changes with or without papilloedema, as previously described (Beare, Taylor et al. 2006; Lewallen, Bronzan et
al. 2008). Clinical grading of retinal changes were recorded as follows: 0-4 for hemorrhages (0=none, 1=mild (1-5 hemorrhages), 2=moderate (>5 & ≤20 hemorrhages), 3=severe (>20 & ≤50 hemorrhages), 4=very severe (>50 hemorrhages)), 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.

8.3.4 Quantification of Biomarkers

Plasma concentrations of biomarkers Ang-1, Ang-2, and sTie-2 (DuoSets, R&D Systems, Minneapolis, MN) were measured by ELISA, “blinded” to other patient data, as previously described (Conroy, Phiri et al. 2010).

8.3.5 Statistical Analysis

Comparisons of continuous variables were performed using the Mann-Whitney U test and Spearman rank correlation coefficient. Comparisons of proportions were performed using Pearson chi-square test, linear-by-linear association, or Fisher’s exact test. Odds ratios (OR) were calculated using Pearson Chi-square or logistic regression models to adjust for covariates. Variables were excluded from multivariate models if >5% of the data was missing (to limit bias and reduction of power); the exception was thrombocytopenia, as platelets are a known source of Ang-1. Classification trees (CRT) were used to identify the biomarker cut-offs. The prognostic accuracy of biomarkers and individual laboratory findings were assessed using receiver operating 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 variable (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 (i.e. misclassifying deaths as survivors) 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.
8.4 RESULTS

8.4.1 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 CM. 50 subjects were retinopathy negative, 103 were retinopathy positive, and 2 died before fundoscopic exams could be performed. Fifty-nine (38.1%) of the children included in this study died. All subjects had plasma samples collected and clinical characteristics recorded at admission. The demographic and clinical characteristics and laboratory findings at admission are summarized in Table 8.1.

8.4.2 Biomarkers of Endothelial Activation are Associated with Malaria Retinopathy

Based on the hypothesis that endothelial activation and dysfunction are central to the pathophysiology of cerebral malaria, markers of endothelial activation were measured in children with retinopathy compared to those without. Venous lactate, a validated biochemical marker of disease severity, was included as a comparator (Waller, Krishna et al. 1995; Newton, Valim et al. 2005). Median Ang-1 levels were decreased (p=0.018), and median Ang-2 and sTie-2 levels were increased (p<0.0001) in subjects with retinopathy compared to those without retinopathy (Figure 8.1). Venous lactate was also more elevated in children with malarial retinopathy than without (p=0.004).

The relationship between endothelial biomarkers and retinopathy was further examined following dichotomization of biomarkers. Biomarker levels (Ang-1 ≤ 6.76ng/mL, Ang-2 > 3.85ng/mL, sTie-2 >67.8ng/mL) were associated with an increased odds of having retinopathy (Table 8.2, Supplementary Table 8.1: 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 (Table 8.1, Supplementary Table 8.1: age, respiratory distress, severe anemia, and thrombocytopenia).
Figure 8.1. 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 hemorrhage, retinal whitening or vessel changes. Analysis by Mann-Whitney (U statistic, p-value), †significant after Holms correction for 4 pair-wise comparisons: *p<0.05, **p<0.01, ***p<0.001. (A) Ang-1 (1969, p=0.018) †, (B) Ang-2 (1288, p<0.0001) †, (C) sTie-2 (1258, p<0.0001) †, and (D) venous lactate (1358, p=0.004) †.
8.4.3 Relationship Between Specific Retinal Changes and the Angiopoietin-Tie2 Axis

The distribution of retinal changes for this population is described in Figure 8.2. Whitening (94.5%) was the most common retinal change followed by vessel changes (76.9%) and hemorrhages (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 in the retinopathy-negative group had papilloedema alone.

We assessed the severity of retinal changes using a standardized clinical score (see Methods) (Lewallen, Harding et al. 1999; Harding, Lewallen et al. 2006). Ang-2 and sTie-2 concentrations correlated positively with the number of hemorrhages, the severity of retinal whitening, and the extent of white vessels and capillaries (Table 8.3: p<0.01). Venous lactate concentrations correlated 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 correlated negatively, albeit weakly, with papilloedema (p<0.05). Overall, Ang-2 and sTie-2 levels correlated with the severity of hemorrhage, retinal whitening, and white vessels (non-perfusion) but were not related to orange vessels (which probably represent parasite sequestration).
Figure 8.2. Venn-diagram depicting the distribution of retinal changes in the CM patients with retinopathy.

A Venn diagram depicting the distribution of retinal changes according to the number of children with hemorrhages, retinal whitening, or vessel abnormalities (orange vessels, white vessels, white capillaries). Data are presented as percentages followed by the number of children in each group. Detailed classification of retinal changes was available for 91 of the 103 retinopathy positive children. A total of 70.3% children had hemorrhages (n=64), 94.5% had retinal whitening (n=86) and 76.9% had vessel abnormalities (n=70).
8.4.4 The Angiopoietin-Tie-2 System is Associated with Disease Severity

The relationship between the endothelial biomarkers and disease severity was investigated using two-way correlations between the biomarkers (Ang-1, Ang-2 and sTie-2) and clinical and laboratory parameters recorded at admission (Table 8.4). Ang-1 was positively correlated with hematocrit 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 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), hematocrit (p<0.001) and platelet count (p<0.001). Finally, increases in Ang-2 were positively associated with pulse rate (p<0.01), respiratory rate (p<0.001), parasitemia (p<0.001), white blood cell count (p<0.01) and venous lactate (p<0.001). Taken together, these data indicate that Ang-2 is associated with several markers of disease severity, whereas Ang-1 was primarily associated with hematocrit and platelet counts.

8.4.5 Biomarkers of Endothelial Activation are Associated with Mortality

We next investigated the hypothesis that markers of endothelial activation would be associated with a poor prognosis. Increased median levels of Ang-2 and sTie-2 at admission, but not Ang-1, were associated with a fatal outcome (Figure 8.3, top panel A-D: p<0.0001). Venous lactate was also elevated in children who subsequently died (p=0.002). In the subset of children who were retinopathy positive, Ang-2 alone was significantly more elevated in fatal cases than in survivors (p=0.027). We generated receiver operating characteristic (ROC) curves to assess the prognostic accuracy of the biomarkers. Ang-2, sTie-2, and venous lactate had comparable areas under the ROC curve (AUC): 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 8.3, E-H). 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 8.3, I-L). 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).

The relationship between endothelial biomarkers and mortality were further explored using logistic regression. Biomarker levels were associated with increased odds of death (Table 8.2,
Supplementary Table 8.2; 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 (Table 8.2, Supplementary Table 8.2: age, respiratory distress, Blantyre coma score, and severe anemia).
Figure 8.3. 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 operating characteristic (ROC) curves and (I-L) decision plots of sensitivity and specificity generated from the ROC curves. Prognostic accuracy was assessed using receiver operating 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.). The vertical line represents the cut-point for dichotomizing the biomarkers by CRT or established criteria (hyperlactatemia).
8.4.6 Incorporating Ang-Tie2 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 the plasma biomarkers. A clinically predictive model of mortality was generated using parameters that 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 biomarker tests to determine whether they would significantly improve predictive accuracy. The clinical model including all three biomarkers had a c-index of 0.79 (0.72-0.84), which was significantly better than the clinical model alone (Figure 8.4, Supplementary Tables 8.3 & 8.4, p=0.03). 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 alone) led to a model that was significantly better than the clinical model alone (c-index (95% CI), p-value: 0.78 (0.68-0.82), p=0.016).
Multiple logistic regression models were generated for clinical variables associated with poor outcomes that are readily available to clinicians (age, Blantyre coma score, respiratory distress, severe anemia) and used to generate a “clinical” model to predict mortality. An additional model was constructed adding in the most informative biomarker, Ang-2, to determine whether biomarkers could enhance clinical prediction. The c-index (equivalent to the AUC for the predicted probabilities) was plotted for both models (c-index (95% CI): clinical model, 0.73 (0.65-0.79); clinical model plus Ang-2, 0.78 (0.71-0.84)) and compared using the method of Delong et al., (p=0.016). See supplementary Tables 8.3 and 8.4 for model validation.
CRT was used as an alternative method to develop a prognostic model. This intuitive approach generates a decision tree that is easy to interpret. CRT divides data into two mutually exclusive groups (“nodes”) in order to maximize the homogeneity within each node. When all variables significantly associated with death were entered into the analysis, a decision tree was generated using Ang-2 at a cut-off of 3.85ng/mL to discriminate between survivors and non-survivors. This model has 93.2% sensitivity to predict death and a misclassification rate of 23.1% (Figure 8.5A). Next, we explored models in which a clinical sign (BCS or respiratory distress) was entered as the first variable to integrate clinical parameters and reduce the number of biomarkers required for testing. In both clinically informed models, Ang-2 was useful for identifying non-survivors in comatose children considered lower risk (BCS 1 or 2 (as opposed to 0); Figure 8.5B, or children without respiratory distress; Figure 8.5C).
Figure 8.5. Classification and regression tree models for the prediction of mortality in children with cerebral malaria

Classification and regression tree analysis (CRT) was performed with mortality as the outcome measure including all significant univariable predictors of mortality with the 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).
8.5 DISCUSSION

In this study we provide evidence linking the angiopoietin-Tie-2 system to the clinical and pathological findings of cerebral malaria, and further suggest that the angiopoietins may be useful prognostic biomarkers in severe malaria. We demonstrate that perturbations in the angiopoietin-Tie-2 system are associated with retinopathic findings, which are likely reflective of pathological changes in the brain (White, Lewallen et al. 2001). Consistent with a role in malaria pathophysiology, elevated admission levels of Ang-2 and sTie-2, and decreased Ang-1 were associated with increased odds of subsequent death. 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 to regulate the vascular endothelium and the best characterised members of this family are Tie-2 ligands Ang-1 and Ang-2. Altered levels of Ang-1, Ang-2 and sTie-2 in this sample of children with severe malaria provide insights into a possible pathophysiologic role of the angiopoietin-Tie-2 system in mediating disease severity and outcome. In this study, elevated Ang-2 was associated with retinopathy (Figure 8.1, Table 8.2) and levels were positively correlated with the number of hemorrhages, the degree of retinal whitening, and the extent of white vessels and capillaries (Table 8.3). In the quiescent endothelium, Ang-2 is stored in intracellular vesicles called Weibel-Palade bodies, and is rapidly mobilized and released following cellular activation (Fiedler, Scharpfenecker et al. 2004). Ang-2 is thought to amplify endothelial activation by opposing the effects of Ang-1 (although it can exert partial agonist activity in the absence of Ang-1 (Yuan, Khankin et al. 2009)). Previous studies have demonstrated that Ang-2 sensitizes the endothelium to sub-threshold levels of TNF (Fiedler, Reiss et al. 2006), increases endothelial cell permeability (Fiedler and Augustin 2006), and promotes the upregulation of cellular adhesion molecules such as ICAM-1 (Fiedler and Augustin 2006). All of these activities would be expected to have detrimental effects in the context of cerebral malaria, since blood-brain-barrier dysfunction and binding of parasitized erythrocytes to cellular adhesion molecules have been implicated in pathogenesis. We hypothesize that Ang-2 promotes a positive feedback loop of endothelial activation, inflammation, and parasite sequestration. However, Ang-2 may simply be a reflection
of endothelial activation and hypoxia (Abdulmalek, Ashur et al. 2001) (Oh, Takagi et al. 1999) and further studies will be required to determine its role in severe malaria.

sTie-2 was also associated with retinopathy and malaria severity. sTie-2 has been identified in supernatants from cultured endothelial cells, is present in normal human serum and plasma (Reusch, Barleon et al. 2001), and has been identified as a biomarker in 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). It is shed from endothelial cells as a result of ectodomain cleavage by matrix metalloproteinases (Onimaru, Yonemitsu et al. 2010), and this cleavage can be stimulated by a variety of physiological factors, including basic fibroblast growth factor and vascular endothelial growth factor (Reusch, Barleon et al. 2001; Findley, Cudmore et al. 2007; Findley, Mitchell et al. 2008). To date, the function of sTie-2 is incompletely understood. sTie-2 can inhibit Ang-2 activity (Roviezzo, Tsigkos et al. 2005), and thus it is plausible that sTie-2 produced during malaria infection counters the potentially detrimental effects of 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 maintain the integrity of endothelial tight junctions through the phosphorylation of Tie-2 (Davis, Aldrich et al. 1996; Milner, Hansen et al. 2009). Like Ang-2 and sTie-2, Ang-1 was associated with retinopathy; however, this relationship was lost following adjustment for thrombocytopaenia. Platelet counts were reduced in retinopathy positive children, consistent with other reports (Lewallen, Bronzan et al. 2008; Chimalizeni, Kawaza et al.). Thus, decreased Ang-1 levels in retinopathy may reflect a decrease in platelet number and/or platelet Ang-1 content. We postulate that activated platelets bound to the damaged endothelium may play a physiologic role, releasing Ang-1 and limiting endothelial injury. 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.

An additional aim of this study was to investigate the prognostic value of endothelial biomarkers in comatose children with CM. Admission levels of sTie-2 and Ang-2 were elevated in fatal cases, and these biomarkers had discriminative value comparable to venous lactate, currently the best available biochemical marker for severe malaria in children (Figure 8.3) (Waller, Krishna et al. 1995; Newton, Valim et al. 2005). Admission plasma Ang-2 concentration remained a
significant independent predictor of mortality after controlling for covariates (age, BCS, respiratory distress, severe anemia; Table 8.2). This suggests that Ang-2 may have clinical utility as a prognostic biomarker. These data could be further used to add prognostic value above and beyond clinical parameters (Figure 8.4). These results are in agreement with those of Yeo et al. which show a significant association between Ang-2 levels and outcome (Yeo, Lampah et al. 2008).

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; Conroy, Lafferty et al. 2009), and correlates with clinical progression or deterioration when measured longitudinally over the course of illness (Yeo, Lampah et al. 2008). Here we show that Ang-2 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 affording a promising molecular target for development of theranostic tools (Bissonnette and Bergeron 2006).

The results of this study, while promising, must be interpreted with some caution. The retrospective case-control design may introduce bias. 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 may reduce the generalisability of the data. These results will need to be confirmed in larger prospective studies. Nonetheless, these data suggest that: i) the angiopoietin-Tie-2 system may be involved in CM pathogenesis; and ii) Ang-2 is a promising prognostic biomarker in severe malaria. Prognostic markers that could identify children with severe malaria at greatest risk of poor outcomes would enable better triage, and allocation of resources, and could select a high-risk group for evaluation of new adjunctive interventions.
### Table 8.1. Demographic and clinical characteristics of population at admission

<table>
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<tr>
<th></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>
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<td>Age (months), median (IQR)</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>
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<td>Hypotension</td>
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<td>4.1%</td>
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<td>3.2%</td>
<td>5.7%</td>
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<td>12.5%</td>
<td>11.9%</td>
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<tr>
<td>Severe anemia</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>Leukocytosis</td>
<td>36.8% c</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>Thrombocytopenia</td>
<td>77.3% d</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>Hypoglycemia</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>Hyperlactatemia</td>
<td>62.6% e</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>

* a n=147, b n=154, c n=144, d n=132, e n=131

d,e included in multivariable logistic regression models as covariates

Variables were dichotomized based on the following: hypotension (pediatric advanced life-support guidelines); malnutrition, weight for age z-scores (WHO 2007 reference values); hyperparasitemia (≥500,000 parasites/µL); severe anemia (<15 % hematocrit); leukocytosis (>12,000 leukocytes/ µL); thrombocytopenia (<150,000 x 10³ platelets/ µL); hypoglycemia (<2.2mmol/L); hyperlactatemia, >5mmol/L; and retinopathy (any one of: hemorrhage, whitening, vessel changes).
Table 8.2. Odds ratios (OR) for positive biomarker tests in retinopathy and mortality

<table>
<thead>
<tr>
<th></th>
<th>Retinopathy</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Low Ang-1</td>
<td>5.9 (2.7-12.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High Ang-2</td>
<td>10.6 (4.6-24.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HighTie-2</td>
<td>11.7 (3.9-35.0)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

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

a,b Adjusted P values: multivariable logistic regression model adjusting for covariates; retinopathy a- age, respiratory distress, severe anemia, thrombocytopenia; mortality b- age, respiratory distress, Blantyre coma score, severe anemia.

See supplementary Tables 8.1 and 8.2 for model validation.
Table 8.3. Plasma concentrations of the Angiopoietin-Tie-2 system and lactate correlate with the severity scores of retinal abnormalities

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Hemorrhage</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. Hemorrhage (0=none, 1=mild, 2=moderate, 3=severe, 4=very severe); Whitening, vessel changes (orange vessels, white vessels, white capillaries), and papilloedema (0=none, 1=mild, 2=moderate, 3=severe).

Orange vessels represent irregular narrowing of vessels and white vessels represent no perfusion.

*p<0.05, **p<0.001
Table 8.4. 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 (mmHg)</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>Parasitemia</td>
<td>0.092</td>
<td>0.432***</td>
<td>0.133</td>
</tr>
<tr>
<td>Hematocrit</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
### Supplementary Table 8.1: Parameters included in logistic regression model of endothelial biomarkers to predict retinopathy

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>Standard error</th>
<th>Wald statistic</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.046</td>
<td>0.014</td>
<td>11.578</td>
<td>0.95 (0.93-0.98)</td>
<td>0.001</td>
</tr>
<tr>
<td>Angiopoietin-1 (Cut-off: 6.76ng/mL)</td>
<td>1.026</td>
<td>0.622</td>
<td>2.721</td>
<td>2.8 (0.8-9.5)</td>
<td>0.099</td>
</tr>
<tr>
<td>Angiopoietin-2 (Cut-off: 3.85ng/mL)</td>
<td>1.458</td>
<td>0.624</td>
<td>5.461</td>
<td>4.3 (1.3-14.6)</td>
<td>0.019</td>
</tr>
<tr>
<td>sTie-2 (Cut-off: 67.8ng/mL)</td>
<td>2.273</td>
<td>0.792</td>
<td>8.249</td>
<td>9.7(2.1-45.8)</td>
<td>0.004</td>
</tr>
<tr>
<td>Respiratory Distress</td>
<td>0.700</td>
<td>0.668</td>
<td>1.100</td>
<td>2.0 (0.5-7.5)</td>
<td>0.294</td>
</tr>
<tr>
<td>Severe anemia</td>
<td>1.013</td>
<td>0.893</td>
<td>1.287</td>
<td>2.8 (0.5-15.9)</td>
<td>0.257</td>
</tr>
<tr>
<td>Thrombocytopenia†</td>
<td>2.324</td>
<td>0.750</td>
<td>9.612</td>
<td>10.2 (2.4-44.4)</td>
<td>0.002</td>
</tr>
<tr>
<td>Constant</td>
<td>-1.643</td>
<td>0.844</td>
<td>3.794</td>
<td>0.2</td>
<td>0.051</td>
</tr>
</tbody>
</table>

Model parameters: n=115 (n=43 retinopathy negative, n=72 retinopathy positive), Hosmer-Lemeshow test (Chi-square, df, p-value: 4.369, 8, 0.822), Nagelkerke R²: 0.670, and percentage correct: 84.3. † Included in analysis despite missing data because it is potential source for Ang-1. Note: analysis with or without thrombocytopenia shows the same trends for all other variables except Ang-1.
Supplementary Table 8.2: Multivariable logistic regression model to predict mortality using clinical parameters plus biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>Standard error</th>
<th>Wald statistic</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.008</td>
<td>0.007</td>
<td>1.311</td>
<td>0.99 (0.98-1.006)</td>
<td>0.252</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>0.899</td>
<td>0.449</td>
<td>4.005</td>
<td>2.5 (1.02-5.9)</td>
<td>0.045</td>
</tr>
<tr>
<td>(Cut-off: 5.89ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>1.359</td>
<td>0.604</td>
<td>5.063</td>
<td>3.9 (1.2-12.7)</td>
<td>0.024</td>
</tr>
<tr>
<td>(Cut-off: 3.85ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sTie-2</td>
<td>0.744</td>
<td>0.402</td>
<td>3.422</td>
<td>2.1 (0.96-4.6)</td>
<td>0.064</td>
</tr>
<tr>
<td>(Cut-off: 67.8 ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory Distress</td>
<td>0.548</td>
<td>0.399</td>
<td>1.881</td>
<td>1.7 (0.8-3.8)</td>
<td>0.170</td>
</tr>
<tr>
<td>Blantyre Coma Score</td>
<td></td>
<td></td>
<td>8.995</td>
<td></td>
<td>0.011</td>
</tr>
<tr>
<td>0 vs. 1</td>
<td>2.038</td>
<td>0.690</td>
<td>8.732</td>
<td>7.7 (2.0-29.7)</td>
<td>0.003</td>
</tr>
<tr>
<td>0 vs. 2</td>
<td>0.613</td>
<td>0.416</td>
<td>2.167</td>
<td>1.8 (0.8-4.2)</td>
<td>0.141</td>
</tr>
<tr>
<td>Severe anemia</td>
<td>0.405</td>
<td>0.458</td>
<td>0.780</td>
<td>1.5 (0.6-3.7)</td>
<td>0.377</td>
</tr>
<tr>
<td>Constant</td>
<td>-3.070</td>
<td>0.752</td>
<td>16.664</td>
<td>0.046</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Model parameters: n=155, Hosmer-Lemeshow test (Chi-square, df, p-value: 8.282, 8, 0.406), Nagelkerke $R^2$: 0.313, and percentage correct: 74.8, c-index: 0.794 (95% CI: 0.724-0.864).
### Supplementary Table 8.3. Multivariable logistic regression model to predict mortality using clinical parameters alone

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>Standard error</th>
<th>Wald statistic</th>
<th>df</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.010</td>
<td>0.006</td>
<td>2.442</td>
<td>1</td>
<td>0.99 (0.98-1.003)</td>
<td>0.118</td>
</tr>
<tr>
<td>Respiratory Distress</td>
<td>0.872</td>
<td>0.366</td>
<td>5.677</td>
<td>1</td>
<td>2.4 (1.2-4.9)</td>
<td>0.017</td>
</tr>
<tr>
<td>Blantyre Coma Score 0 vs. 2</td>
<td>1.771</td>
<td>0.609</td>
<td>8.445</td>
<td>1</td>
<td>5.9 (1.8-19.4)</td>
<td>0.004</td>
</tr>
<tr>
<td>Blantyre Coma Score 1 vs. 2</td>
<td>0.570</td>
<td>0.395</td>
<td>2.086</td>
<td>1</td>
<td>1.8 (0.8-3.8)</td>
<td>0.149</td>
</tr>
<tr>
<td>Severe anemia</td>
<td>0.736</td>
<td>0.438</td>
<td>2.823</td>
<td>1</td>
<td>2.1 (0.9-4.9)</td>
<td>0.093</td>
</tr>
<tr>
<td>Constant</td>
<td>-1.134</td>
<td>0.449</td>
<td>6.371</td>
<td>1</td>
<td>0.3</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Model parameters: n=155, Hosmer-Lemeshow test (Chi-square, df, p-value: 7.878, 8, 0.445), Nagelkerke $R^2$: 0.209, and percentage correct: 67.1, c-index: 0.725 (95% CI: 0.641-0.808).
Supplementary Table 8.4: Multivariable logistic regression model to predict mortality using clinical parameters plus Ang-2

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>Standard error</th>
<th>Wald statistic</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.010</td>
<td>0.007</td>
<td>1.998</td>
<td>0.99 (0.98-1.004)</td>
<td>0.157</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>1.755</td>
<td>0.589</td>
<td>8.888</td>
<td>5.8 (1.8-18.3)</td>
<td>0.003</td>
</tr>
<tr>
<td>(Cut-off: 3.85ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory Distress</td>
<td>0.620</td>
<td>0.385</td>
<td>2.590</td>
<td>1.9 (0.9-4.0)</td>
<td>0.108</td>
</tr>
<tr>
<td>Blantyre Coma Score</td>
<td></td>
<td></td>
<td>8.028</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 vs. 1</td>
<td>1.800</td>
<td>0.641</td>
<td>7.873</td>
<td>6.0 (1.7-21.3)</td>
<td>0.005</td>
</tr>
<tr>
<td>0 vs. 2</td>
<td>0.560</td>
<td>0.406</td>
<td>1.906</td>
<td>1.8 (0.8-3.9)</td>
<td>0.167</td>
</tr>
<tr>
<td>Severe anemia</td>
<td>0.523</td>
<td>0.448</td>
<td>1.358</td>
<td>1.7 (0.7-4.1)</td>
<td>0.244</td>
</tr>
<tr>
<td>Constant</td>
<td>-2.413</td>
<td>0.672</td>
<td>12.900</td>
<td>0.090</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Model parameters: n=155, Hosmer-Lemeshow test (Chi-square, df, p-value: 5.760, 8, 0.674), Nagelkerke R²: 0.292, and percentage correct: 72.3, c-index: 0.780 (95% CI: 0.706-0.854)
Chapter 9
Summary and Future Directions

9.1 Malaria in Pregnancy

9.1.1 C5a in Placental Malaria

9.1.1.1 Summary

Based on previous work highlighting the importance of C5a in the pathogenesis of experimental cerebral malaria and in murine models of recurrent pregnancy loss (Girardi, Yarilin et al. 2006; Patel, Berghout et al. 2008), we sought to evaluate the role of C5a in placental malaria. In Chapter 2 we investigated the role of C5a in malaria using an in vitro system: first investigating whether *P. falciparum* could activate C5 and then asking whether an inflammatory byproduct of parasite rupture, *Pf*GPI, could affect C5a receptor (C5aR; CD88) expression. Since we found increased C5a in serum exposed to *P. falciparum* and increased C5aR induction on monocytes, we pursued co-stimulation experiments where we treated mononuclear cells with C5a and *Pf*GPI. The accumulation of mononuclear cells in the placenta is one of the most well established histological findings associated with poor outcomes in PM (Rogerson, Pollina et al. 2003), with alterations in inflammatory cytokines and chemokines a common finding (Fried, Muga et al. 1998; Moormann, Sullivan et al. 1999; Abrams, Brown et al. 2003; Rogerson, Pollina et al. 2003; Suguitan, Leke et al. 2003; Chaisavaneeyakorn, Lucchi et al. 2005). We saw a pronounced interaction between C5a and *Pf*GPI where a number of inflammatory proteins (IL-6, IL-8, MCP-1, MIP1β, MIP1α, TNF and IL-10) and an anti-angiogenic protein (sFlt-1) were synergistically induced. This interaction was dependent on the C5aR, as levels of these proteins returned to baseline levels of *Pf*GPI stimulation following C5aR blockade. As a proof-of-concept, we measured C5a levels in a group of PG from Kenya with placental malaria (PM) and found increased C5a levels in both peripheral and placental plasma. Based on these promising findings we wanted to further investigate the relationship between C5a and angiogenesis. This was possible in the large case-control study of women from Malawi with PM where changes in biomarkers could be associated with birth outcome.
In the Malawian study, C5a levels were significantly elevated in both PG and MG women with placental malaria. There were also significant changes in angiogenic factors Ang-1, Ang-2, sFlt-1 and sEng in the placental blood. Increased levels of systemic C5a (>100ng/mL) were associated with an increased odds of delivering an infant that was small-for-gestational-age. 100ng/mL corresponded to the concentration of C5a that induced the synergistic proinflammatory and anti-angiogenic response in vitro (~10nM). In order to test our hypothesis that C5a may drive dysregulated angiogenesis in PM, we turned to a multivariate statistical technique that can model multiple dependent and independent variables simultaneously and use composite measures to estimate concepts, like angiogenesis. We investigated a model where C5a was upstream (i.e. an initiating event) of the observed dysregulated angiogenic response that contributed to fetal growth restriction. This model fit a scenario where C5a was produced by mononuclear cells (rather than being activated by PEs and acting as a chemoattractant for mononuclear cells), thus providing the first evidence (albeit indirect) that C5a may be activated in a C3-independent manner in malaria. This is consistent with reports that activated immune cells can activate C5 directly (discussed further below).

In order to provide direct experimental evidence to support the human data, a murine model of PM was used. Ablation of C5a-C5aR signaling resulted in the partial rescue of fetal weight and was characterized by a shift in angiogenic proteins towards a more pro-angiogenic state. These changes corresponded to structural and functional changes in placental vascular remodeling where C5aR^{-/-} mice had an increased number of fetal vessels and decreased vascular resistance in the presence of malaria infection compared to wild type animals. Together, these data support the assertion that C5 activation can impair fetal growth in PM by preventing the vascular remodeling that is necessary to support normal fetal growth.

9.1.1.2 Future Directions

While the data from Chapters 2 and 3 come together to present a cohesive story of dysregulated complement activation and its impact on angiogenesis in PM, a few questions remain regarding the source of C5a and the physiologic impact of altered angiogenic factors on fetal development over the course of pregnancy.

Elucidating the pathways leading to C5a production in PM could lead to an improved understanding of early mediators of disease pathology and may identify potential targets for
intervention. Results from the structural equation model in Chapter 3 suggest that mononuclear cells in the placental intervillous space can activate C5. This is consistent with other reports describing direct C5 cleavage from activated leukocytes (Ward and Hill 1970; Vogt 1996; Vogt 2000; Huber-Lang, Younkin et al. 2002; Fukuoka, Xia et al. 2008). C5 can also be directly activated by platelets or pathogen derived proteases (Maruo, Akaike et al. 1997; Huber-Lang, Sarma et al. 2006). Recently, collagen activated platelets were shown to activate C5 in a thrombin-dependent manner and these platelets also showed a concomitant upregulation of the C5aR (Martel, Cointe et al. 2011). As placental malaria is associated with thrombocytopenia and it has been postulated that coagulopathy may be a central component of malaria pathogenesis (Mbanya, Tayou Tagny et al. 2007; Francischetti, Seydel et al. 2008; Tan, McGready et al. 2008), it would be interesting to examine interactions between coagulation and complement activation in the context of PM. Increased tissue factor expression has been associated with poor outcomes in a murine model of PM and the majority of placental mononuclear cell infiltrates in PM are positive for tissue factor (Imamura, Sugiyama et al. 2002; Poovassery, Sarr et al. 2009), thus it is possible that platelet activation in the placenta may contribute to C5a production, inflammation and dysregulated angiogenesis.

It will also be important to validate the Malawian findings from Chapter 3 in a prospective longitudinal study. Repeated assessment of malaria infection (at scheduled antenatal visits and during any febrile episode) alongside functional readouts of placental insufficiency, including increased placental vascular resistance (assessed using Doppler ultrasonography) and fetal growth, could be correlated with aberrations in complement activation and angiogenesis. Using this design, it would be possible to model the relationship between complement activation, dysregulated angiogenesis, inflammation and functional changes in blood flow and fetal growth restriction at various points in pregnancy. Important considerations when trying to model the effect between C5a and angiogenesis on fetal growth would be the timing, duration, and magnitude of biomarker changes. In addition, alternative models of PM pathogenesis defined a priori (including additional inflammatory parameters) could be compared with the structural equation model presented in Chapter 3 and to identify the model that best explains the data.

Results from Chapter 2 and 3 suggest that CD88 is the dominant C5a receptor in placental malaria, as C5aR blockade in vitro completely abrogated the C5a-induced synergistic inflammatory and anti-angiogenic response to PfGPI. Further, blockade of C5L2 in the murine
model of PM did not provide any additional improvement in fetal outcomes in addition to C5aR deletion. These findings support early reports that suggested C5L2 was a non-signaling receptor with potential anti-inflammatory effects through its clearance of C5a from the circulation (Okinaga, Slattery et al. 2003; Scola, Johswich et al. 2009).

**9.1.2 Biomarkers of Placental Malaria**

**9.1.2.1 Summary**

During pregnancy, it can be difficult to ascertain when a woman has placental malaria due to the propensity of parasites to sequester in the intervillous space. Data from Ghana suggest that peripheral blood microscopy may have sensitivity as low as 42% to detect placental malaria (Mockenhaupt, Ulmen et al. 2002). Among women who are correctly identified as having malaria in pregnancy, it is difficult to know whether malaria has led to intrauterine growth restriction or other manifestations of fetal distress. As diagnostics in pregnancy are limited in resource constrained settings, the identification of biomarkers present in the maternal circulation that have diagnostic utility (to detect PM and/or fetal growth restriction) could be transformative.

We first approached this problem with a candidate biomarker in mind, sEng. As a study from Gabon found children with severe malaria had elevated sEng levels which correlated with disease severity (Dietmann, Helbok et al. 2009). Further, a number of studies have identified sEng as a putative predictive biomarker for preeclampsia (Levine, Lam et al. 2006; Romero, Nien et al. 2008); therefore, we asked whether sEng would be a biomarker of malaria infection and fetal growth restriction in pregnancy (Chapter 4). Because sEng is being developed as a putative biomarker in pregnancy, it would be an ideal candidate biomarker to translate for use in malaria in the developing world. Unfortunately, we observed modest dynamic range in sEng levels in women from either Cameroon or Malawi and these findings would seem to preclude the use of sEng as a biomarker in the context of malaria infection during pregnancy.

Despite this, there were interesting findings in the study that may be relevant to understanding pathogenic conditions in pregnancy: sEng was elevated in all primigravidae, regardless of infection status. This may explain, at least partly, why primigravidae are at increased risk of pregnancy complications (including preeclampsia). sEng levels were also elevated with malaria infection in women from both Cameroon and Malawi independent of maternal gravidity or
gestational age. Elevated levels of sEng were also observed in primigravidae with PM who delivered growth restricted (but not preterm) low birth weight infants. These data are important because there are few studies showing significant associations between host proteins and LBW outcomes (Fried, Muga et al. 1998; Rogerson, Brown et al. 2003; Kabyemela, Fried et al. 2008; Silver, Zhong et al. 2010), and even fewer that differentiate between adverse outcomes of different etiology (fetal growth restriction, preterm delivery) (Moormann, Sullivan et al. 1999; Suguitan, Cadigan et al. 2003). The agreement between findings in Cameroon and Malawi, two geographically distinct populations with different malaria endemicities and background levels of HIV lends credence to the idea of angiogenic dysregulation reported in Chapter 3.

Finally, in Chapter 5 we examined a panel of angiogenic and inflammatory proteins as potential host biomarkers of occult PM (i.e. we excluded all cases of PM that were identifiable by peripheral blood smear leaving only the cases of “hidden” PM). We identified three putative biomarkers in the maternal peripheral blood that were altered in occult PM: sFlt-1, CRP and leptin. We devised a simple scoring system of dichotomizing the biomarkers as abnormal (1-point) or normal (0-points) and evaluated the discriminatory ability of various models (including clinical parameters associated with occult PM). We found that biomarkers could improve the identification of occult PM compared to clinical parameters alone. This may be increasingly important as drug resistance to SP, the main IPTp drug, increases (as is the case in Malawi).

9.1.2.2 Future Directions

As mentioned above, a prospective study that could longitudinally document PM infections, along with information on angiogenic factors, placental vascular resistance and fetal outcome, would be powerful in enhancing our understanding of angiogenic dysregulation during PM. Such a study design would also be a robust way to compare different methods (e.g. point-of-care biomarker or rapid diagnostic tests) of identifying occult or submicroscopic PM. At each antenatal visit (or febrile episode), blood could be collected for biomarker assessment, RDT, malaria smear and parasite quantification by real-time qRT-PCR. Early studies using RDTs to detect PM showed mixed results (Leke, Djokam et al. 1999; Malhotra, Dent et al. 2005; Singh, Saxena et al. 2005); however, improved heat-stability, performance, and acceptance of RDT in remote areas where trained microscopists are lacking suggest it may be worth reassessing the utility of RDTs for detecting occult PM. Advances in molecular diagnostics have led to the
development of platforms capable of measuring multiple analytes (e.g. parasite antigen + biomarkers) thus making it possible to combine biomarkers with parasite antigen to potentially improve identification of occult PM and identify pregnancies at risk of poor outcomes. In the meantime, RDTs could easily be integrated into routine antenatal care that included intermittent screening and treatment of malaria in pregnancy.

9.2 Endothelial Activation in CM

In this thesis, three studies (Chapter 6-8) are presented that examine endothelial activation and angiogenic biomarkers in severe and cerebral malaria. In the first (Chapter 6), we measured Ang-1 and Ang-2 in whole blood samples obtained from Thai adults with uncomplicated or severe malaria. This study demonstrated that these proteins are robust measures of endothelial activation and disease severity that are quantifiable in unprocessed whole blood samples. This study represented a logical follow-up to previous studies identifying Ang-1 and Ang-2 as potential biomarkers of malaria disease severity and mortality (Yeo, Lampah et al. 2008; Lovegrove, Tangpukdee et al. 2009). Because the whole blood data indicated that the angiopoietins would be good candidate biomarkers that could be incorporated into a lateral flow immunochromatographic device, we went on to validate these biomarkers in a population of Malawian children with retinopathy-validated CM.

In Chapter 7 we compared the discriminatory ability of the angiopoietins alongside other putative biomarkers (mostly of endothelial origin) in malaria. We asked how levels differed in uncomplicated malaria versus retinopathy-confirmed CM and a non-malaria febrile illness control group that also had signs of decreased consciousness. In this study, we selected only children who survived their infection so that we could compare biomarker levels at admission to those at convalescence. In contrast, in Chapter 8 we focus only on severe malaria and compare levels of angiopoietins in children with CM with and without retinopathic findings and relate admission levels of biomarkers with clinical measures of disease severity, retinal indicators of disease pathology, and mortality.
9.2.1 Insights into Pathophysiology

9.2.1.1 Summary

When considering all data on endothelial activation and CM together, a consistent pattern emerges of endothelial activation and dysfunction in severe and fatal malaria. In the two studies comparing different clinical syndromes, Ang-1 was the preferred biomarker (highest AUC) to differentiate between uncomplicated malaria (and other febrile illness) and cerebral malaria in both Thai adults and Malawian children. However, in those with severe disease, Ang-1 was not as informative. Ang-1 did not correlate with the cumulative organ injury score in Thai adults and it was correlated with only a few markers of disease severity in Malawian children in Chapter 8 (only BCS, platelet count and hemoglobin levels). Thus, there appears to be a pronounced drop in Ang-1 levels in severe disease, but among those with severe disease, Ang-1 seems to offer little additional information.

Conversely, Ang-2 appears to be an independent and quantitative measure of disease severity and prognosis in malaria. Ang-2 was correlated with the cumulative organ injury score in Thai adults with severe malaria and was associated with retinopathy (Chapter 7, 8) and mortality (Chapter 8) in Malawian children with clinical CM. Ang-2 was also correlated with a number of clinical measures of disease severity, including lactate levels, white blood cell counts, hemoglobin, respiratory rate, platelet count, etc. sTie-2, which was only measured in the latter two chapters, was correlated with Ang-2 and elevated in retinopathy and mortality, but did not have the same dynamic range or prognostic utility as Ang-2.

Together, the angiopoietin data support a model of endothelial activation in severe malaria characterized by decreased Ang-1, increased Ang-2 and increased shedding of their cognate receptor, Tie-2. While these findings highlight the importance of the angiopoietin-Tie-2 system in malaria pathogenesis, they represent a systemic view of endothelial activation. Questions remain regarding endothelial activation at the tissue and cellular level in malaria.

9.2.1.2 Future Directions

As the endothelium represents a heterogeneous organ system where different vascular beds show differential susceptibility to exogenous stimuli, studies designed to evaluate organ-specific vascular pathologies could provide invaluable insight into specific manifestations of disease. The
endothelium in the brain represents a highly restrictive barrier supported by underlying pericytes. One outstanding question worth addressing is the source of Ang-1 (platelet vs. pericyte). The positive correlation between Ang-1 and platelet count suggests that at least some of the Ang-1 measured is platelet derived. However, it is unclear what biological significance, if any, reduced platelet Ang-1 would have. The results herein show that Ang-1 is an excellent discriminator between uncomplicated and severe malaria. A recent report from Malawi examining a large cohort of children with CM (n=1811) and meningitis (n=521) suggested that platelet counts could differentiate between these two clinically similar syndromes (Chimalizeni, Kawaza et al. 2010) and platelet accumulation has been observed in the cerebral vasculature in fatal CM (Grau, Mackenzie et al. 2003). These data plus the preliminary results showing good discrimination between CM and CNS controls in Chapter 7 suggest that Ang-1, if platelet derived, may a specific marker of severe malaria. In this case, Ang-1 may represent a good biomarker to differentiate between comas of different etiology, but may not provide information on endothelial function per se. On the other hand, studies examining Ang-1 alongside parameters of platelet activation including platelet count, size, granularity, etc. may increase our understanding of CM pathology. If studies indicate that Ang-1 is platelet, rather than pericyte derived, then it will be important to ascertain whether decreased Ang-1 is causally involved in pathology, or is just a biomarker of pathophysiology. A causal association between decreased Ang-1 and the development of severe malaria may point to potential adjunctive therapies in severe malaria.

Alternatively, decreased levels of Ang-1 in CM may reflect decreased pericyte production of Ang-1. It is possible that malaria can impair the bidirectional signaling at endothelial-pericyte interactions. In an acute TLR-2 induced model of vascular leak in mice, pericytes exhibited structural changes, were more loosely attached to the endothelium, and reduced in number (Fuxe, Tabruyn et al. 2011). Further, the transient reduction in pericyte coverage corresponded with extravasation of microspheres in areas with reduced or no pericyte coverage (Fuxe, Tabruyn et al. 2011). Based on these findings, it would be interesting to examine pericyte coverage in murine models of cerebral malaria, where endothelial-pericyte interactions could be visualized and quantified using retinal whole mounts. The interaction of the endothelium and underlying pericyte layer could be further investigated by quantifying Ang-1 transcript levels in dermal biopsy samples from subjects with uncomplicated vs. cerebral malaria using real-time qRT-PCR.
In unstimulated cultured endothelium, Tie-2 is broadly expressed at the plasma membrane; however, following the *in vitro* stimulation of endothelial cells with Ang-1, Tie-2 is translocated to cell-cell junctions where it forms homotypic Tie-2-\textit{trans}-associated complexes (Fukuhara, Sako et al. 2008; Saharinen, Eklund et al. 2008). It is unknown whether a similar pattern of Tie-2 expression occurs \textit{in vivo} or how endothelial activation (though decreased Ang-1 and/or increased Ang-2) affects localization of Tie-2. Studies using experimental murine models and dermal biopsy samples obtained from individuals with uncomplicated vs. severe falciparum malaria could provide insight into Tie-2 localization during different endothelial activation states.

**9.2.2 Utility as Prognostic Biomarkers**

Measurement of circulating biomarkers can provide unique information on pathogenic processes during disease. This information can be used to design new interventions or to better understand disease systems. However, the ultimate goal for a biomarker is for it to improve clinical decision making. Thus, in the final data chapter (Chapter 8) we examined the ability of Ang-1, Ang-2 and sTie-2 to predict fatal outcomes in cerebral malaria and compared the predictive ability of the biomarkers to that of an established prognostic marker, venous lactate.

**9.2.2.1 Summary**

By univariable analysis, Ang-2 and venous lactate both had moderate prediction of mortality and were associated with many parameters of disease severity. However, when we attempted to integrate the biochemical and biomarker data with clinical predictors of disease severity, Ang-2 (not lactate) was able to improve clinical prediction. In order to verify the importance of Ang-2 in this subset of samples, we used two complementary approaches, logistic regression and classification using CRT. While Ang-2 was an independent predictor of mortality in this cohort, it was biased towards severely ill children. Nonetheless, these data support the utility of Ang-2 as a marker of disease severity and outcome in severe malaria.

**9.2.2.2 Future Directions**

In Chapter 8, we show that Ang-2 is independently predictive of mortality and that it adds significant value to a clinically based predictive model. Interestingly, venous lactate did not improve prediction in this model. This is in agreement with a large multicentre trial conducted in
African children that found acid base status, while associated with mortality, did not appreciably improve clinically prediction (Newton, Valim et al. 2005). Moving forward, efforts should be made to include side-by-side comparisons of promising biomarkers in order to identify the best predictors. It will also be useful to evaluate biomarkers in other infectious diseases and conditions of critical illness to assess the applicability of the biomarkers in other life-threatening conditions. While it may be preferable to have markers that are highly specific for certain diseases, this is unlikely in the context of host biomarkers. Rather, the inherent redundancy of the host immune system makes it likely that common pathways of disease pathogenesis may be similarly elevated in conditions of critical illness and thus Ang-2 may be a good biomarker of malaria, as well as sepsis, and other conditions characterized by endothelial activation and systemic inflammation (Parikh, Mammoto et al. 2006; Orfanos, Kotanidou et al. 2007; Giamarellos-Bourboulis, Kanellakopoulou et al. 2008; Yeo, Lampah et al. 2008). It will be important to integrate these tests alongside traditional pathogen-specific markers to ensure appropriate treatment of the associated infectious agent is maintained, while improving supportive or adjunctive treatment. Different combinations and permutations of biomarkers could be applied to different disease states as “biosignatures” in specific diseases to enhance specificity and identify individuals likely to benefit from targeted adjunctive therapy. In addition, surrogate biomarkers could be introduced in studies testing adjunctive therapies enabling smaller studies.

Rapid diagnostic tests that include biomarkers are amenable for use in resource poor settings because they circumvent the need for expensive equipment, consistent power supply and skilled personnel—all of which are often in short supply in resource constrained settings. Recent advances in molecular diagnostics can facilitate the development of low cost and high quality point-of-care biomarker platforms (Bissonnette and Bergeron 2006) and could lead to significant improvements in the identification and management of malaria.
References


