Anti-Malarial Strategies Among Vulnerable Populations: exploring bed net underutilization among internally displaced persons and novel adjunctive therapies for cerebral malaria

by

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ABSTRACT

Background: Despite declining incidence globally, malaria morbidity and mortality remain elevated among internally displaced persons (IDPs) and children under five, particularly in sub-Saharan Africa. Malaria prevention and treatment efforts in the Democratic Republic of the Congo (DRC) are hindered by armed conflict, which has resulted in the forced displacement of an estimated 1.5 million individuals. Among children under five, cerebral malaria (CM) represents a severe manifestation that may result in death or developmental disability. This thesis has two major focuses: 1) to describe the burden of malaria and reasons for the underutilization of bed nets, a key intervention in malaria control efforts, among IDPs in Eastern DRC; and 2) to test recently licensed pharmaceuticals as putative neuroprotective agents in an *in vitro* model of CM.

Methods: 1) A cross-sectional survey was conducted in Lushebere IDP camp in Eastern DRC to describe the burden of malaria in the population. A second cross-sectional survey was conducted in Birambizo IDP camp in Eastern DRC, supplemented with qualitative descriptions by IDP camp residents, neighbouring villagers, and health workers from the camp, to describe reasons for use and disuse of bed nets among IDPs. 2) An *in vitro* model of the blood brain barrier (BBB) was created using endothelial and astrocyte cells in a transwell system to test select pharmacological agents in their ability to prevent BBB breakdown, measured as a function of changes in transendothelial electrical resistance (TEER). Three challenges were independently used to compromise endothelial monolayer integrity: tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF) and *Plasmodium falciparum* infected red blood cells (Pf-IRBCs). Pharmacological agents are recently licensed and were tested at physiologically plausible concentrations.

Results: The proportion of IDP camp residents who tested positively for malaria via rapid diagnostic test was high, particularly among children under five (61% of febrile children under 5 in Lushebere, and 58% of children under 5 in Birambizo). Despite free bed net distribution campaigns throughout both camps, bed net ownership and use was low (36% in Lushebere and 29% in Birambizo). Focus group discussions revealed several pragmatic barriers to bed net use within the camp setting and competing needs for

nutrition which often drove individuals to sell or exchange their nets in order to feed their families. In our *in vitro* studies of CM, three pharmacologic agents were successful in rescuing BBB break down induced by either TNF, VEGF, or Pf-IRBCs: fingolimod, sunitinib, and pazopanib. If successful in future *in vivo* studies and human clinical trials, these recently licensed agents may be repurposed for use as adjunctive therapies in CM.

Conclusions: These studies highlight the burden of malaria among two key vulnerable populations: IDPs and children under five. Our findings call attention to the need for new and improved malaria control and treatment efforts within these populations. Current malaria vector control and cerebral malaria adjunctive therapies are incompletely effective for IDPs in regions of sub-Saharan Africa and children under five, respectively. Global efforts to further reduce malaria associated morbidity and mortality should not forget vulnerable and often hard-to-reach populations, and efforts should be tailored to meet the individual needs of these large populations if progress is to be made.

PREFACE

Some of the research conducted for this thesis forms part of an international research collaboration, led by my supervisor, Dr. Michael Hawkes of the University of Alberta. Data from Chapter 2 was published as Brooks HM, Paul J, Katsuva M, Claude KM, Houston S, Hawkes MT. Malaria in an Internally Displaced Persons Camp in the Democratic Republic of the Congo. Clinical Infectious Diseases. 2017 Jun 15. I was responsible for data analysis and manuscript preparation. MH was the corresponding author who conceptualized and designed the study, oversaw collection of data, and critically reviewed the manuscript. MKJP and KMC collected the data, and SH critically reviewed the manuscript. Data from Chapter 3 has been accepted and is in press and will be published as Brooks HM, Katsuva M, Claude KM, Mocanu V, Hawkes MT. Use and disuse of malaria bed nets in an internally displaced persons camp in the Democratic Republic of the Congo: a mixed methods study. PLoS ONE. 2017. I contributed to the study design, and was responsible for data analysis and manuscript preparation. MH was the corresponding author who conceptualized and designed the study, oversaw collection of data, and critically reviewed the manuscript. MKJP and KMC collected the data. VM contributed to data analysis and critically revised the manuscript. The literature review in Chapter 4 (sections 4.1 and 4.3) was published as Brooks HM, Hawkes MT. Repurposing Pharmaceuticals as Neuroprotective Agents for Cerebral Malaria. Current clinical pharmacology. 2017 Jul 4. Dr. Michael Hawkes and I were jointly responsible for the conceptualization and preparation of the manuscript.

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LIST OF ABBREVIATIONS

ACT artemisinin combination therapy
AM astrocyte medium
ANC antenatal care
BBB blood brain barrier
CM cerebral malaria
CNS central nervous system
CSF cerebral spinal fluid
DCA dichloroacetate
DRC Democratic Republic of the Congo
ECIS electrical cell impedance sensing
ECM experimental cerebral malaria
ELISA enzyme-linked immunosorbent assay
EM endothelial cell medium
EPO erythropoietin
FGD focus group discussion
HAST human astrocyte cell
HBMEC human brain microvascular endothelial cell
HCMEC/D3 human cerebral microvascular endothelial cell
HRP2 histidine-rich protein-2
ICAM-1 intracellular adhesion molecule-1
IDP internally displaced person
iNO inhaled nitric oxide
IPTp intermittent preventative therapy in pregnancy
IRS indoor residual spraying
ITN insecticide treated bed net
LLIN long lasting insecticide treated net
NO nitric oxide
NGO non-governmental organization
NVU neurovascular unit
P. falciparum *Plasmodium falciparum*
PCA principle component analysis
PE parasitized erythrocyte
PfEMP1 *Plasmodium falciparum* endothelial membrane protein 1
Pf-IRBC *Plasmodium falciparum*-infected red blood cells
PlGF placental growth factor
PTX pentoxifylline
RBC red blood cell
RCT randomized controlled trial
RDT rapid diagnostic test
S1P sphingosine-1-phosophate
S1PR sphingosine-1-phosophate receptor
SDG sustainable development goal
sICAM-1 soluble ICAM-1
SK sphingosine kinase
sVEGFR1 soluble VEGF receptor-1
TKI tyrosine kinase inhibitor
TNF tumour necrosis factor
TW transwell
uRBC uninfected red blood cell
VEGF vascular endothelial growth factor
VEGFR vascular endothelial growth factor receptor
WHO World Health Organization
ZO zonula occludens
ZO-1 zonula occludens protein 1

CHAPTER 1 – INTRODUCTION TO MALARIA

1.1. ETIOLOGY

Malaria is a leading cause of child mortality worldwide, accounting for an estimated 429,000 deaths annually [1]. Approximately 90% of malaria cases occur in sub-Saharan Africa, where children under 5 account for 70% of global malaria deaths [1]. Most deaths due to malaria are caused by *Plasmodium falciparum*, the predominant parasite species in Africa, and the focus of this thesis. Other *Plasmodium* species that can cause malaria include *P. vivax, P. ovale, P. malariae,* and *P. knowlesi.* These parasites are transmitted to humans through the bites of female *Anopheles* mosquitoes, which act as the disease vector.

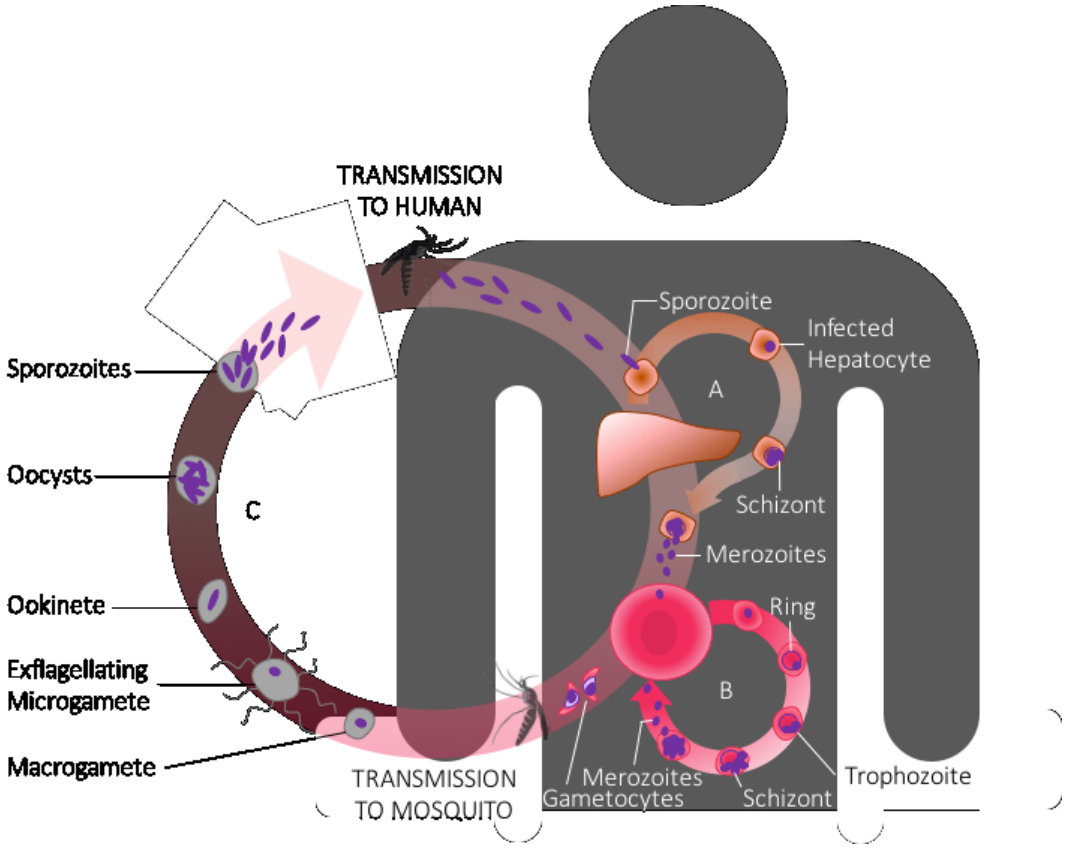

Figure 1.1 Lifecycle of *Plasmodium falciparum*. During the course of its lifecycle, *P. falciparum* must infect two hosts, the mosquito and the human, and go through various cycles including A) the exo-erythrocytic cycle in the human liver, B) the erythrocytic cycle in human red blood cells, and C) the sporogenic cycle in mosquitoes.

The *P. falciparum* life cycle requires successive infection of two hosts: humans and female *Anopheles* mosquitoes (Figure 1.1). In the mosquito, the parasite undergoes its sporogenic cycle, whereby sporozoites are generated; sporozoites can then be released from the mosquito's saliva and injected into the human host as it takes its next blood meal. In humans, the parasite first replicates within the liver, then infects red blood cells (RBCs) and travels throughout the body. When in the blood stage, the parasite causes symptoms of malaria. Mosquitoes then ingest *P. falciparum* gametocytes, the sexual blood stage forms, as they take a blood meal from an infected human. Transmission of malaria is highest in regions where the environment promotes mosquito breeding, such as the tropics, and regions where mosquitoes are more likely to bite humans rather than other mammals. For this reason, worldwide and countrywide differences exist in the prevalence of malaria.

1.2. EPIDEMIOLOGY

Globally, there have been large reductions in the number of malaria cases and deaths over the past two decades; however, malaria remains an important public health threat. Malaria is most prevalent in impoverished areas with limited resources for disease identification, treatment, and reporting. According to the World Health Organization (WHO) Malaria Report 2015, 106 countries remain at risk of malaria transmission [2]. An increasing number of countries are moving towards malaria elimination, yet it is estimated that 114 million individuals were infected with malaria in sub-Saharan Africa in 2015 alone [1]. The global burden of morbidity and mortality is dominated by sub-Saharan African countries, especially the Democratic Republic of the Congo (DRC) and Nigeria, which together account for more than 35% of the total estimated global malaria deaths [2]. These data demonstrate that despite global efforts, millions of individuals remain at risk of infection and lack access to services required for prevention and treatment.

1.2.1 Vulnerable Populations

Among those most vulnerable to infection are pregnant women, children under five years, and displaced individuals. Pregnancy reduces a woman's immune response to various infectious diseases, including malaria, and may result in harm to both the mother and the fetus if infection occurs [3-5]. Children under the age of five have yet to develop an effective adaptive immune response, and are

therefore particularly susceptible to infection and rapid disease progression [6]. Displaced individuals are very mobile and are therefore difficult to reach and often lack stable health infrastructure, therefore prevention, diagnosis, and treatment efforts are compromised [7-9]. The research contained in this thesis focuses on children under five and displaced individuals who are affected by malaria.

1.2.2 Children Under Five

It is estimated that malaria claims the life of a child every two minutes [1]. Despite impressive reductions in the number of malaria deaths since 2000, an estimated 306,000 malaria deaths occurred in children under five years of age, in 2015 alone [2]. In 2015, malaria was no longer the leading cause of death among children under five in sub-Saharan Africa; however, malaria remains a major cause of childhood morbidity and mortality in sub-Saharan Africa, responsible for 10% of all deaths among children under five in the region [2].

In malaria-endemic regions, severe malaria is mainly a disease of children under the age of five, becoming less common with increasing age due to protection from specific acquired immunity. Features of severe malaria that are more common in children than adults include severe anemia, hypoglycemia, and cerebral malaria (CM). The duration of severe malaria is much shorter in children than in adults (1-2 days versus 5-7 days) and therefore rapid diagnosis and treatment are essential [10]. A large proportion of illness among children under five in sub-Saharan Africa occurs in rural and remote areas where access to health care facilities is lacking; therefore, many children are left untreated, contributing to high rates of mortality among infected children [11]. In children with severe malaria who seek medical attention, most deaths occur within 48 hours of admission [11]. Even with treatment, 5-30% of children who survive CM experience neurological sequelae [10, 11].

Due to the disproportionate burden of malaria morbidity and mortality carried by children, governmental efforts in malaria endemic countries have often focused on this population. Reducing the burden of malaria among children under five is a priority that was identified by the United Nations Sustainable Development Goal (SDG) 3 under target 3.2 to end preventable deaths of children under five years of age by 2030, and target 3.3 to end the malaria epidemic by 2030 [12]. Unfortunately, gaps in

intervention coverage are widespread. In 2014, 74-86% of infected children under five in sub-Saharan Africa did not receive treatment [2]. In 2015, only 78% of suspected malaria cases among children under five received a parasitological diagnostic test, and only 16% of confirmed cases received first-line antimalarial treatment [2]. Despite government and international efforts to increase access to anti-malarial interventions and reduce the burden of malaria among children under five years, there remains much room for progress.

1.2.3 Internally Displaced Persons

Children make up more than half of the refugee population and a higher proportion of internally displaced persons (IDPs) [13, 14]. Globally, over 65 million individuals were forced into displacement by the end of 2015 [15]. High rates of infectious disease have been reported among forcibly displaced populations, which may be caused by numerous factors including environment, poverty, and violent conflict [16, 17]. Displaced individuals often live in suboptimal living conditions, elevating their risk of water-borne and vector-borne infectious diseases [18]. Insufficient access to water, sanitation, and hygiene results in increased morbidity and mortality, particularly among children under five. Among IDPs, malaria remains one of the most significant infectious diseases [8, 16, 19-21]. Displacement camps tend to be placed in locations favorable for mosquito breeding, further contributing to the high transmission and prevalence of malaria within these populations [8]. Populations moving from an area of low to high transmission may be more susceptible to infection than local residents, due to lack of acquired immunity. Establishment of medical facilities within displacement camps is often slow and short-lived due to the threat of violence [8]. Resource mobilization by international and non-governmental organizations is often reactive rather than proactive due to the unpredictable nature of violent conflict within complex humanitarian emergencies. These limitations prevent early diagnosis and treatment of malaria, and compromise prevention efforts, thereby threatening malaria control among large vulnerable populations. Additionally, surveillance and monitoring data among these populations is lacking, therefore data surrounding incidence, prevalence, and mortality rates are often unavailable [14].

Following decades of civil and international conflict, the DRC has more than 1.5 million IDPs [22]. The incidence of malaria in the DRC is estimated to be over 6 million cases annually, and malaria is the leading cause of childhood mortality in the country [22]. Chapter 2 of this thesis details an observational study examining the burden of malaria in a vulnerable and high-incidence population of children living in an IDP camp in eastern DRC.

1.3. CLINICAL MANIFESTATIONS

Malaria control is further complicated by the wide variety of symptoms that may be experienced following infection, which can lead to misdiagnosis and under-reporting of infections. Primary symptoms of uncomplicated malaria infection are nonspecific and include headache, fatigue, and myalgia, followed by fever, chills, perspiration, and malaise. The disease may progress to severe malaria within hours to days, and usually manifests with one or more of the following symptoms: coma, severe anemia, respiratory distress, or acute renal failure [23].

Many factors influence disease progression from uncomplicated to severe malaria, including the *Plasmodium* species, an individual's level of innate and acquired immunity, and the timing and efficacy of diagnosis and treatment [6]. Most forms of severe malaria are caused by *P. falciparum* infection. The WHO criteria for severe malaria are based on clinical and laboratory parameters [11], requiring that patients have one or more of the following features: impaired consciousness, prostration, convulsions, respiratory distress, acute pulmonary edema, circulatory collapse or shock, acute kidney injury, clinical jaundice, and/or abnormal bleeding. Laboratory indices of severe malaria include severe anemia, hypoglycemia, acidosis, hyperlactatemia, renal impairment, and hyperparasitaemia.

1.3.1 Cerebral Malaria

A major central nervous system manifestation of infection with *P. falciparum* is CM, which is characterized by asexual forms of the parasite detected by peripheral blood smears, repeated seizures, and coma lasting more than 30 minutes without an alternative explanation. The pathologic processes leading to CM are not fully elucidated; however, widely accepted mechanisms include parasite sequestration, release of infected red blood cell contents, activation of endothelial cells, increased inflammatory responses, and

ultimately dysfunction of the neurovascular unit (NVU) [24, 25]. CM is associated with a high mortality rate and long-term neurocognitive deficits in survivors [26]. In the absence of laboratory diagnostics, distinguishing severe malaria from other fatal febrile conditions is often inaccurate, increasing the risk of mortality in infected individuals.

1.4. DIAGNOSTICS

Due to the non-specific clinical features of both uncomplicated and severe malaria, diagnosis is challenging. Prompt diagnosis is essential in preventing the development of severe malaria. Current WHO guidelines recommend that all suspected cases of malaria have a parasitological test to confirm diagnosis, either by microscopy or by using a rapid diagnostic test (RDT) [23]. The use of parasitological tests ensures that only confirmed cases receive treatment, however in some resource-poor settings these diagnostics are used infrequently and results are sometimes ignored. In the WHO Africa Region, diagnostic testing has increased dramatically from 36% of suspected cases being tested in 2005 to 65% in 2014 [2]. This increase is likely due to an increase in availability of RDTs. Even so, in settings with high transmission rates and low resources, many individuals do not seek medical attention and those who receive treatment without diagnosis may be contributing to increases in *P. falciparum* resistance to anti-malarial treatments.

1.5. TREATMENT

Once diagnosis is confirmed, WHO guidelines recommend treatment with artemisinin combination therapy (ACT) for uncomplicated malaria and parenteral artesunate followed by ACT treatment for severe malaria [27, 28]. Despite the availability of efficacious drugs that rapidly eliminate parasites, malaria continues to cause deaths. Many individuals lack access to medical facilities where diagnosis and treatment are possible, and resource limitations often lead to drug shortages during high transmission seasons. Despite the best available anti-parasitic agent, parenteral artesunate, severe malaria is associated with a mortality rate of 8.5% [28]. In survivors of CM, up to 30% experience neurological sequelae [10]. The search for effective adjunctive therapies has therefore been ongoing in an effort to further reduce the morbidity and mortality associated with CM.

1.5.1 Adjunctive therapies

Adjunctive therapies are additional agents that modify the physiologic processes of malarial infections, by acting directly on specific pathways that are altered by malaria, or by acting on end-stage factors produced by malarial infections. These therapies are used in combination with first line treatments, which act to clear the parasite from the infected individual. Many interventions have shown promise as adjunctive therapies in murine models of severe malaria [29], but none have been shown to reduce mortality in human studies, to date [30]. Strategies have included modifying the immune response, iron chelation, correction of acidosis, reduction of intracranial pressure, prevention of seizures, prevention of cytoadherence, and modulating the blood brain barrier (BBB). Chapter 4 of this thesis describes experiments designed to test recently licensed pharmaceuticals as putative neuroprotective agents for CM.

1.6. PREVENTION

As discussed above, access to diagnostic and treatment services is often lacking in regions where they are most needed. Preventative measures against malaria are therefore an essential component of global efforts to reduce the burden of malaria. Vector control is the main mode of malaria prevention, and is achieved primarily using insecticide treated bed nets (ITNs) or indoor residual spraying (IRS). These interventions are effective due to the night-biting nature of the disease vector, the female *Anopheles* mosquito. Other widely used interventions include chemoprevention to suppress blood-stage infection in humans, intermittent preventative treatment during pregnancy (IPTp), mass drug administration, and case management, which includes prompt diagnosis and treatment of infections.

Despite these preventative measures, gaps in intervention coverage are widespread, and are widened by weaknesses in health systems. An estimated 269 million individuals continue to live in malaria endemic regions of sub-Saharan Africa without ITNs or IRS [2]. In 2015, only 31% of pregnant women in 36 African countries received three or more doses of IPTp [1]. Reasons for low coverage are complex, and are compounded by resource limitations such as low gross national income and low total domestic government spending per capita in malaria endemic countries. The majority of the international spending

on malaria control focuses on commodities rather than addressing fundamental issues such as health system strengthening and health education [1, 2]. Globally, challenges in malaria control efforts are numerous and comprehensive strategies are required to reduce malaria-associated morbidity and mortality. Chapter 3 of this thesis details a mixed methods study examining the under utilization of bed nets among IDPs in eastern DRC.

1.7. RESISTANCE

Further threatening malaria control efforts are increases in resistance. Two major forms of resistance are that of parasites to anti-malarials, and of mosquitoes to insecticides. *P. falciparum* resistance to anti-malarial treatments has threatened reductions in global control efforts since the early 1990s, and is one of the major factors that has pushed the development of improved diagnostics and treatments. *P. falciparum* resistance to current first-line ACT drugs has been detected in five countries in South-East Asia [1]. Cambodia has seen the greatest prevalence of *P. falciparum* drug resistance, with high rates of treatment failure for four different ACTs [1]. Insecticide resistance of mosquitoes has also increased, reducing the effectiveness of ITNs and IRS. Of the malaria endemic countries with monitoring data, 82% reported resistance to at least one insecticide, and 68% reported resistance to two or more insecticide classes [1].

1.8. RESEARCH OBJECTIVES

To address challenges in anti-malarial strategies within vulnerable populations, this research has two main focuses. The first is to better understand the burden of malaria and the underutilization of bed nets among IDPs in sub-Saharan Africa (thesis Chapters 2 and 3). The second is to accelerate the development of novel adjunctive treatments for CM, a major contributor of child morbidity and mortality in sub-Saharan Africa (thesis Chapter 4).

CHAPTER 2 – MALARIA IN AN INTERNALLY DISPLACED PERSONS CAMP IN THE DEMOCRATIC REPUBLIC OF THE CONGO

Data from this Chapter has been previously published [31].

2.1. INTRODUCTION

The global malaria burden is not evenly distributed, and identifiable vulnerable populations with high malaria incidence are disproportionately affected. Among these high-risk groups, displaced individuals are a key unreached population. Internal displacement has significant effects on the health of affected populations both directly as a result of violence and injury, and indirectly due to disrupted health systems and services, and increases in transmission rates of infectious diseases. Almost two thirds of refugees, internally displaced persons (IDPs), and victims of humanitarian emergencies live in regions where malaria is endemic [32]. Displaced individuals are among the poorest in the world, living in conditions susceptible to a variety of neglected tropical diseases, of which malaria is the most common [33, 34]. The Democratic Republic of the Congo (DRC) in particular faces extreme poverty, poor infrastructure, and armed conflict, which has caused substantial population displacement and may contribute to high rates of malaria transmission in the country [34].

Following decades of international and civil conflict, the DRC has more than 1.5 million IDPs [22], who often live in crowded displacement camps with little access to sanitation, clean water, food or healthcare [35]. Under these conditions, malaria prevention and treatment efforts are compromised, leading to elevated malaria transmission. Historically, individuals living in IDP camps rely on support from international humanitarian agencies; however, programs targeting specific diseases or health issues such as malaria, malnutrition, and diarrhea often have little impact due to the extent of factors that must be addressed to manage these health issues. Furthermore, there are few medical facilities available to treat individuals living in displacement camps, and non-governmental organizations (NGOs) are often forced to remove themselves from these areas due to violent conflict.

2.2. RESEARCH OBJECTIVE

With increasing population displacement and violent conflict in malaria endemic areas, disease surveillance and continuous efforts to improve malaria prevention are essential. The purpose of this study was to document the burden of malaria in IDP camps in the DRC, particularly among children under five. We conducted an observational study describing the burden of malaria within a large IDP camp in eastern DRC.

2.3. METHODS

Study setting

Lushebere is the largest displacement camp in the region of Masisi, North Kivu, DRC. The camp was created in September of 2013 when approximately 1,100 inhabitants from Ufamandu and Rubaya fled their homes due to violent conflict. This was followed by a second wave of approximately 1,500 individuals who arrived between January and February of 2014. The closest and only available medical centre that accommodates these IDPs is called the Centre de Santé Kitsule, which is supported by 5 international organizations and serves a 55 km^2 region inhabited by approximately 23,679 individuals, of which 2,580 live in Lushebere.

Design

This descriptive observational study enrolled patients presenting for medical treatment of febrile illness to the Centre de Santé Kitsule between January and July 2014. Consenting patients were included if they had a fever and were from Lushebere IDP camp (i.e., were registered on a census of the Lushebere IDP camp, conducted March 2014). All 751 patients were tested using a histidine-rich protein-2 (HRP2)-based rapid diagnostic test for malaria (Paracheck-Pf®; Orchid Biomedical Systems, Goa, India). Participant information was abstracted from medical consultation records, including age, literacy, length of time spent in Lushebere, clinical symptoms, classification of malaria episode, treatment given, bed net ownership and use, literacy, and outcome (death or survival). Proportions and percentages, along with their Chi squared p-values, were calculated for statistical significance.

Ethics approvals and permissions

All participants provided informed consent. Ethics approval was obtained from Comité d'Éthique du Nord Kivu (Université Catholique du Graben, ref 002/TEN/2012), the University of Alberta Human Research Ethics Board (ref Pro00055619), and from the Médecin Chef de Zone, within the DRC Ministry of Health.

2.4. RESULTS

Of 751 IDPs treated between January and July 2014 at the health centre, 323 (43%) tested positive for malaria by rapid diagnostic test, including 169/279 (61%) of children under five years of age. Using camp census data (Table 2.1), we estimated that the incidence of medically attended malaria was at least 210 per 1,000 at risk per year overall and 910 per 1,000 at risk per year in children under five. Of the patients with malaria, 292/323 (90%) had uncomplicated disease and 31/323 (9.6%) had severe disease. There were four deaths, including two deaths in children under five. Patient characteristics, disaggregated by malaria test status, are shown in Table 2.2. Distribution of malaria cases according to patient age and bed net ownership and use are also shown (Figure 2.1).

Table 2.1 Characteristics of internally displaced persons living in Lushebere in March 2014[1].

	First Displacement Wave (N=1111)	**Second Displacement Wave (N=1469)**	**p-value**
Age			<0.0001
<5 years	97 (8.7)[2]	221 (15)	
≥5 years	1014 (91)	1248 (85)	
Sex			0.043
Male	428 (39)	624 (42)	
Female	683 (61)	845 (58)	
Bed net ownership			<0.0001
Yes	1055 (95)	677 (46)	
No	56 (5)	792 (54)	

[1]Data are from the camp census. [2]Values are n (%) unless stated otherwise.

Table 2.2 Characteristics of patients from Lushebere IDP camp presenting to the health centre for management of acute febrile illness, according to malaria rapid diagnostic test (RDT) status.

	Malaria RDT positive (N=323)	**Malaria RDT negative (N=428)**	**p-value**
Age			<0.0001
0-5	169 (61)[1]	110 (39)	
5-10	34 (38)	55 (62)	
10-15	38 (28)	98 (72)	
15-20	23 (28)	58 (72)	
20-25	13 (27)	36 (73)	
25-30	16 (34)	31 (66)	
30+	30 (43)	40 (57)	
Sex			0.34
Male	175 (41)	247 (59)	
Female	148 (45)	181 (55)	
Bed net			
Ownership	106 (23)	346 (77)	<0.0001
Use	24 (9.0)	245 (91)	<0.0001
Duration Lived in Camp			<0.0001
> 6 months	44 (12)	319 (88)	
≤ 6 months	279 (72)	109 (28)	
Literacy[2]			0.34
Literate	54 (24)	171 (76)	
Illiterate	36 (20)	147 (80)	

[1]Values are n (%) unless stated otherwise. [2]Literacy rates exclude individuals 14 years of age and younger.

More than half, 452/751 (60%), of febrile patients owned a bed net and of these, 267/452 (59%) reported sleeping under the net. Patients who did not sleep under a bed net were more likely to test

positive for malaria than those who did (299/484 (62%) vs 24/267 (8.9%), p<0.0001) (Figure 2.1B). Recent settlers in the IDP camp (i.e., the second wave of IDPs) had a higher proportion of malaria than those from the first wave (72% vs 12%, p<0.0001). In a multivariable logistic regression model, adjusting for sex and literacy and accounting for potential confounding effects, age under 5 (OR_A 2.97 (95% CI 1.84-4.81), p<0.0001), lack of bed net use (OR_A 15.63 (95% CI 9.09-27.03), p<0.0001), and recent arrival in the camp, (OR_A 18.18 (95% CI 11.63-28.57), p<0.0001) remained significant predictors of malaria.

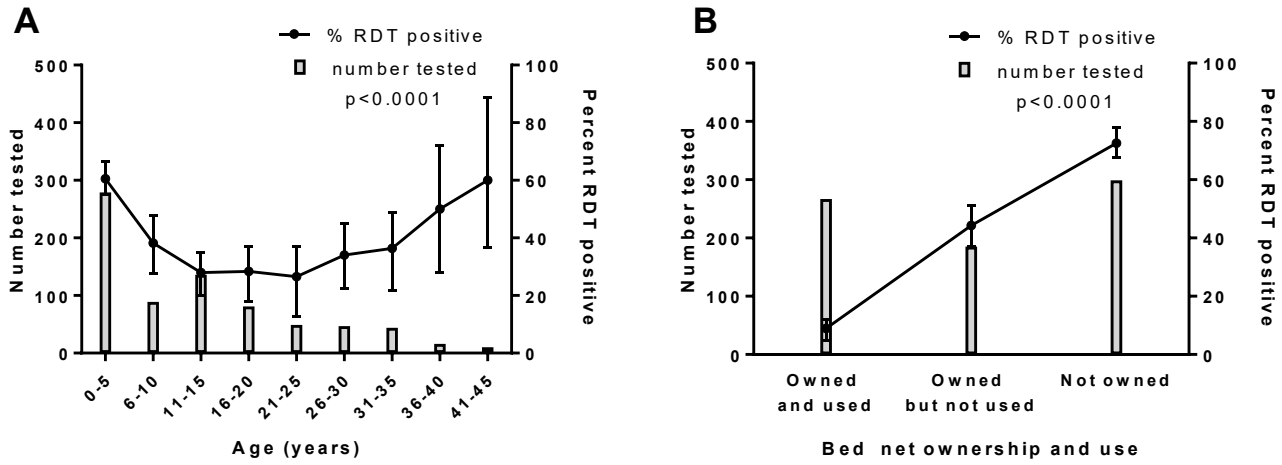

Figure 2.1 Malaria diagnosis according to A) age, and B) bed net ownership and use. Bed net ownership was based on patient self-report of at least one bed net in the household. Use was defined as the index patient sleeping under the bed net the night prior to the clinic visit. Error bars represent 95% confidence intervals.

2.5. DISCUSSION

These results highlight the overwhelming burden of malaria among IDPs at a remote camp in Eastern DRC. The incidence of malaria in Lushebere was estimated to be 3-fold greater than the WHO Africa region and that of the DRC [2], calling for action to address this preventable yet potentially fatal infectious disease within this population. Our study is noteworthy as a description of RDT-confirmed malaria in IDP camps deep within the zone of ongoing human insecurity. Studies from this area and among displaced

populations are challenging; these data represent a rare and valuable snapshot of malaria in an area of human insecurity.

Our findings are consistent with previous studies describing malaria as an important cause of child morbidity and mortality in complex humanitarian crises [16, 33, 36-39]. Children under five years of age made up the greatest proportion of patients presenting to the medical facility and the group with the highest risk of malaria. Four deaths occurred, including 2 in children under five, illustrating the significant contribution of malaria to child mortality in the region, as previously recognized [16, 37, 38]. Literacy, previously associated with improved health markers [36], was not associated with malaria in our study.

An important element of malaria prevention is the use of vector control measures, including bed nets as a physical and chemical barrier to night-biting *Anopheles* mosquitoes [40, 41]. As expected, bed net use was associated with a lower probability of malaria infection in our study. However, uptake of this proven intervention was suboptimal (64% of febrile children in our study did not own or did not use a bed net). Additionally, a lower percentage of individuals in the second displacement wave owned a bed net.

Limitations of our study include the diagnostic modality, since 1) RDTs are less sensitive than polymerase-chain reaction-based testing, and 2) RDTs measure HRP2 antigen, which may persist for up to a month after recently treated malaria infection. Due to resource constraints in IDP camp settings, microscopy and molecular diagnostic methods were not available. Additionally, a longer sampling period would have provided a larger sample size and measurement over a greater variety of seasons throughout the calendar year.

2.6. CONCLUSIONS

Our report of febrile illness managed at an IDP camp in the DRC demonstrates the overwhelming burden of malaria in a vulnerable population forcibly displaced by violent conflict. Previous studies have shown that malaria is an important cause of child morbidity and mortality in complex humanitarian crises [16, 33, 36-39]. In our study, the incidence of malaria and non-malarial febrile illness were highest among children under five. These findings are consistent with previous reports showing that preventable and treatable infections, including malaria, are major causes of morbidity and mortality among civilian

populations affected by violent conflict, disproportionately affecting children under 5 [16, 37, 38]. Conditions within displacement camps may promote transmission of other infectious diseases such as pneumonia and diarrhea [7, 18, 35, 42]. Furthermore, close proximity of shelters, providing a dense population for malaria transmission, lack of water drainage, lack of appropriate hygiene and sanitation infrastructure, and lack of access to health care may increase the incidence and severity of vector-borne diseases like malaria [18]. The following Chapter will examine reasons for underutilization of bed nets among IDPs in the DRC.

CHAPTER 3 – BARRIERS TO BED NET USE AMONG INTERNALLY DISPLACED POPULATIONS IN THE DEMOCRATIC REPUBLIC OF THE CONGO: A MIXED METHODS STUDY

Data from this Chapter is accepted and currently in press with PLoS ONE.

3.1. INTRODUCTION

Recent global estimates suggest that violent conflict and natural disasters resulted in~20 million refugees and ~40 million IDPs fleeing their homes in 2016 [14, 43]. Whereas most refugees were from Syria, Afghanistan and Somalia, the majority of IDPs were in the DRC, Colombia and Iraq [44-46]. Children make up more than half of the refugee population and a higher proportion of IDPs [13, 14]. These mobile populations, fleeing their homes due to violent conflict, are difficult to reach and often lack stable health infrastructure, therefore disease prevention, diagnosis, and treatment efforts may be compromised. Often living in crowded camps, these vulnerable populations generally have limited access to quality shelter, sanitation, clean water, stable food supply, and healthcare [35]. Under these conditions, malaria prevention and treatment efforts are challenging and malaria transmission is elevated [8, 39, 47]. Furthermore, few medical facilities are available to diagnose and treat individuals living in displacement camps.

The DRC has more than 1.5 million IDPs, as a result of decades of civil and international conflict [22]. The incidence of malaria in the DRC is estimated to be over 6 million cases annually, and malaria is the leading cause of childhood mortality in the country [22]. Despite the proven efficacy of bed nets as a preventative measure against malaria [41], they are under-utilized among IDPs [8, 31, 39, 47]. Due to the insecurity of regions where IDPs often reside, bed net availability and accessibility is often compromised, even with non-governmental organization (NGO) mass distribution efforts.

3.2. RESEARCH PURPOSE

We and others have previously reported that the burden of malaria is disproportionately high in IDP camps, and that bed nets are under-utilized [31, 47, 48]. The purpose of this study was to explore the factors influencing bed net ownership and use in an IDP camp with universal free bed net distribution,

using mixed qualitative and quantitative methods. We first sought to understand the reasons for the lack of bed net ownership and use in this high-risk population through focus group discussions (FGDs) with key informants, including IDPs, neighboring villagers, and health care providers working in the displacement camp. Results arising from thematic analysis of FGDs were then used to design and implement a door-to-door survey of a random subset of IDP households to quantify bed net use and disuse, as well as explanatory factors.

3.3. METHODS

Study setting

This study was conducted in the IDP camp of Birambizo and the nearby village of Kizimba, in the Walikale district of North Kivu province in the DRC. Birambizo is one of four IDP camps in the Birambizo health zone, in the region of Rushurui, North Kivu, DRC. Approximately 255,530 individuals live within the 1,967 km^2 region, of which over 73,000 are IDPs. The Walikale region of the DRC has faced many years of violence and armed conflict. A recent investigation in this region found the leading cause of mortality in children under 5 years of age to be malaria [33].

For the past decade, the Birambizo health zone has faced political and ethnic based conflict, leading to the destruction of homes, banks, schools, medical centers, and the degradation of human security. The health zone is now effectively militarized, which adds to the tension and conflict in the region. Birambizo IDP camp is made up of a constantly evolving number of IDPs, who arrive and leave as a function of continuous conflict and in search of a safer area. At the time of our research, approximately 13,700 IDPs lived in Birambizo IDP camp. Following 4 attacks directed against humanitarian agencies in December 2014 and March 2015, all NGOs in the region have now stopped permanent activities in this area.

Design

We use mixed qualitative (FGDs) and quantitative (cross-sectional survey) methods.

Focus group discussions. FGDs were conducted in Birambizo and Kizimba in April, June, and December of 2015. Key informants, including IDPs living in Birambizo IDP camp, residents from communities neighboring the displacement camp (Kizimba, Budei and Nyangutu), and health care

providers serving the displacement camp participated in FGDs. Overall there were 10 FGDs (Table 3.1), each comprised of 3-6 individuals [49]. Nine FGDs involved residents of the IDP camp or neighboring village, and one involved health workers (nurses). FGDs were conducted in the local languages (Kiswahili, Kinyarwanda and Kinande) by members of the IDP camp with supervision from our research collaborators who were trained in qualitative methods. Group discussions were recorded and transcribed verbatim for subsequent translation and analysis. FGD questions were elastic, open-ended and probing, allowing participants to shape the discussion. Questions were adapted over the course of the FGDs, aiming to explore emerging themes in greater depth. FGDs were continued until a point of saturation, when no new themes emerged in discussions [50]. Thematic analysis was used to identify, analyze and report themes in the data. Themes that emerged from the focus groups were used to inform quantitative survey content. Two investigators (VM and HJ) read the transcripts several times and noted preliminary ideas, producing initial codes by highlighting relevant data, generating themes by collating codes across the data set, and refining themes. Representative quotations, as well as statements of particular interest, were extracted and organized according to themes to support the qualitative findings.

Cross-sectional survey. Major themes from FGDs were incorporated with questions from a standardized malaria indicator survey toolkit [51] to create a novel 50 item questionnaire for the quantification of barriers to bed net ownership and use among IDPs. Community health workers, trained in malaria rapid diagnostic testing as previously described [52], visited a random sample of 100 family units ("households") within the Birambizo IDP camp. Random sampling of temporary households in the camp was performed using a census created by NGOs providing services in the area. All households on the census were eligible for inclusion. Testing for *P. falciparum* infection using a HRP2-based rapid diagnostic test (RDT) (Paracheck-Pf®) was offered to all household residents. All participants who tested positive for *P. falciparum* were treated with the artemisinin-based combination therapy according to WHO recommendations [23]. Heads of households were asked questions relating to household composition, demographics, bed net ownership and use (see full survey in appendix).

Statistical analysis

A standard sample size calculation indicated that 95 households would be needed to estimate the proportion of households that own a bed net to within ±10%, with 95% confidence, assuming that the proportion of bed net ownership was 34%, based on previous studies in the area [47]. For descriptive statistics, binomial 95% confidence intervals were calculated for proportions. Comparative statistics were computed using non-parametric methods (Mann-Whitney U-test) for continuous variables and Chi squared or Fisher's exact test for dichotomous variables, as appropriate. Correlations between continuous variables were analysed by the non-parametric Spearman's rank correlation coefficient. Household wealth was approximated as follows: ownership of household assets or household construction characteristics were coded as binary variables and weighted using principal component analysis (PCA). This method was modified from Filmer and Pritchett [53], and has been validated as a measure of household consumption and poverty, and has been used in previous studies in sub-Saharan Africa [54]. Eleven assets or household characteristics from the questionnaire were included to determine a wealth index; this index was then used to divide the cohort into households above and below the median wealth index. Measures that we included were: household characteristics (electricity, tarpaulin vs mud wall construction) and asset ownership (bicycle, motor vehicle, radio, telephone, television, refrigerator, chicken, cow and goat). Statistical software SPSS version 19 (IBM, USA) was used for the analysis.

Ethics approvals and permissions

All participants provided written informed consent. Ethics approval was obtained from Comité d'Éthique du Nord Kivu (Université Catholique du Graben, ref 002/TEN/2012), the University of Alberta Human Research Ethics Board (ref Pro00055619), and regionally from the Médecin Chef de Zone, within the DRC Ministry of Health.

3.4. RESULTS

Focus Group Discussions

We explored several potential barriers to the use of bed nets for malaria prevention through FGDs, which were grouped thematically: (1) awareness of malaria as a cause of morbidity and mortality; (2)

knowledge of bed nets as an effective malaria preventative measure; (3) access and availability of bed nets; (4) pragmatic barriers to bed net use; and (5) competing needs that compel IDPs to trade or sell the bed net. Here we present each theme, together with representative quotations, translated into English from the language of the FGD.

(1) Awareness of malaria as a cause of morbidity and mortality. Participants were generally aware that malaria is a major cause of mortality among young children. One participant recounted his personal loss related to malaria: '*I take [malaria] seriously because I recently lost my eldest son of three years [of age] to this vile disease' (IDP).* Nonetheless, gaps in knowledge about malaria were noted by health care providers in the IDP camp. One FGD participant, a health care worker in the IDP camp, felt that self-treatment and/or the use of traditional herbal remedies was a factor contributing to severe malaria in the camp: '*We live in a rural area where the level of knowledge remains [primitive]' (Nurse).* Another nurse gave the following opinion: '*Many of the cases we see are complications of uncomplicated malaria cases that were [mismanaged] at home' (Nurse).*

Table 3.1 Characteristics of participants included in focus group discussions (FGDs).

	Number of individuals (N=55)	**Residence**	**Sex**	**Children < 5 in the residence**	**Bed net ownership**
FGD 1	6	IDP camp	F	Yes	Yes
FGD 2	6	IDP camp	F	Yes	No
FGD 3	6	IDP camp	M	Yes	No
FGD 4	3	IDP camp	M	Yes	-
	3	IDP camp	F	Yes	-
FGD 5	3	IDP camp	F	Yes	Yes
	3	IDP camp	F	Yes	No

FGD 6	3	IDP camp	F	Yes	Yes
	3	Villages	F	Yes	Yes
FGD 7	3	IDP camp	F	Yes	No
	3	Villages	F	Yes	No
FGD 8	5	IDP camp	F	Yes	No
FGD 9	5	Villages	F	-	-
FGD 10	3	Villages (Nurses)	-	-	-

(2) Knowledge of bed nets as an effective malaria preventative measure. Participants were widely aware that bed nets prevent malaria: '*Bed nets are effective because the displaced people who use them don't often fall ill' (IDP).* Some cited positive personal experience with bed nets: '*Because I use a bed net regularly, I rarely fall ill, and even my baby, compared to the neighbour's baby who doesn't use a bed net' (IDP).* Others went beyond free distribution programs to purchase bed nets for their family: *'My wife had the chance to receive a bed net when she was pregnant and we still use it. I bought two more bed nets for our children' (IDP).* FGD participants from outside the IDP camp also recognized the value of bed nets: *'The effectiveness of bed nets was proven in our village because without using the bed net, we risk getting sick every month or even two to three times per year' (Villager).* Health information on bed nets was routinely provided in the camp, as noted by several respondents: '*Information about the bed nets is always given at the health centre to pregnant women, but there are those who don't receive the information about the usefulness of bed nets in malaria prevention, especially certain pregnant women who don't attend their antenatal visits' (IDP).* FGD participants trusted this information provided by health workers: '*The nurse from the health centre was telling the truth during the antenatal visit about the protection bed nets give against illness' (IDP).*

(3) Access and availability of bed nets. FGD participants generally had access to bed nets through free distribution programs, sponsored through the DRC government health services as well as NGOs: '*NGOs ensure the distribution of blankets, tarps, bed nets and other items for the IDPs' (Villager)*. One mechanism of bed net distribution was through antenatal care (ANC) visits: '*We all, IPD or not, receive a bed net during antenatal visits to protect us against malaria' (Villager).* Nonetheless, gaps in the distribution programs were reported by some participants: '*I didn't get the chance to benefit from a bed net because the quantity of nets distributed was not sufficient' (IDP).*

(4) Pragmatic barriers to bed net use. Given that malaria awareness was high, bed nets were perceived as useful, and were available free of charge, why did IDPs not use them more frequently? Uniquely challenging living conditions in the IDP camp may compromise the utility and effectiveness of bed nets. FGD participants noted pragmatic limitations, such as size of the dwelling and the mud floor: '*The state of our tents doesn't encourage the use of bed nets because the tent is small and the net falls on the floor, so each time we have to wash it but lack of soap is an issue' (IDP).* Space constraints in the tent meant that bed nets required daily installation at dusk and dismantling at dawn. The bed nets were easily soiled in the muddy environment, required frequent washing, tore easily and developed holes, resulting in short life spans: *'We must clean the nets at least once a week [which] damages it, we also don't have permanent water sources in the camp, and soap is an issue' (IDP); 'With the candle that lights our night, my bed net now has many burn holes because the tent is [too] small to separate the net from the candle' (IDP).* IDPs usually slept on mats on the mud floor, such that bed nets could not be tucked under a mattress to provide a physical barrier against mosquitoes: *'It's difficult to use the nets well […] We have no beds or mattresses to tuck the nets under, we have no bedrooms' (IDP).* Sleeping conditions for children under five (a target high-risk group for malaria prevention) are often cramped; children often slept between parents in tents intended for two users and a second tent was often required for additional family members. One FGD participant summarized the impracticalities of bed nets in the IDP camp as follows: '*NGOs should think more about food before any bed net distribution because the bed nets that [they] give out aren't even suited to our tents' (IDP).*

Other factors were not specific to the IDP camp, but nonetheless influenced bed net acceptability. Some complained of the chemical smell: '*The bed nets give off a nauseating smell that disrupts our sleep and affects our breathing' (IDP).* Others expressed anxieties about adverse health effects: *'Some say the nets are toxic when they give off odour' (IDP)*.

(5) Competing needs. FGD participants were challenged to explain why so few IDPs owned a bed net, despite their free distribution. Extreme poverty among IDPs drives some to sell their bed nets, sometimes because of food insecurity. In the FGD involving health workers, one nurse explained: '*We ensure free bed net distribution to pregnant women and young children, but the issue is that many of these women sell their nets' (Nurse).* IDPs corroborated this explanation with their own, sometimes poignant, personal testimonials: *'I used to use a bed net regularly before I became displaced. Nowadays, I don't use it because I sold the net I received at the antenatal clinic because of food problems' (IDP); 'I traded [our bed net] for food because my children were hungry and I didn't have money to solve this problem' (IDP); 'We told our wives to sell the bed net they received in the market because we are heads of households without work. We can't fulfill our basic duty, that's why we sell some of our belongings so that the children can eat' (IDP)*.

Further probing revealed that bed nets were not the only traded commodity. Participants reported that residents of nearby communities purchased a range of goods donated to the IDPs, and employed IDPs for inexpensive farming labour. Thus, one FGD participant from a neighboring community noted that bed nets could be purchased cheaply from IDPs: *'At the market it costs around $2.5 but [from] IDPs the bed nets can be bought even for $0.5 [...] for me the cost is acceptable because even at the market it's the price of one or two bottles of beer' (Villager).* Another noted the value-for-cost of donated items: *'We buy several donated goods from the IDPs, because they sell them cheaper and the quality is good' (Villager).*Yet another noted the range of products available in the illicit market of donated goods: *'[IDPs] sell goods at a cheap price, for example casseroles, tarps, and flour' (Villager).*In addition to demand for cheap quality goods, an appetite for a commerce in bed nets existed among IDPs on the "supply-side" as well: '*We sell our bed nets within the camp or at the market on Thursdays, because there are always lots*

of people in Thursday' (IDP). Another participant described the widespread entrepreneurship around the bed net trade: '*Everyone buys bed nets: business people, NGO workers in the camp, even other IDPs in order to resell them when there is a shortage' (IDP).* The trade in bed nets was not condoned by authorities and, in one participant's description, took on a humorously clandestine aspect: *'Officially the sale [of bed nets] is prohibited; that's why the sale happens from ear to mouth or in a hidden corner of the market'* (IDP).

We further explored dynamics and tensions between IDPs and the neighboring community, which may influence the retention of bed nets in the camp. In addition to the benefit of low-price, high-quality donated goods, competing needs of IDPs and villagers sometimes lead to strain between the two populations. One FGD participant from a neighboring community noted: '*We get along easily, but these interactions also give rise to several conflicts, namely cases of rape, pregnancy, stealing of cattle or poultry' (Villager).* Another noted: *'There is a big problem of theft in the village related to [IDP] presence' (Villager).* Selectively helping IDPs in a setting of widespread material poverty led to a perception of unfairness: *'It's unfair to ensure distribution of a necessary product only to displaced people knowing that we face the same nutritional difficulties as them (Villager).* Another community member remarked on the burden borne by the neighboring village, and urged international donors not to forget surrounding communities in their zeal to help IDPs: *'[We] encourage the donors in their goodwill [towards IDPs], but [we ask that they] think of us as well because a house that has visitors spends [more] money' (Villager).*

The rich data generated from FGDs disclosed complexities in the use and disuse of malaria bed nets in an IDP camp. Of note, however, this very focus on bed nets was thrown into sharp relief by one participant's sobering perspective on root causes of malaria: '*I think NGOs and the government should do everything in their power to return us to our villages by putting an end to the armed conflict and we will be able to fight against malaria because we will be in our own houses, [which are] better than a tent' (IDP).*

Cross-sectional Survey

Overall malaria control indicators

One hundred family units ("households") in the IDP camp, consisting of 411 individuals, were surveyed between November 2016 and March 2017. Participants had been displaced for a median of 30 months (range 19-36 months) and had been living in the camp for most of this time (median 30 months, range 25-36 months). Indicators for malaria control in the IDP camp, based on standard survey indicators [55], are shown in Table 3.2. Overall, the burden of malaria was high (45/78 (58%) children <5 were positive for malaria by RDT), and bed net utilization was low (29/100 (29%) households owned a bed net, and 85/411 (20%) individuals slept under a bed net the previous night).

Table 3.2 Indicators for malaria control in Birambizo IDP camp[1].

Vector Control	
Proportion of households with at least one bed net	29/100 (29%)
Proportion of households with at least one bed net for every two people	0/100 (0%)
Proportion of population with access to a bed net within their household	139/411 (34%)
Proportion of population that slept under a bed net the previous night	85/411 (20%)
Proportion of children under five years old who slept under a bed net the previous night	32/78 (41%)
Proportion of existing bed nets used the previous night	29/29 (100%)
Case management – Health seeking behaviour and accurate diagnosis	
Proportion of children under five years old with fever in the last month for whom advice or treatment was sought	10/15 (67%)
Proportion of children under five years old with fever in the last month who had a RDT	10/15 (67%)
Morbidity indicator	
Parasite Prevalence: proportion of children aged 6-59 months with *P. falciparum* infection	45/78 (58%)

[1]Modified from [55].

Household composition, structure of shelter, and bed net ownership.

Median household size was 4 individuals (range 2-8 individuals), with median 1 (range 1-2) child under 5. There was a median of 2 (range 1-4) shelters for each household. A positive correlation between the number of individuals per household and the number of shelters was observed (Spearman's rho=0.75, p<0.0001). Nearly all shelters were made of tarpaulin walls (94%), tarpaulin roof (100%), and mud/dirt floor (99%). No households had access to electricity, and all shared a latrine with other families. Households within the camp were characterized by extreme material poverty, with no households owning a refrigerator, television, car, or cow. Exposure to community violence was common, with at least one family member being victim of theft or physical violence in 48/100 (48%) households.

Twenty-nine (29%) households owned a bed net, and no household owned more than one bed net. No households had sufficient bed nets to accommodate all family members, allowing one bed net for every 2 individuals [55]. Table 3.3 shows the characteristics of households, disaggregated by bed net ownership. Households with one or more children under five years of age were more likely to own a bed net than those without (27/69 (39%) vs 2/31 (6.5%), p=0.0007). Households with a shelter made of durable material (mud walls) were more likely to own a bed net than those with tarpaulin walls (5/6 (87%) vs 24/94 (25%), p=0.0075).

Table 3.3 Factors associated with household bed net ownership.

	Overall (N=100)	**Households with a bed net (N=29)**	**Households without a bed net (N=71)**	**p-value**
Duration of displacement in months, median (range)	30 (19-36)	30 (29-36)	29 (19-35)	0.005
Number of individuals per household, median (range)	4 (2-8)	5 (3-8)	4 (2-7)	0.0019
Number of children under 5 per household				0.0001

None	31 (31)[1]	2 (6.9)	29 (41)	
One	60 (60)	21 (72)	39 (55)	
Two	9 (9)	6 (21)	3 (4.2)	
Characteristics of shelter				
Flooring				0.29
Dirt	99 (99)	28 (97)	71 (100)	
Wood boards	1 (1)	1 (3)	0 (0)	
Roofing				
Tarpaulin	100 (100)	29 (100)	71 (100)	-
Walls				0.0075
Tarpaulin	94 (94)	24 (83)	70 (99)	
Mud	6 (6)	5 (17)	1 (1.5)	
Electricity	0 (0)	0 (0)	0 (0)	-
Asset ownership				
Refrigerator	0 (0)	0 (0)	0 (0)	-
Television	0 (0)	0 (0)	0 (0)	-
Radio	12 (12)	3 (10)	9 (13)	>0.99
Cell phone[2]	2 (2)	0 (0)	2 (3)	>0.99
Bicycle	8 (8)	2 (7)	6 (8)	>0.99
Vehicle	0 (0)	0 (0)	0 (0)	-
Watch[2]	13 (13)	4 (14)	9 (13)	>0.99
Livestock	14 (14)	2 (7)	12 (17)	0.34
Wealth index[3]				0.84
Above median	78 (78)	23 (79)	55 (77)	
Below median	22 (22)	6 (21)	16 (23)	

[1]Values are n (%) unless stated otherwise. [2]If any member of the household owned a cell phone or a watch, the household was classified as owning this asset. [3]Assets that did not contribute to the wealth index include those that were not owned by any household, namely refrigerator, television, and vehicle.

Survey participants, P. falciparum infection, and bed net use

The median age of the 411 study participants was 23 (range 1-75), 78/411 (19%) were under the age of 5, and 177/411 (43%) were female. At the time of the survey, 61 (15%), 50 (12%), and 26 (6%) of

individuals were symptomatic with headache, fever, and/or myalgia, respectively, and 69 (17%) tested positive for malaria by RDT. Symptoms were statistically significantly associated with RDT positivity ($p < 0.0001$ for all comparisons). In the target group of children under five, fever was reported in 40/78 (51%) and RDT was positive in 45/78 (58%) at the time of the survey. Thus, age<5 was associated with higher odds of fever (OR 16 (95%CI 8.4-29) $p<0.0001$) and RDT positivity (OR 18 (95%CI 9.5-32), $p<0.0001$), relative to older children and adults. Because RDT positivity may persist for up to 4 weeks after resolved *P. falciparum* infection [56, 57], we performed a subgroup analysis to account for possible false-positive results. RDT was positive in 6/15 (40%) of children with a reported fever in the past month, and 39/63 (62%) of children without a reported fever in the past month ($p=0.15$).

The night prior to the survey, 85/411 (20%) individuals had slept under a bed net, including 31/78 (40%) children under five. Children under five were more likely to use a bed net than older children or adults (OR 3.4 (95%CI 2.0-5.8), $p<0.0001$). Table 3.4 shows individual-level characteristics, disaggregated by bed net use. Besides age, no other factors were associated with bed net use.

Bed nets currently owned and in use

Of the 29 bed nets currently in use by study participants, 21 (72%) were obtained free of charge at the IDP camp health center during an ANC visit, and 8 (28%) were received free of charge from NGOs. No bed nets had been purchased. The median duration since the net was received was 25 months (range 14-30 months). Most (21/29 (72%)) of the nets were washed regularly and 15/29 (52%) had developed holes.

Table 3.4 Individual-level factors associated with bed net use.

	Overall	Used bed net[1]	Did not use bed net	p-value
All participants (N=411)		**N = 85**	**N = 326**	
Age, median (range)	23 (1-75)	16 (1-36)	24 (1-75)	0.002
Sex				0.69
Male	234 (57)[2]	50 (59)	184 (56)	
Female	177 (43)	35 (41)	142 (44)	
Children under 5 (N=78)		**N = 31**	**N = 47**	
Age, median (range)	2.5 (1-4)	2.6 (1-4)	2.4 (1-4)	0.24
Sex				0.44
Male	54 (69)	23 (74)	31 (66)	
Female	24 (31)	8 (26)	16 (34)	
Maternal education				0.85
None	39 (50)	16 (51)	23 (49)	
Primary	17 (22)	8 (26)	9 (19)	
Secondary and above	22 (28)	7 (23)	15 (32)	
Symptoms at the time of survey				
Fever	40 (51)	17 (55)	23 (49)	0.61
Headache	8 (10)	5 (16)	3 (6)	0.25
Myalgia	9 (12)	4 (13)	5 (11)	>0.99
RDT result positive	45 (58)	18 (58)	27 (57)	0.96
Fever in the past month	15 (19)	5 (16)	10 (21)	0.77
Sought care	10 (67)	4 (80)	6 (60)	0.15
Diagnosed with malaria	10 (67)	4 (80)	6 (60)	0.15
Tested with RDT or microscopy	10 (67)	4 (80)	6 (60)	0.15
Received antimalarial drug	10 (67)	4 (80)	6 (60)	0.15

[1]Bed net use is defined as sleeping under the bed net the night prior to the questionnaire. [2]Values are n (%) unless stated otherwise.

Bed nets no longer owned or used

Asked to recall any bed nets they had owned since coming to the IDP camp, participants reported a total of 146 bed nets that they no longer owned. Of these, 120 (82%) were received from NGOs and 26 (18%) were received from the IDP camp health center. None of the bed nets had been purchased. All of these nets had been sold (82%) or exchanged (18%) either in the camp (27%) or in the neighboring village market (73%) at a median price of $2.15 (range $1-3). None had been discarded, repurposed, or worn out. The majority of nets were bought or accepted by residents of the neighboring community (80%), however other IDP camp residents (12%) and government workers (8%) also accepted or bought the nets. The reasons given for selling or exchanging a bed net are given in Table 3.5. The most common reasons related to pragmatic considerations including installation and size of the net, rather than lack of awareness of their utility, or monetary value of the net.

Table 3.5 Factors associated with bed net disuse.

	Bed nets previously owned[1] (N=146)
Reasons for bed net disuse	
Causes irritations/coughing	100 (68)[2]
Not shaped well	78 (53)
Difficult installation	74 (51)
Gives off a chemical	70 (48)
Too many holes	64 (44)
Smells badly	54 (37)
Gets dirty quickly	43 (29)
Needed money	24 (16)
Too small	23 (16)
Too hot	16 (11)
Can suffocate/causes difficulties breathing	0 (0)
Can’t tuck under mattress	0 (0)

Not efficacious	0 (0)
Other	0 (0)
Don't know	0 (0)
Outcome of net	
Sold	119 (82)
Exchanged	27 (18)
Duration since exchange/selling of net, median months (range)	26 (2-34)
Place of exchange/selling of net	
Village market	107 (73)
Camp	39 (27)
Who bought/accepted net	
Villager	117 (80)
Displaced individual	17 (12)
Government worker	12 (8)
NGO	0 (0)
Price net was sold for, median (range)	$2.15 (1-3)

[1]Previous bed nets are those that households had reported owning since their displacement but had now been sold or exchanged. [2]Values are n (%) unless stated otherwise.

Table 3.6 Factors associated with current and previous bed net ownership.

	Bed nets currently owned[1] (N = 29)	**Bed nets previously owned[2] (N=146)**	**p-value**
Age of bed net in months, median (range)	25 (14-30)	28 (12-39)	0.012
Source of bed net			<0.0001
NGO	8 (28)[3]	120 (82)	
Hospital/ Health Centre	21 (72)	26 (18)	
Received during ANC visit	21 (72)	24 (16)	<0.0001
Received during distribution campaigns	-	119 (82)	-

[1]Current bed nets are those that were used the night prior to the questionnaire. [2]Previous bed nets are those that households had reported owning since their displacement to the IDP camp but had now been sold or exchanged. Values are n (%) unless stated otherwise.

Table 3.6 compares bed nets owned and in use at the time of the survey to those that had been sold or exchanged. Bed nets received at the health centre were more likely to still be in use than those received from an NGO (OR 12.1, 95%CI 10.7-18.9). Bed nets received during an antenatal care visit were more likely to still be owned and in use (OR 13.3, 95%CI 5.3-33.6).

3.5. DISCUSSION

Our study of bed net use and disuse in an IDP camp in Eastern DRC unveils complexities and limitations of an established malaria control strategy applied in a challenging environment. Studies from this area and among displaced populations are scarce; therefore, these data offer rare insights into an under-studied population. Our findings may be useful to governments and NGOs operating in complex humanitarian crises in the tropics, where malaria is exacerbated by violence and displacement [47], and plays a major role in child mortality [16, 33, 36-39].

An important element of malaria prevention is the use of vector control measures, including bed nets, as a physical and chemical barrier to night-biting female *Anopheles* mosquitoes [40, 41]; however, it is less clear how to deploy this strategy in the challenging environment of an IDP camp. In this study, we investigated barriers to bed net use among IDPs in a region with ongoing human insecurity. Despite a high burden of malaria and free distribution of bed nets, only 29% of IDP households owned a bed net, compared to 16-75% in other studies in IDP camps in Eastern DRC and 8-90% in community-based surveys and surveillance data from sub-Saharan Africa [31, 39, 47, 48, 58]. In our random survey of an IDP camp in Eastern DRC, 20% of individuals slept under a bed net the previous night, compared to 16-25% in another IDP camp in the DRC [47] and 6-70% in other African studies [59]. Point prevalence of *P. falciparum* infection among children under 5 in our study was 58%, compared to 0.4-78% in other studies in Sub-Saharan Africa [39, 59]. Taken together, these data indicate that the Birambizo IDP camp appears to be representative of other IDP camps in the area, with high malaria burden and low bed net use.

A large number (146/175 (83%)) of the total number of bed nets reported by IDP camp residents had been sold or exchanged and were no longer available for malaria prevention in the population for which

they were intended. The reasons given for dispensing of a bed net were solicited in both FGDs (qualitative methodology) and survey questionnaires (quantitative methodology). Lack of awareness of malaria burden or bed net effectiveness did not emerge in either FGDs or surveys as a major reason for dispensing of bed nets. Unlike participants from a community-based survey in Kenya [60], no participants in our study reported repurposing their bed net for other uses such as a blanket or curtain.

Both qualitative and quantitative methods pointed to substantial pragmatic limitations of bed nets in the IDP camp. Sleeping arrangements (multiple shelters, lack of beds/mattresses), cramped space inside the tent, mud floors and need for frequent washing, difficulties in hanging a bed net inside, and the development of holes were given as practical reasons why bed nets may not be used. Similar pragmatic barriers have been previously identified in community-based African studies exploring bed net underutilization [61, 62]. Characteristics of the bed nets themselves, independent of the IDP camp context (e.g., unpleasant odor or chemical irritation) were other common reasons for bed net disuse. These reasons are consistent with other community survey results in African countries with free insecticide-treated bed net (ITN) and long-lasting insecticide treated net (LLIN) distribution campaigns [61-63]. Due to a lack of space within the camp and an immediate need for the nets, new bed nets are likely to be used without following manufacturer's instructions for a 24-48 hour drying period, which may be responsible for the initial irritations and chemical odour reported by FGD and survey participants.

In FGDs, the position of economic vulnerability of IDPs, relative to neighboring communities, appeared to fuel a trade in bed nets and other donated goods. Selling bed nets to fill an immediate need for food or income was a noteworthy finding; however, quantitative survey results suggest that this was the reason for dispensing of only 16% of the bed nets that were no longer in use. We suspect that social acceptability bias may have led to an under-reporting of financial motives for bed net sale/trade in survey questionnaires. Of note, selling or trading nets is strongly discouraged by authorities, and door-to-door surveys were conducted by members of the health center. On the other hand, FGDs were led by IDP camp members themselves with special attention to participant engagement and confidentiality; therefore, participants may have been more open and honest in their discussions.

Commercial transactions of donated goods within the IDP camp and with the surrounding community explained, at least in part, the large number of bed nets that had been sold or exchanged. FGDs revealed a dynamic exchange of goods and services between the camp and neighboring community, including farm labour and the exchange of donated quality household goods such as bed nets. Most bed nets were sold to neighboring villagers, at a price well below retail value. In addition to sympathy for the general plight of the IDPs, resentments were expressed by some FGD participants from the surrounding community, who were not beneficiaries of free commodities distributed in the camp by external agents (NGOs), and who sometimes experienced theft at the hands of the IDPs. Recognizing that freely distributed bed nets were not supposed to be sold or bartered, the trade in donated bed nets was often clandestine, from "mouth to ear" or in a "hidden corner of the market".

Children under five carry a disproportionate burden of malaria [16, 37, 38]; this was reflected in our study where fever and RDT positivity were more prevalent in this age group. Appropriately, bed nets were preferentially used for this age group, as evidenced by higher household ownership of bed nets in households with at least one child under five, and higher utilization of bed nets by children under five. Bed net distribution during ANC visits, as done in Birambizo IDP camp and in other IDP camps and regions of Africa [64-66], appears to be a rational strategy to target the youngest camp residents, and appears to be at least partially successful in Birambizo. Higher rates of bed net retention were observed when bed nets were obtained in the context of an antenatal clinic visit. Still, there remain significant gaps in bed net coverage for the under five age group, since 47/78 (60%) children under five did not sleep under a bed net the night prior to the survey and the burden of malaria was concentrated in this group.

Unlike previous studies conducted by our research group among IDPs in sub-Saharan Africa [31, 47, 67], maternal education was not associated with bed net use among children under five in our study population. We hypothesize that this finding is a result of the extreme poverty and long duration of displacement that is unique to our population. We propose that under the horrible living conditions faced by IDPs in Birambizo, competing nutritional needs are so severe that any available finances are directed towards feeding children rather than malaria prevention, despite knowledge of malaria as a deadly disease

for children. Nutritional needs were repeatedly discussed by FGD participants, and is not an uncommon need among displaced populations, however we are unaware of other IDP camps in tropical environments facing the same level of violent conflict and nutritional deprivation whilst simultaneously being severely affected by malaria. Furthermore, we were surprised to find that bed nets did not have a statistically significant protective effect among children under five. Again, we propose that this is due to the conditions of the displacement camp, where it is near impossible to maintain bed nets in good condition and use them appropriately. Additionally, due to the long duration of displacement experienced by our study population, it is likely that individuals of higher wealth and education were better able to relocate and resettle into neighbouring communities and find employment. Thus, our study population may contain a higher number of individuals with lower education levels and extreme poverty compared to other study populations. Our data suggests that this may be true, based on the extremely low wealth of our population and few mothers with secondary education or higher. It is possible that a full spectrum of wealth and education is necessary among study populations in order to observe the protective effects of maternal education and bed nets on malaria.

As highlighted by both the qualitative and quantitative results presented here, barriers to bed net use among IDPs are numerous, and call into question the usefulness of bed net distribution campaigns as currently implemented in IDP camps in sub-Saharan Africa. Bed nets in more stable contexts in Africa are an evidence-based approach to malaria control with proven effectiveness, ease of use and distribution [1, 41]. However, in the context of complex humanitarian emergencies where households are mobile, living conditions are cramped, and malaria transmission is elevated, bed net distribution campaigns may be less effective.

Modifications to bed net design may make them more user-friendly within the small shelters of displaced populations. Alternatively, indoor residual spraying (IRS) could be used; however, IRS can be operationally challenging and economically unsustainable [1, 68-70]. Within Birambizo IDP camp, IRS was carried out when NGOs were permanently operating within the camp, however violent conflict interrupted NGO efforts at maintaining IRS interventions. One other possible vector control method that

could be well suited to the IDP camp setting is the use of insecticide-treated tarpaulin for IDP shelters. A recent review [68] highlights evidence suggesting the effectiveness of insecticide-treated durable wall lining over multiple years, however phase III trials are still underway to provide evidence as to the operational implementation of this novel vector control method within displaced populations [71-73]. It is also important to note that emerging insecticide resistance in mosquitoes may account for some of the observed ineffectiveness of bed nets within our study population. Studies on insecticide resistance within the mosquito population of the area would be required to confirm these suspicions, however it is important not to dismiss the possibility as resistance has been increasingly detected [2].

Our study is noteworthy as a qualitative description of barriers to bed net utilization and a quantitative description of RDT-confirmed malaria in an IDP camp deep within a zone of ongoing human insecurity. Nonetheless, this study has several limitations. It was conducted at a single, densely populated IDP camp within a region of high malaria transmission. Therefore, results should not be extrapolated to low transmission settings or areas with marked seasonal malaria transmission. The wealth index is specific for the IDP context and cannot be compared to other wealth indices due to the extreme poverty and subsequently low asset ownership of IDPs. Similarly, FGDs were conducted with a small population of DRC IDP camp residents, and therefore should only be extrapolated to other IDP camp settings with caution. Despite reaching saturation within our qualitative data, our participants were purposively selected and thus we inevitably have not collected a full range of qualitative descriptions for bed net disuse. Translation of our initial FGD transcripts from an outside source and including participants from different IDP camps and villages could have broadened our data. Furthermore, it is well-recognized that the HRP2-based RDT (Paracheck-Pf®) used in our cross-sectional study will test positively up to four weeks after parasite clearance [56, 57], such that individuals who had infections in the past month would test positively. Additionally, the HRP2-based RDT may have limited sensitivity at low parasite density, which would lead to underestimations in point prevalence, particularly among non-symptomatic participants [74]. The use of additional diagnostic tools such as microscopy and/or polymerase chain reaction would be desirable; however, given the resource limitations in our study setting, this was not feasible. Our

resource limitations also limited our data such that community health workers were responsible for collecting data using the door-to-door quantitative survey (as they were trained to use the RDTs), likely introducing bias into our survey data as participants did not have an established relationship with the researcher and in that situation, gaining trust from participants can be difficult. Thus, survey answers may not have been wholly truthful. Lastly, emerging insecticide resistance [1] was not addressed, but could be a factor threatening the efficacy of bed nets within the context of our study population, where bed nets are not consistently used and bed net distribution campaigns are unpredictable.

3.6. CONCLUSIONS

This study, from a complex chronic humanitarian crisis in the DRC, illustrates complexities and limitations of bed net distribution as a malaria control strategy in an IDP camp. Engineering improvements on current bed net design, to accommodate the special needs of a tented camp, might be considered to improve their acceptability and retention in this unique setting. Alternative vector control methods (e.g., larval breeding control, indoor residual spraying, insecticide-treated tarpaulin, and others) may be considered in addition to or in lieu of bed nets in the IDP camp setting. Addressing competing needs and food insecurity will be important to ensure that donated goods, including bed nets, are not sold or exchanged.

IDPs in remote areas in the tropics, further isolated by violent conflict, and afflicted by malaria among numerous other threats to their health and well-being, pose a formidable public health challenge. Given the unique situation in an IDP camp, malaria control strategies borrowed from other contexts may not be directly transferrable. Our findings call for an improved, thoughtful, and tailored approach to address the burden of malaria in IDP camps in the tropics and suggest that bed net distribution alone is not a panacea.

CHAPTER 4 – CEREBRAL MALARIA

Material from this Chapter (sections 4.1 and 4.3) has been previously published [75].

4.1. INTRODUCTION

One of the most severe consequences of malaria in children is cerebral malaria (CM). In highly endemic areas in sub-Saharan Africa, CM affects children under 5 during a critical period of brain development. Clinical diagnosis is challenging due to the non-specific nature of the symptoms, which may be mistaken for benign viral infections or life-threatening bacterial infections such as pneumonia, sepsis, and meningitis [76, 77]. Despite efficacious drugs that rapidly eliminate parasites, CM continues to cause death and developmental disability, with up to 30% of CM survivors experiencing lasting neurocognitive deficits [10]. The pathologic processes leading to CM are not fully elucidated; however, widely accepted mechanisms include parasite sequestration, release of infected red blood cell contents, activation of endothelial cells, increased inflammatory responses, and ultimately dysfunction of the neurovascular unit (NVU) [24, 25].

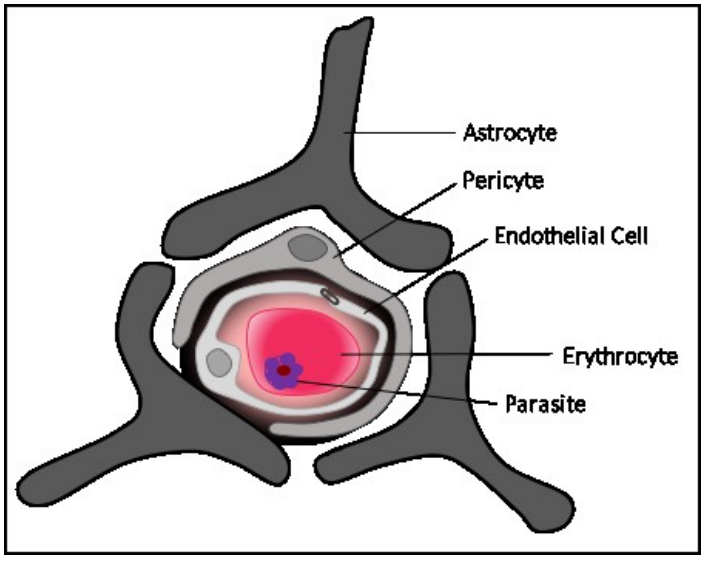

Figure 4.1 Simplified cross section of the neurovascular unit (NVU). The 3 main cell types that make up the NVU are endothelial cells, astrocytes, and pericytes. The red shaded area represents the

microvasculature. The white area surrounding the astrocytes represents the brain parenchyma. Parasites are present in red blood cells circulating in the brain microvasculature during cerebral malaria.

The NVU is comprised mainly of 3 cell types: endothelial cells, astrocytes, and pericytes (Figure 4.1). The endothelium is the site of parasitized erythrocyte (PE) sequestration, as well as the mediator of fluid extravasation in the central nervous system (CNS) [78, 79]. During infection with *P. falciparum*, the endothelium undergoes functional changes, becoming activated [79]. In the activated state, endothelial cells express a variety of cellular adhesion molecules, which promote the binding of inflammatory cells such as neutrophils and macrophages, and the cytoadherence of PEs [80-87]. Cytoadherence, rupture and release of PE contents, and/or host cytokines may lead to activation of cell signalling pathways that alter the junctional permeability of endothelial cells, leading to transcellular and paracellular leakage, and ultimately disrupting and destabilizing the NVU [88, 89]. Pro-coagulant factors in children with severe malaria also contribute to endothelial cell activation and microvascular thrombosis [90]. Factors such as soluble endoglin, endothelins, and matrix metalloproteinases are also found to be significantly elevated in patients with severe malaria, corroborating the joint roles of inflammation and endothelial activation in severe malaria [91-93]. Cerebral edema in CM is believed to result from these processes.

Clinical, radiographic, and pathology studies provide a body of evidence implicating cerebral edema as a major pathologic feature of CM. Brain swelling occurring during CM may be cytotoxic, vasogenic, or due to increased intravascular fluid [89]. Cytotoxic edema occurs when dysfunction of the sodium-potassium ATPase ion pump, due to hypoxia or nutrient deprivation, leads to osmotic fluid redistribution across cell membranes and accumulation of intracellular water in neurons or glial cells [89]. Vasogenic edema occurs when NVU dysfunction and subsequent fluid movement from the intravascular space into the brain interstitium leads to intercellular edema [89]. Finally, brain swelling may be due to sludging of blood flow with PE sequestration, which results in increased intravascular fluid volume [89]. In children, increasingly severe pathologic features on autopsy (PE sequestration, intravascular and extravascular pathology) are associated with progressive increases in brain edema [89]. Intracranial pressure, measured

by manometry of cerebrospinal fluid (CSF) is elevated in both adults and children with CM [94-96]. Brain weight at autopsy was elevated in 86% of children with severe CM pathology, 70% of patients with sequestration alone, and 62% of patients with no sequestration [26]. Similarly, brain swelling was observed on CT scans in 63% of CM patients in an Indian study [94]. In a recent study from Malawi, 84% of children who died of CM had MRI evidence of brain swelling compared to 27% of children who survived [97]. Thus, several lines of evidence point to cerebral edema as a major pathologic process in CM. Novel adjunctive therapies that target the endothelium to reduce cerebral edema may therefore improve outcomes in patients with CM.

Here, we provide a narrative review of current recommended malaria treatment (anti-parasitic agents), and host-directed adjunctive therapies which have been tested for CM. We next discuss potential areas for future development of pharmacologic agents, with particular attention to potentially neuroprotective agents that stabilize the endothelium. We begin with a description of the anatomy and function of the NVU, and highlight selected molecular pathways involved in endothelial activation and dysfunction. We then describe the shortcomings of current CM therapeutics and propose two major regulatory pathways that govern endothelial permeability, sphingosine-1 phosphate (S1P) and vascular endothelial growth factor (VEGF), which could be targeted with recently licensed pharmaceuticals. Lastly, we describe research conducted *in vitro* to mimic the NVU and test the ability of select candidate molecules to prevent dysfunction of the NVU in response to inflammatory, edemagenic, and infectious challenge.

4.2. HYPOTHESIS

Our hypothesis was that novel neuroprotective agents would reduce BBB dysfunction in experimental models of CM. We tested this hypothesis by measuring electrical resistance of cells in response to inflammatory, edemagenic, and infectious challenges in the following two models 1) an in vitro BBB model, using primary endothelial cells co-cultured with primary astrocytes; and 2) an in vitro BBB model using immortalized endothelial cell monolayers.

4.3. BACKGROUND

Malaria is spread by female *Anopheles* mosquitoes infected with *Plasmodium* spp. The dominant pathogen in Africa is *P. falciparum*, an intra-erythrocytic parasite that exerts its pathogenicity on the CNS without direct contact with neurons. The disease is characterized by fever, myalgia, and headache in its uncomplicated form, but may progress to central nervous system (CNS) involvement with convulsions, altered consciousness, coma, and even death. The endothelium plays a central role in the pathologic processes in CM.

4.3.1 Structure and function of the NVU and its role in CM

The NVU consists of multiple cellular and extra-cellular components. This functional unit is composed of groups of neurons and their associated astrocytes which together interact with smooth muscle cells and endothelial cells on micro vessels [98]. Endothelial cells differ by organ site and the endothelial cells of the brain capillary are highly specialized for regulating the passage of substances [99]. The anatomical basis of the NVU lies in the tight junctions between endothelial cells and their low pinocytotic activity [100, 101]. Brain homeostasis is controlled by tight junctions which regulate the paracellular permeability between brain endothelial cells of the NVU [99]. Interactions between adjacent endothelial cells require various junctions and components, which together form a cobblestone mosaic layer lining the microvasculature [102, 103]. Tight junctions form a continuous circumferential belt around the endothelial cell and regulate the paracellular passage of solutes and ions and restrict the free movement of lipids and proteins from apical and basolateral cell surfaces [104]. The brain endothelium has more complex tight junctions than the capillary beds outside the CNS [98]. Several molecular components make up the tight junctions between endothelial cells, including occludin, claudin, and the zonula occludens (ZO) proteins. Adherens junctions initiate cell-cell contacts and are considered essential for the formation of tight junctions [105]. These junctions bridge cell-cell contact points to the intracellular actin cytoskeleton. They may appear as bands encircling the cell (zonula adherens) or as points of attachment to the extracellular matrix (adhesion plaques). In the NVU, the interaction of transmembrane VE-cadherin dimers is responsible for establishing adherens junctions. Regulation of

endothelial cell junctions and vascular permeability can be modulated by vasoactive compounds, such as inflammatory cytokines, thrombin, histamine, and VEGF, that induce transient gaps between endothelial cells and facilitate paracellular permeability [106]. Disruption of cell adhesion between brain capillary endothelial cells plays a major role in the onset and progression of CNS disorders [99].

P. falciparum is an intra-erythrocytic parasite which exerts its pathogenicity on the CNS without direct contact with neurons. In order to generate CNS injury in malaria, the interface between the vessel lumen and the brain parenchyma must be disrupted. Thus, the endothelium and closely associated components of the NVU have central roles in malaria pathogenesis. In children with CM, focal loss of immunostaining for zonula occludens protein 1 (ZO-1), occludin, and vinculin is spatially associated with PE sequestration [107]. Similarly, adults with fatal CM have a loss of tight junction proteins and leakage of plasma proteins across the microvasculature [108]. Endothelial integrity decreases in murine models of malaria in the brain, lungs and kidneys [83, 109-114]. Three mechanisms have been shown to cause increased permeability of the endothelial layer: paracellular leakage, transcellular leakage, and leakage due to endothelial cell death [89]. The integrity of the NVU can be altered by PEs that induce metabolic acidosis, thereby opening tight junctions [85]. This effect is reproducible *in vitro* when parasite metabolism generates low pH in culture media [85]. Experiments using endothelial monolayers co-cultured with PEs from CM patients have shown decreased mRNA transcripts for tight junction proteins [115]. Other experiments with human brain endothelial cell monolayers have demonstrated a decrease in transendothelial electrical resistance of the monolayers upon direct contact with PEs or *P. falciparum* supernatant [116, 117]. Recent evidence suggests that the rupture of parasites and release of contents, rather than cytoadhesion alone, is necessary to induce brain endothelial monolayer disruption [117].

4.3.2 Current Adjunctive therapies for CM.

Current WHO guidelines recommend treatment with artemisinin combination therapy (ACT) for uncomplicated malaria and parenteral artesunate followed by ACT treatment for severe malaria [27, 28]. Artesunate has clear advantages over quinine, the previous standard primary treatment for severe malaria [28, 118]. Artemisinin derivatives are generally safer and have fewer serious side effects, and patients

treated with artesunate have lower mortality rates than those treated with quinine [28, 118]. Clearance of parasites varies according to disease severity and geographical location, but is more rapid in individuals treated with artesunate compared to quinine [119-121]. A recent trial found an average clearance time of 16.5 hours compared to 21.7 hours when using artesunate versus quinine as treatment, respectively [122].

In South East Asia, increasing parasite resistance to artemisinin derivatives threatens the efficacy of first line treatments [123]. Even under optimal conditions, the mortality rate for severe malaria treated with artesunate is 8.5% [28]. In an effort to reduce severe malaria fatalities and to prevent neurological complications in CM survivors, numerous adjunctive therapies have been tested.

In CM, the host immune response has an important role in disease progression, motivating the study of therapies which attempted to alter specific and potentially deleterious immune responses. Many of these interventions have shown promise in murine models of severe malaria [29], but none have been shown to reduce mortality in human studies, to date [30]. Previous attempts included: modifying the immune response, iron chelation, correction of acidosis, reduction of intracranial pressure, prevention of seizures, prevention of cytoadherence, and modulating the BBB. These have been reviewed previously [30] and here we briefly discuss progress to date with adjunctive strategies that target cerebral edema and the BBB.

Mannitol, an osmotic diuretic that lowers intracranial pressure, was put forward as a therapy for clinical management of CM owing to its ability to regulate the movement of fluids from brain parenchyma into microvascular circulation. Mannitol was able to reduce intracranial pressure in children with CM who had moderately elevated levels of intracranial pressure, but not in patients with severely elevated levels, and it did not reduce mortality [124, 125]. In a randomized controlled trial (RCT) with adult CM patients, without monitoring intracranial pressure, prophylactic mannitol was found to have deleterious effects, and prolonged coma duration in survivors [94]. This particular RCT was not powered to assess differences in mortality; however, there was a trend towards increased mortality in the treatment group compared to the placebo control [94, 126].

Decreased bioavailable nitric oxide (NO) is associated with CM and may contribute to endothelial dysfunction. Hence, there is increasing interest in interventions that restore normal NO signalling during

malaria infection. An RCT of inhaled NO (iNO) versus room air placebo as adjunctive treatment to artesunate in 180 children with severe malaria was conducted in Uganda. No difference in mortality nor in circulating levels of Ang-2, a surrogate marker of malaria severity, were detected [127]. Similarly, a trial of iNO in 90 children with CM did not show a difference in mortality or biomarkers of endothelial activation [128].

In addition to its role in stimulating the production of erythrocytes, the peptide hormone erythropoietin (EPO) has a neuroprotective function, increasing endothelial barrier stability. Murine models have shown promising results using EPO, which led to more rapid clinical recovery, increased survival, and neuroprotection [129]. However, recombinant human EPO therapy is associated with clinical complications including thromboembolic disease [130-132].

Recent findings suggest that rupture of infected erythrocytes activates β-catenin, leading to disruption of brain endothelial cells through increased transcription of factors that disrupt endothelial cell-cell junctions [117]. Inhibition of β-catenin through angiotensin II type 1 receptor (AT1) or angiotensin II type 2 receptor (AT2) modulators may reduce BBB dysfunction in CM and may therefore serve as adjunctive therapeutics. In experimental cerebral malaria (ECM) models using C57BL/6J mice, both the AT1 blocker irbesartan and the AT2 agonist compound 21, have reduced CM-associated mortality when used along with chloroquine, compared with infected mice treated with chloroquine alone [117].

In an attempt to improve outcomes in CM, several adjunctive agents targeting the endothelium have been explored, though none have yet demonstrated clinical efficacy in human studies. Additional pathways regulating endothelial stability and activation warrant further study as potential targets for drug development.

4.3.3 Endothelial Pathways as Targets in CM.

Cytoadherence of PEs or rupture and release of PE contents in the microvasculature leads to activation of phosphorylation pathways that alter the junctional permeability of endothelial cells, ultimately disrupting and destabilizing the NVU [88, 89]. Numerous factors have been shown to affect the

permeability of the BBB, including VEGF, S1P, NO, bradykinin, histamine, serotonin, glutamate, purine nucleotides, adenosine, platelet-activating factor, phospholipase A2, arachidonic acid, prostaglandins, leukotrienes, interleukins (IL-1α, IL-1β, IL-6), and tumour necrosis factor (TNF). Several of these mediators have been implicated in CM pathology [109, 133-135] and select pathways will be discussed.

4.3.3A Sphingosine-1-Phosphate

S1P is a lipid that regulates angiogenesis, vascular stability, and permeability through its interaction with its cognate G protein-coupled receptor, S1PR. S1P has been implicated in several physiological and disease processes including atherosclerosis, diabetes, osteoporosis, Alzheimer's, and many malignancies [136, 137].

Two sphingosine kinase isoenzymes exist (SK1 and SK2) which are responsible for the production of S1P from sphingosine. These enzymes display distinct cellular localization patterns and fulfill different roles. SK1 is mainly localized in the cytosol, but has also been found to translocate to the plasma membrane following phosphorylation by ERK [138]. In contrast, SK2 is present in several intracellular compartments including the nucleus, mitochondria, and endoplasmic reticulum (ER). Subcellular localization of SK1 and SK2 determines the outcomes of S1P signalling. When S1P is formed by SK1 at the plasma membrane, it can easily be exported from the cell to act in an autocrine or paracrine fashion [139-141]. S1P produced by SK2 is likely to be quickly degraded due to its proximity to ER-bound S1P lyase and S1P phosphatases which degrade and dephosphorylate S1P, respectively. In the nucleus, S1P acts as an endogenous inhibitor of histone deacetylases, thereby regulating gene transcription [136].

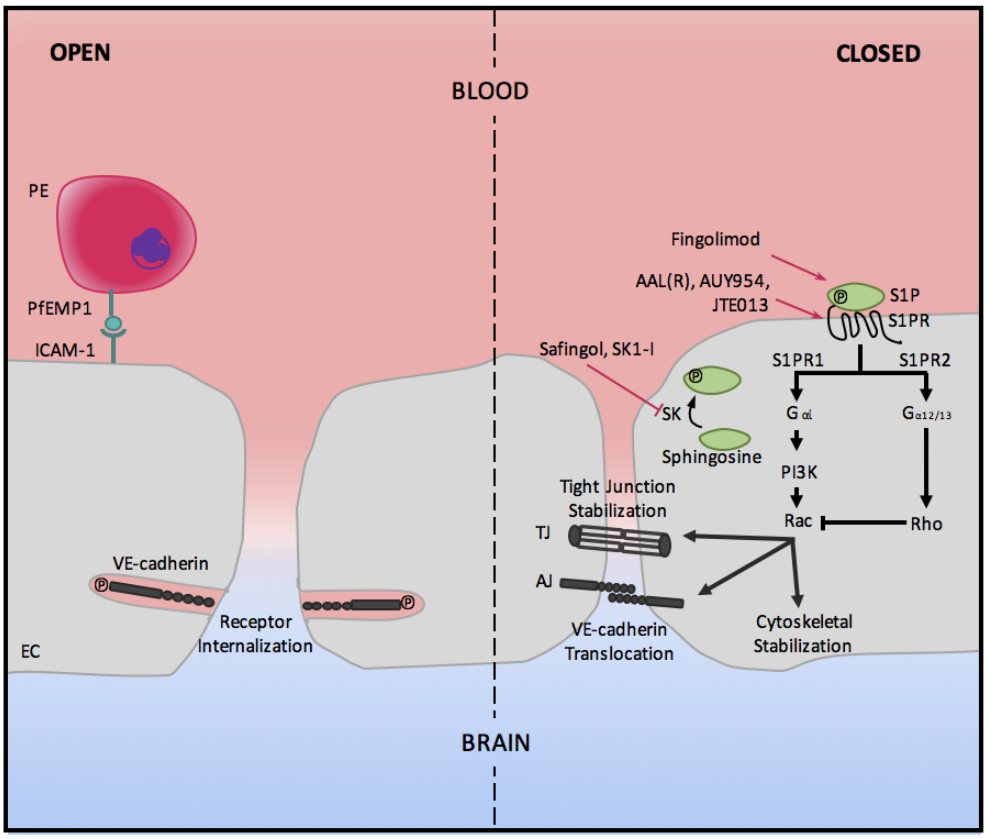

Figure 4.2 Sphingosine-1 phosphate (S1P) signalling and downstream effects on endothelial barrier stability. During cerebral malaria, parasitized erythrocyte (PE) binding to ICAM-1 or other cell surface receptors on endothelial cells (ECs) results in destabilization of the endothelial monolayer. S1P signalling through its cognate G-protein coupled receptors (S1PRs) results in stabilization of the endothelial barrier by tight junction (TJ) stabilization, VE-cadherin translocation, and cytoskeletal stabilization. Pharmacologic modulators of the S1P pathway include fingolimod, AAL(R), AU7954, JTE013, safingol, and SK1-I. AJ, adherens junction; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; PI3K, phosphatidylinositol 3-kinase; SK, sphingosine kinase.

Although S1P has been implicated in intracellular-mediated signalling [142], most S1P effects have been attributed to its function as an extracellular signalling molecule [137]. The cell surface receptors for S1P are transmembrane G protein-coupled receptors, classified into 5 subtypes: S1PR1-5. Downstream signalling pathways stimulated by S1P vary depending on the Gα subunit that is activated, which varies

according to the profile of S1PR expression on a given cell type. S1P signalling may therefore produce pleiotropic responses on several pathways, including those involved in cell survival, vascular tone, and endothelial tight junction integrity [143]. S1PR1–3 are expressed on smooth muscle and endothelial cells, where they regulate vascular homeostasis and vascular permeability [142, 144]. The actions of S1P on endothelial cells are mediated through the coordinated action of S1PR1 and S1PR2 [145]. S1P dynamically regulates endothelial barrier tightness, causing rapid opening followed by closing and strengthening of the barrier [145]. Acting through S1PR1, S1P promotes adherens junction and tight junction assembly, and cytoskeletal rearrangements, leading to enhanced vascular barrier function (Figure 4.2). Intracellular events following stimulation of S1PR1 include activation of the G(i)/Akt/Rac pathway, leading to cytoskeleton stabilization and the maintenance of tight junctions and adherens junctions [146]. Rearrangement of endothelial cell cytoskeleton results in cellular spreading, reduction of gaps between neighboring endothelial cells and increases the transendothelial electrical resistance [147]. Furthermore, S1P increases the translocation of VE-cadherin to adherens junctions *in vitro* [148]. Finally, after S1P stimulation, the tight junction protein ZO protein-1 is redistributed to cell-cell junctions and forms functional complexes with α-catenin [149]. S1P signalling pathways also interact with inflammatory signalling pathways at the endothelium, through the endothelial protein C receptor (EPCR). EPCR ligation and S1P1 transactivation results in cytoskeletal rearrangement and stabilization of endothelial monolayer integrity [150-152]. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) binds to EPCR, implicating S1P in BBB permeability upon parasite sequestration, as reviewed previously [150, 153]. Of note, S1P has been shown to play a protective role in both human CM and ECM [154]. Acting through S1PR2, S1P stimulates the Rho pathway and induces cytoskeletal contractions as well as phosphorylation and internalization of VE-cadherin [155]. Thus, S1P plays a dual role in vascular permeability, depending on its receptor, resulting in dynamic effects on endothelial permeability [145].

A recently licensed first-in-class pharmacologic modulator of the S1P pathway is fingolimod (FTY720). The compound was isolated from the fungus *Isaria sinclairii* [156], and is structurally similar to S1P, allowing it to act as a substrate for SK2. Once phosphorylated, the small molecule can function as

an agonist to four of the five S1P receptors: S1PR1, S1PR3, S1PR4, S1PR5 [137]. Fingolimod binding to S1P receptors results in activation of S1P1R, thereby reducing inflammatory responses by T-cell sequestration to the lymph nodes and ultimately increasing adherens junction assembly and stabilizing endothelial cell-cell contacts [144, 157]. In endothelial cells, fingolimod enhances VE-cadherin adherens junction assembly and thus endothelial barrier function by inducing VE-cadherin translocation to focal contact sites between cells [158]. On the other hand, fingolimod did not increase transendothelial electrical resistance or tight junction protein expression in rat brain microvascular endothelial cell culture exposed to inflammatory conditions *in vitro*, emphasizing the dual role of S1P on vascular permeability, depending on its receptor [159]. In addition to its endothelial effects, fingolimod attenuates excitotoxicity and neuroinflammation through inhibition of p38 MAPK stress signalling pathway in microglia [160]. Fingolimod has been FDA-approved for several diseases, including multiple sclerosis, dermatitis, arthritis, and allergies [158, 161-163].

Other small molecules that have not yet been FDA-approved, but which affect the SK/S1P pathways, include safingol, an SK inhibitor used against solid tumors [164]; SK1-I, an SK1 inhibitor used against glioblastomas and leukemia [165, 166]; THI, an S1P lyase inhibitor for lung injury and ischemia/reperfusion [167, 168]; AAL(R), an S1PR1 and S1PR3 agonist used against Influenza viruses [169]; AUY954, an S1PR1 agonist used during transplantations and to treat sepsis [170-172]; and JTE013, an S1PR2 agonist used for anaphylaxis, atherosclerosis, and cancer [173, 174]. Current and future SK/S1P agonists may enhance treatment options for diseases regulated by SK/S1P-dependent components.

Given the central role of the NVU in CM pathogenesis, pharmacologic modulation of S1P pathways in the brain endothelium may improve outcomes in CM. In ECM, fingolimod has been shown to improve survival and reduce BBB leakage in mice infected with the murine malaria species, *P. berghei* ANKA [154, 175, 176]. The mechanisms underlying these observations have not been elucidated, but may involve altered trafficking of leukocytes into the CNS or changes in BBB permeability to macromolecules independent of inflammatory cell infiltrates. Although no clinical trials of fingolimod or other S1P modulators in CM have yet been conducted, this is a promising agent for the adjunctive treatment of CM.

Because the agent has already been licensed for use in humans for other indications, repositioning of the drug indication for CM may be feasible at relatively low cost and low risk.

4.3.3B Vascular Endothelial Growth Factor

VEGF family members are major mediators of vasculogenesis and angiogenesis during human development and in pathological conditions. This growth factor family was originally described in highly vascularized tumours, where its expression increased in response to hypoxia [177, 178]. VEGF activity is restricted mainly to cells of the vascular endothelium; however, it does have effects on a limited number of other cell types. VEGF exerts potent mitogenic effects on the vascular endothelium through the inhibition of apoptosis, chemotaxis, and induction of blood vessel permeabilization.

Several VEGF isoforms are recognized, including VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor (PlGF). These growth factors are homodimeric polypeptides, produced by mast cells, that act mainly via binding to extracellular domains of transmembrane tyrosine-kinase receptors (VEGFRs), inducing receptor dimerization and subsequent autophosphorylation of the tyrosine residues in the intracellular catalytic domains [179]. This leads to an active receptor conformation, thereby activating a phosphorylation cascade, with signal transduction ultimately resulting in biological responses such as cell proliferation, migration, vasculogenesis, and endothelial permeability [179, 180].

There are 3 VEGF receptors, VEGFR1 (*flt-1*), VEGFR2 (*KDR/flk-1*), and VEGFR3 (*flt-4*), with overlapping but distinct expression patterns. VEGFR2, expressed on endothelial cells, mediates most responses to VEGF, including increases in endothelial permeability; all VEGF isoforms can bind this receptor with varying affinities [176, 181]. VEGFR1 negatively regulates VEGFR2 through its higher affinity binding to VEGFA and in part through the binding of free VEGF by soluble VEGFR1 [182]. VEGFR1 is also involved in the migration of monocytes and macrophages [180, 182]. VEGFR3, also expressed on endothelial cells, is specific for VEGFC and VEGFD and is important for lymphatic vessel development [183].

The VEGF/VEGFR2 signalling pathway has multiple effects, including calcium signalling, MAPK signalling, and FAK/Paxillin focal adhesion signalling. Two major mechanisms have been implicated in

vascular permeability increases due to VEGF/VEGFR2 signalling transductions: the creation of transcellular endothelial pores, and the transient opening of paracellular endothelial junctions [180, 184]. With respect to paracellular pathways, VEGF/VEGFR2 signalling regulates endothelial cell-cell contact by phosphorylation and internalization of VE-cadherin, an adherens junction protein [180]. In confluent, resting endothelial cells, VE-cadherin interaction with VEGFR2 is long lasting, and maintains the cells in a quiescent state. The transmembrane domain of VE-cadherin binds directly to the transmembrane domain of VEGFR2 [182, 185]. When VE-cadherin is bound to VEGFR2, the receptor is retained at the cell membrane, where it is dephosphorylated by DEP-1 phosphatase [180, 186]. VEGFR2 eventually becomes ubiquinated and degraded. Upon VEGF binding, VEGFR2 is released from VE-cadherin, stimulating Src phosphorylation cascades which ultimately result in VE-cadherin endocytosis due to serine phosphorylation [180]. During CM infection, VEGF levels are increased in the brain and soluble VEGFR2 levels are decreased [187]. Studies in specific cell lines HMC-1 and KU812 have demonstrated parasite-induced increases in VEGF secretion from mast cells [188]. Due to the involvement of the vascular endothelium and VEGF/VEGFR pathways in CM, agents targeting this pathway may be used as adjunctive therapies for CM (Figure 4.3).

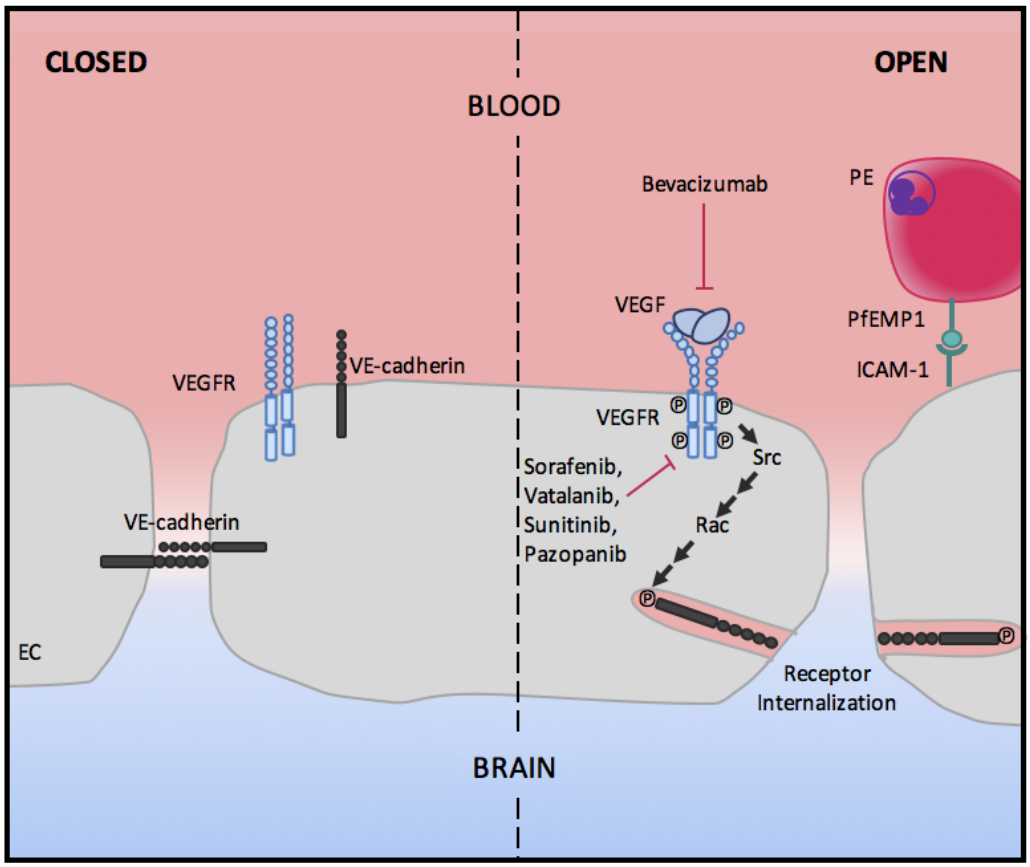

Figure 4.3 Vascular endothelial growth factor (VEGF) signalling and downstream effects on endothelial barrier stability. Under normal conditions, the brain endothelial barrier remains closed due to stable interactions between adherens junction protein VE-cadherin on neighboring endothelial cells (ECs). During cerebral malaria infection, parasitized erythrocyte (PE) binding to ICAM-1 or other cell surface receptors on ECs destabilizes the endothelial monolayer. VEGF binding to its cognate receptor (VEGFR) leads to a phosphorylation cascade through Src and Rac, ultimately resulting in VE-cadherin phosphorylation and subsequent internalization. As a result, the endothelial barrier opens, allowing for blood and associated particles to leak into the brain parenchyma. Pharmacologic modulators of the VEGF/VEGFR pathway include bevacizumab, sorafenib, vatalanib, sunitinib, and pazopanib. PfEMP1, *P. falciparum* erythrocyte membrane protein 1.

The VEGF signalling pathway has been the focus of investigation and cancer drug development due to its important role in angiogenesis and tumour neovascularisation. Many types of solid tumours express high levels of VEGFRs, including glioma carcinomas, breast carcinomas, ovarian carcinomas, and gastrointestinal tract carcinomas [183]. Consequently, inhibition of VEGF/VEGFR pathways has been studied as a potential therapy for cancers, and has been clinically validated with FDA-approved pharmacologic agents. Two major classes of compounds blocking VEGF/VEGFR exist: monoclonal antibodies, and small molecules that block VEGFR tyrosine kinase activity [179]; both types are discussed below.

Bevacizumab is a monoclonal antibody that selectively binds to and neutralizes VEGFA, and was FDA-approved for the treatment of recurrent glioblastoma [189-191]. Sorafenib (Nexavar) is a potent small-molecule inhibitor of VEGFR2, VEGFR3, and Raf serine/threonine kinase isoforms. Sorafenib was FDA-approved for the treatment of renal cell carcinoma, hepatocellular carcinoma, differentiated thyroid carcinoma, and for gastrointestinal stromal tumors [192-194]. Vatalanib is a small-molecule receptor tyrosine kinase inhibitor (TKI) that inhibits VEGFR2, which was approved for the treatment of advanced non-small cell lung cancer [194]. Sunitinib (SU11248, Sutent) is a small-molecule receptor TKI that targets all three VEGFRs, and was approved by the FDA for treatment of renal cell carcinoma, neuroendocrine cancer, and resistant gastrointestinal stromal tumours [194-197]. Pazopanib (GW786034, Votrient) is another small-molecule receptor TKI that inhibits all three VEGFRs, and which was approved for treatment of renal cell carcinoma and soft tissue sarcoma [194]. Pazopanib dosing in children has been established, making this molecule an attractive candidate for rapid translation to clinical trials in CM [198].

One licensed TKI, imatinib, has been tested in experimental models of malaria, providing proof-of-principle that targeting this pathway may one day improve outcomes in patients with malaria. Imatinib (Gleevec, Glivec) is a TKI that targets ABL1 kinase, which is induced by VEGFR-2 signalling [199]. Imatinib was FDA-approved for the treatment of chronic myelogenous leukemia [200-203]. Imatinib modulated VEGF-induced vascular permeability in endothelial cells and in murine models of malaria

[199, 204]. Imatinib has not yet undergone clinical trials in humans with CM. Other TKIs may also show promise as modulators of endothelial permeability in CM.

We hypothesized that select molecules discussed would reduce *Plasmodium*-induced endothelial permeability and BBB leak in our *in vitro* model of CM. Mimicking the NVU *in vitro* is possible by culturing endothelial cells and astrocytes under specific conditions [116, 205, 206]. Two molecular pathways were examined *in vitro*, which have been shown to play central roles in endothelial activation: (1) sphingosine-1-phosphate (S1P) and its cognate receptor (S1PR1); and (2) vascular endothelial growth factor (VEGF) and its receptor-2 (VEGFR2) signalling. Candidate molecules that interact with these pathways include: S1PR1 modulator fingolimod (FTY720), and VEGFR2 inhibitors pazopanib, sunitinib, and imatinib. If effective, these agents could be applied to *in vivo* studies of ECM and subsequently in clinical trials to the treatment of children with CM. The *in vitro* methods used will next be described, followed by results and conclusions.

4.4. MATERIALS AND METHODS

Cell lines

Human cerebral microvascular endothelial cells (HCMEC/D3) were used in our monoculture BBB model *in vitro*. HCMEC/D3 (Millipore, Temecula, CA, USA) were cultured in endothelial growth medium (EndoGRO, Millipore) and expanded in 75-cm^2 tissue culture flasks (Corning Life Science, Tewksbury, MA, USA) coated with rat collagen type I (Millipore) at 37 °C with 5% CO_2 until confluency (5-7 days), then detached with a trypsin (0.05% wt/vol)–EDTA (0.02% wt/vol) solution (Gibco Thermo Fisher Scientific, Waltham, MA, USA). Human brain microvascular endothelial cells (HBMECs) and human astrocyte (HAST) cell lines were used in our co-culture *in vitro* BBB model. HBMECs are known to mimic barrier properties of vessels in the CNS, and in co-culture with astrocytes, the barrier function of endothelial cells increases significantly. HBMECs (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in endothelial cell growth medium (EM, ScienCell) and expanded in 75-cm^2 tissue culture flasks (Corning Life Science) coated with fibronectin (Sigma Aldrich, Saint Louis, MO, USA) at 37 °C with 5% CO_2 until confluency (5-7 days), then detached with a trypsin (0.05% wt/vol)–EDTA (0.02%

wt/vol) solution (Gibco Thermo Fisher Scientific). HAST cells were cultured in astrocyte medium (AM, ScienCell) and expanded in 75-cm^2 tissue culture flasks coated with poly-L-lysine (Sigma Aldrich) at 37 °C with 5% CO_2 until confluency (5-7 days) then detached with a trypsin (0.05% wt/vol)–EDTA (0.02% wt/vol) solution.

In vitro BBB model

Confluent endothelial monolayer. HCMEC/D3 were seeded at a density of $1.0x10^5$ on the apical side of polycarbonate coated transwell (TW) inserts with 0.4 μm pores (Corning) in EndoGRO media, and incubated at 37 °C with 5% CO_2 for up to 8 days. Each day following cell seeding, media was replaced and TEER was measured using an STX2 electrode and EVOM2 meter (World Precision Instruments, Sarasota, FL, USA). At least three replicates were measured for each condition in a single experiment, and experiments were repeated at least three times. TEER in $\Omega*cm^2$ was calculated by subtracting the resistance of a TW without cells from a TW containing cells in the same types of media as the blank TW, and by subsequent correction for surface area. Normalized resistance was calculated using the following equation:

$$\frac{TEER\ (\Omega * cm^2)}{Average\ TEER\ of\ control\ wells\ (\Omega * cm^2)} - 1$$

where control wells are those containing only cells and no challenge or pharmacological agent.

Endothelial-astrocyte co-culture. To mimic the cell-cell interactions which contribute to the BBB, HAST were seeded at a density of $1.0x10^5$ to the basal side of polycarbonate coated TW membrane inserts with 0.4 μm pores, and after 2 hours the membrane was gently flipped and placed in a 24-well plate containing AM and incubated at 37 °C with 5% CO_2. Two days later, $1.0x10^5$ HBMECs were seeded in EM to the apical side of the TW inserts and incubated at 37 °C with 5% CO_2 for up to 8 days (Figure 4.4). Each day following endothelial cell seeding, media was replaced and TEER was measured using an STX2 electrode and EVOM2 meter.

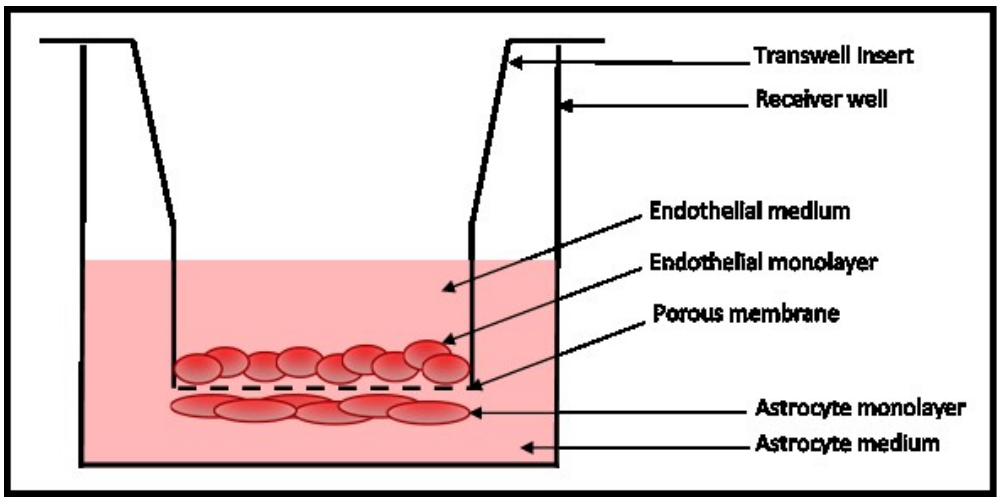

Figure 4.4 *In vitro* blood brain barrier model. Endothelial cells, seeded on the apical side of transwell inserts, could come into direct contact with astrocytes seeded on basal sides of transwell inserts via the transwell porous membrane. Cells were cultured in their respective mediums and transendothelial electrical resistance was measured daily following media changes.

Inflammatory, edemagenic, and infectious challenge

Endothelial monolayers were challenged with TNF (Thermo Fisher Scientific), VEGF (Gibco), or *P. falciparum* parasitized infected red blood cells (Pf-IRBCs) generously donated by the Yanow Lab. TNF was added to cells at concentrations of 10, 20 and 50 μg/mL. VEGF was added to cells at concentrations of 10, 20, 50, and 100 ng/mL. Pf-IRBCs were cultured in RPMI 1640 medium (Gibco) supplemented with 0.225% sodium bicarbonate, 40ug/ml Gentamicin sulphate, 11mM glucose, 200um hypoxanthine, 0.5% ALUMAX II solution; a subset of ICAM-1 binding parasites were selected by multiple rounds of panning on HCMEC/D3 cells; parasites were synchronized by selective sorbitol lysis of mature stages, then parasitized erythrocytes were purified by VarioMACS magnetic purification (Miltenyi Biotec Inc., Auburn, CA, USA) and either cryopreserved following a previously published protocol [207], or used directly. Parasitemia of Pf-IRBCs used was 7.5-13% and purity was 65-92%. Pf-IRBCs were added to the endothelial monolayers at a concentration of 5-15 RBCs per endothelial cell. Parasites settled onto

HBMECs through gravity, as previously described [208], for up to 24 hours. Each day following cell seeding, media was replaced and TEER was measured using an STX2 electrode and EVOM2 meter.

Pharmacologic rescue of barrier dysfunction

We next tested the ability of select small molecules to modulate endothelial permeability induced by TNF, VEGF, or Pf-IRBCs. TNF was added to confluent HCMEC/D3 monolayers (day 4) or HBMEC monolayers in co-culture with HASTs (day 4) at a concentration of 20 μg/mL. VEGF was added to confluent HCMEC/D3 monolayers (day 4) at a concentration of 20 ng/mL. Pf-IRBCs were cultured and purified as stated above, and added to confluent HCMEC/D3 monolayers (day 3-5) at a concentration of 5-15 RBCs per endothelial cell. Candidate molecules, their targets, plasma concentrations, and suppliers are listed in Table 4.1. Compounds were tested at physiologically plausible concentrations and were added 24 hours after inflammatory, edemagenic, or infectious challenge. Each day following cell seeding, media was replaced and TEER was measured using an STX2 electrode and EVOM2 meter. All experiments were conducted with three replicates for each condition, and were independently repeated three times, except for the following conditions which were repeated twice: HCMEC/D3 + pharmacological agents, HCMEC/D3 + TNF + imatinib, and HCMEC/D3 + VEGF + pharmacological agents.

Table 4.1 Pharmacological agents interacting with target pathways.

	Plasma Concentration	**Supplier**
S1PR1 Agonist		
Fingolimod	3.66 ng/mL [209]	Sigma Aldrich
VEGFR2 Antagonists		
Sunitinib	101 ng/mL [210]	Focus Biomolecules
Pazopanib	58 μg/mL [211]	Focus Biomolecules
Imatinib	2.1 μg/mL [212]	Sigma Aldrich

ELISA Assay

In a subset of experiments, supernatants from apical and basal sides of TW inserts were saved and used for ELISA analysis. Briefly, supernatants were collected from the same well on consecutive days and protein expression levels were analyzed using commercially available solid phase sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) according to specifications and protocol set by the manufacturer. Levels of Ang-2, soluble VEGFR1, and soluble ICAM-1 were quantified.

4.5. RESULTS

Monolayers of confluent immortalized endothelial cells and primary endothelial cell/astrocyte co-cultures recapitulate BBB function in vitro.

The HCMEC/D3 cell line is an immortalized cell line known to mimic properties of primary cells [213, 214], and has been widely used in *in vitro* BBB model systems [205, 213-217]. However, a more complex system can be mimicked by co-culturing primary endothelial and astrocytes, as astrocytes within the NVU influence mature brain endothelial phenotypes and barrier integrity [213, 214]. The use of primary human cell lines is desirable as it most closely represents the BBB in humans, however primary cell culture is challenging and cell differentiating properties are not preserved for many passages, thus limiting cell growth. For these reasons, we first used the HCMEC/D3 monoculture to test permeability disruption by TNF, VEGF, or Pf-IRBC and subsequent rescue by pharmacological agents. Co-culture was used to further validate these findings, when possible.

In order to validate our *in vitro* model of the BBB, we used TEER as an index of barrier function, representing a measure of ion flux across the confluent cell monolayer. Barrier resistance, as indicated by TEER measures, was similar between HCMEC/D3 monocultures and HBMEC co-cultures with HAST. These results are in accord with previous reports in which no significant increases in TEER were observed by co-culturing endothelial and astrocyte cells in TW model systems [213]. In both *in vitro* BBB models, TEER increased until cells reached confluency (day 3-4) and remained stable until approximately day 8,

after controlling for blank TWs (Figure 4.5). These findings suggest that our model BBB system mimics features of the BBB by restriction of ion flux.

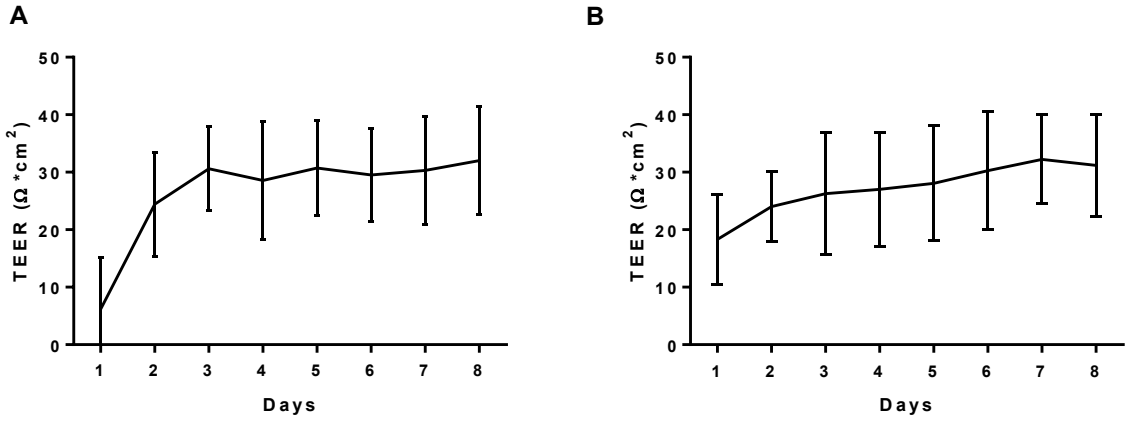

Figure 4.5 Stability of *in vitro* blood brain barrier model as a function of transendothelial electrical resistance (TEER). The A) HCMEC/D3 monoculture and B) HBMEC/HAST co-cultures reached confluency by day 3, after which time TEER remained stable until approximately day 8. Day 1 denotes the first time TEER was measured, 24 hours after endothelial cells were seeded to transwells. TEER was measured daily following media changes. Data shown represent pooled results from at least 3 independent experiments.

Permeability of in vitro BBB is increased with TNF, VEGF, and parasitized erythrocytes.

As an inflammatory cytokine, TNF increases endothelial cell permeability by destabilizing endothelial cell junctions [214, 218]. TNF and other cytokines have been implicated in the pathogenesis of CM [219, 220], and therefore TNF was a positive control for barrier disruption. The addition of TNF to confluent HCMEC/D3 monolayers resulted in a time ($p<0.0001$) and concentration ($p<0.0001$) dependent decrease in electrical resistance (Figure 4.6). The addition of 20 µg/mL of TNF was sufficient to induce a significant decrease in TEER after 48 hours ($p<0.0001$) in both monoculture and co-culture models; similar results have been demonstrated in other BBB models of malaria [216, 221].

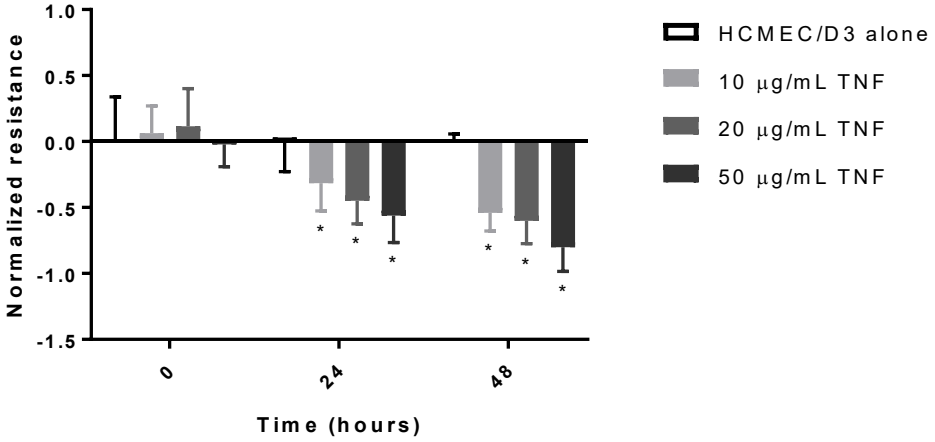

Figure 4.6 Concentration and time dependent decrease in endothelial monolayer integrity following challenge with tumour necrosis factor (TNF). At time 0, endothelial monolayers were challenged with varying concentrations of TNF. Transendothelial electrical resistance (TEER) was measured daily following media changes, and normalized resistance was calculated relative to control wells containing only media and endothelial cells. Data shown represent pooled results from at least 3 independent experiments with at least 3 replicates per condition. *$P < 0.0005$ compared with respective control condition.

Endothelial cell permeability is not only affected by inflammatory mediators such as TNF, but also by pro-coagulant factors such as thrombin and angiogenic factors such as VEGF. VEGF is a known inducer of BBB breakdown [106, 176, 222, 223] and was used to further validate our *in vitro* BBB model. As an endogenous mediator of endothelial permeability, VEGF is elevated in the plasma of patients with CM [187]. Likewise, in our model, the addition of VEGF to confluent HCMEC/D3 monolayers resulted in time ($p<0.0001$) and concentration ($p<0.0001$) dependent decrease in TEER (Figure 4.7). The addition of 20 ng/mL VEGF was sufficient to induce a significant decrease in TEER after 24 hours ($p<0.0001$) in our monoculture model.

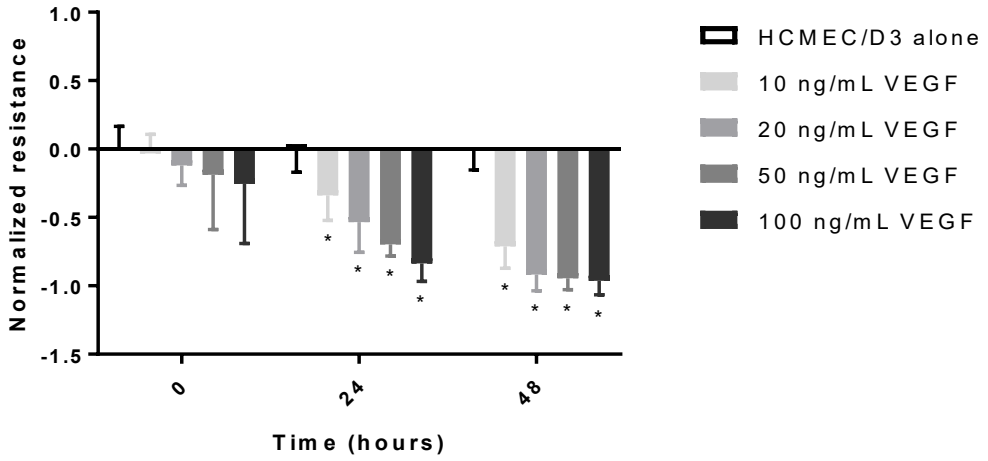

Figure 4.7 Concentration and time dependent decrease in endothelial monolayer integrity following challenge with vascular endothelial growth factor (VEGF). Endothelial monolayers were challenged with varying concentrations of VEGF at time 0. Transendothelial electrical resistance (TEER) was measured daily, following media changes. Normalized resistance was calculated relative to control wells containing only media and endothelial cells. Data shown represent pooled results from at least 3 independent experiments with 3 replicates per condition. *$P < 0.0001$ compared with respective control condition.

To assess the effect of Pf-IRBCs on BBB endothelial integrity, purified 3D7 parasitized erythrocytes at the trophozoite/schizont stage were added to confluent HCMEC/D3s. Pf-IRBCs at this stage have increased expression of extracellular proteins that mediate endothelial cell binding and have a greater effect on barrier integrity [116]. The 3D7 strain was chosen for its high ICAM-1 binding properties [116]; endothelial binding was further increased by panning infected parasites on HCMEC/D3 monocultures up to 4 times prior to the purification and addition of Pf-IRBCs to the BBB models. Infected RBCs were added at a ratio of 5-15 RBCs per endothelial cell. Previous *in vitro* studies have reported a time and concentration dependent decrease in electrical resistance of endothelial cells following challenge with Pf-IRBCs [116]. In our HCMEC/D3 monoculture model, these results were replicated, showing time ($p<0.0001$) and concentration ($p<0.0001$) dependent decrease in TEER (Figure 4.8 A). Even when using

Pf-IRBCs that had not been selected through panning, Pf-IRBCs induced a decrease in endothelial electrical resistance (data not shown). Uninfected RBCs had no effect on endothelial monolayer resistance with no statistically significant difference between HCMEC/D3 cells alone and different ratios of uRBCs to endothelial cells (p=0.30, Figure 4.8 B).

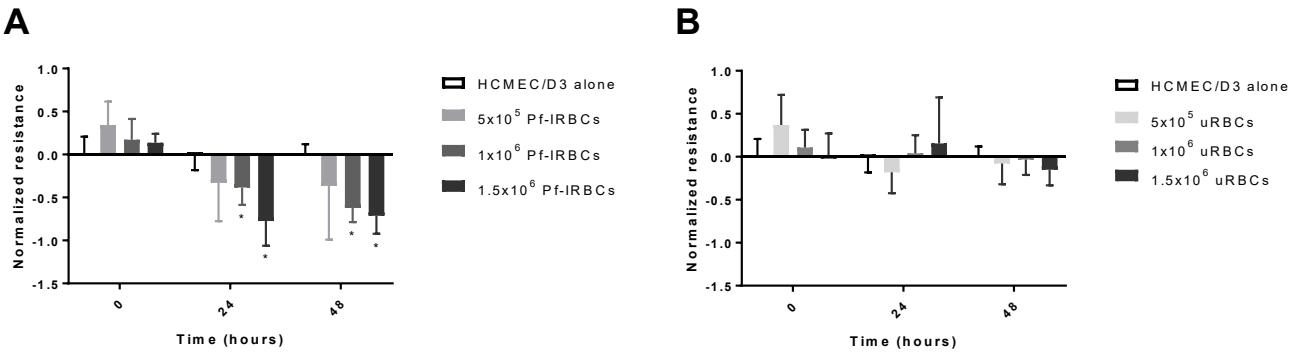

Figure 4.8 Dysfunction of endothelial monolayer integrity as a result of challenge with *P. falciparum*-infected red blood cells (Pf-IRBCs). Endothelial monolayers were challenged with varying concentrations of A) Pf-IRBCs or B) uninfected red blood cells (uRBCs) at time 0, and TEER was measured daily following media changes. Normalized resistance was calculated relative to control wells containing only media and cells. Data shown represent pooled results from at least 3 independent experiments with at least 3 replicates per condition. *$P < 0.0005$ compared with respective control condition.

Taken together, these findings indicate that our *in vitro* model recapitulates key aspects of the pathophysiologic response of the NVU during CM, including increased permeability with endogenous inflammatory cytokine TNF, mediator of vascular permeability VEGF, and parasitized erythrocytes in direct contact with endothelial cells.

ELISA quantification of Ang-2, soluble ICAM-1 and soluble VEGFR1.

As further validation of our *in vitro* model, ELISA quantification of Ang-2, soluble ICAM-1 (sICAM-1) and soluble VEGFR1 (sVEGFR1) was conducted. The selected molecules are known to increase following endothelial activation: Ang-2 is released from Weibel-Palade (WP) bodies exocytosed

by activated endothelial cells, and both sICAM-1 and sVEGFR1 levels are increased following endothelial cell surface shedding in response to inflammation (TNF) [84, 224]. Preliminary results showed that levels of Ang-2 remained stable 24 hours following the addition of TNF to endothelial/astrocyte co-cultures (p=0.055, Figure 4.9 A) whereas sICAM-1 levels increased (p=0.016, Figure 4.9 B), as did levels of sVEGFR1 (p=0.016, Figure 4.9 C).

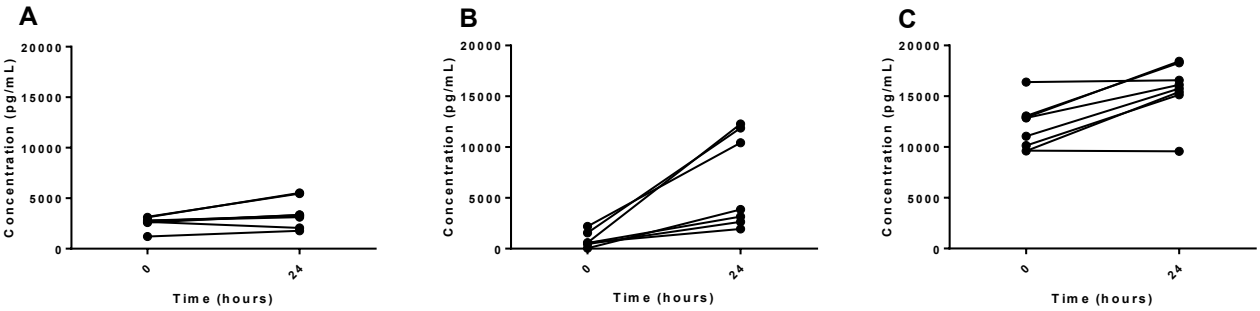

Figure 4.9 Quantification of markers of endothelial activation following challenge of co-cultures with tumour necrosis factor (TNF). At time 0, TNF was added to confluent endothelial monolayers in co-culture with astrocytes. Levels of A) Ang-2, B) sICAM-1, and C) and sVEGFR1 were measured at time 0 and 24 hours following the addition of TNF. Data are matched for each well.

Licensed S1P modulator fingolimod rescues BBB dysfunction in vitro.

Given the effects of S1P on vascular endothelial cell permeability [145-155] and the protective role of S1P in human and experimental CM [154], we hypothesized that fingolimod, a licensed agonist of S1PR1, would rescue BBB dysfunction in our *in vitro* BBB model. Twenty-four hours following inflammatory challenge of confluent HCMEC/D3 monolayers with TNF, fingolimod significantly increased TEER (p<0.0001). Rescue of TNF-induced BBB dysfunction persisted 48 hours after fingolimod was added to cell monolayers (p<0.0001, Figure 4.10 A). Fingolimod alone had no statistically significant effect on endothelial stability (p=0.27 after 48 hours). In our co-culture model, fingolimod was also successful in rescuing TNF-induced BBB dysfunction both after 24 hours (p=0.0027) and 48 hours (p=0.0022) (Figure 4.10 B).

We next tested the ability of fingolimod to stabilize the endothelial monolayer in response to Pf-IRBC-induced disruption of endothelial integrity. Twenty-four hours after the addition of Pf-IRBCs to HCMEC/D3 monolayers, fingolimod was added to endothelial cell monolayers, resulting in a subsequent increase in TEER that remained significant 24 hours ($p<0.0001$) and 48 hours ($p=0.0003$) after the small molecule was added to the monolayer (Figure 4.10 C).

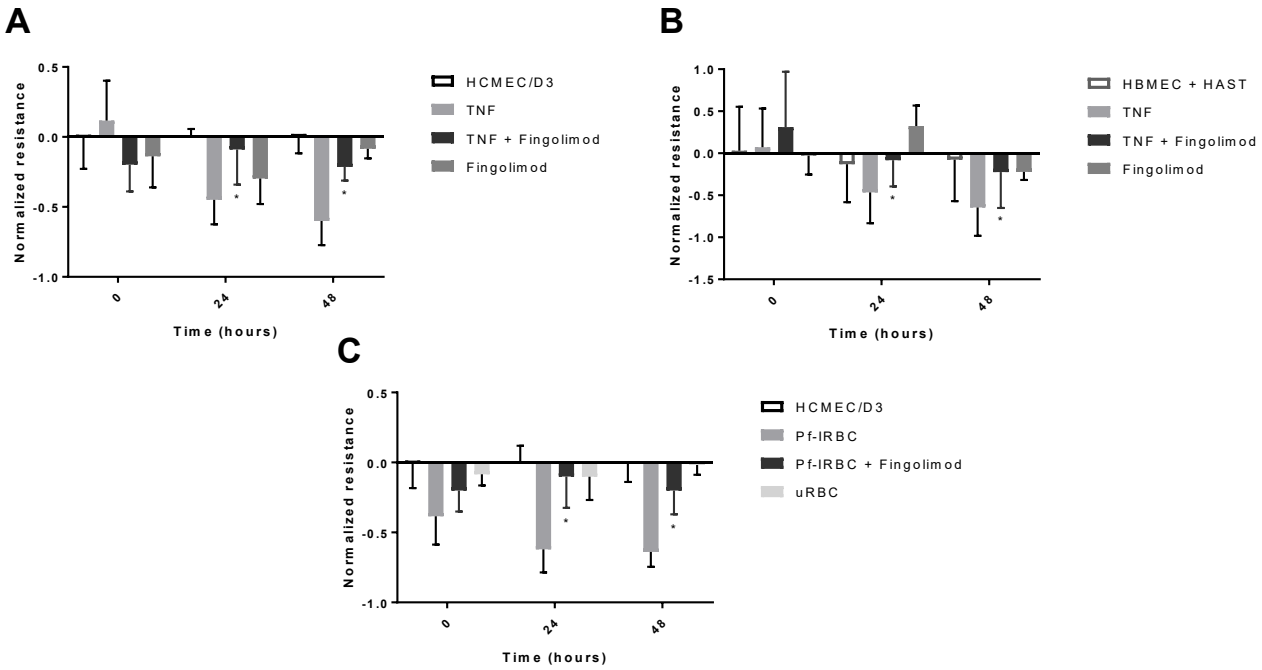

Figure 4.10 Fingolimod rescues *in vitro* blood brain barrier (BBB) dysfunction induced by tumour necrosis factor (TNF) and *P. falciparum*-infected red blood cells (Pf-IRBCs). Challenges were applied to confluent monolayers for 24 hours, after which time fingolimod was added (time 0). Electrical resistance was measured daily following media changes. In response to BBB dysfunction by TNF, fingolimod rescued endothelial integrity in A) our endothelial monoculture and B) endothelial/astrocyte co-culture models of the BBB. Following challenge of endothelial monocultures with C) Pf-IRBCs, fingolimod again rescued endothelial barrier integrity. Normalized resistance was calculated relative to control wells containing only media and cells. Data shown represent pooled results from at least 3 independent experiments. uRBC, uninfected red blood cell. *$P < 0.005$ compared with respective control condition.

Licensed tyrosine kinase inhibitors sunitinib and pazopanib rescue BBB function in vitro.

Given the importance of VEGF signalling in endothelial permeability [180, 184, 187, 188], we hypothesized that licensed pharmacologic agents targeting the VEGF/VEGFR2 pathway would rescue BBB dysfunction in response to cellular challenge. When tested on HCMEC/D3 endothelial monolayers, VEGFR2 antagonists sunitinib and pazopanib were successful in rescuing endothelial barrier integrity following challenge with TNF, VEGF, and Pf-IRBCs. Following endothelial barrier disruption with TNF, sunitinib significantly increased TEER ($p=0.0001$ after 24 hours and $p<0.0001$ after 48 hours, Figure 4.11 A), as did pazopanib ($p=0.0052$ after 24 hours and $p<0.0001$ after 48 hours, Figure 4.12 A). The pharmacological agents alone had no statistically significant effect on TEER after 48 hours (sunitinib $p=0.071$, pazopanib $p=0.83$).

When tested on endothelial/astrocyte co-cultures, TNF-induced dysfunction of the BBB was again rescued by the addition of tyrosine kinase inhibitors sunitinib (Figure 4.11 B) and pazopanib (Figure 4.12 B). Increases in TEER were significant at 24 and 48 hours after the addition of sunitinib ($p=0.002$ and $p=0.0003$, respectively) and pazopanib ($p=0.0001$ and $p<0.0001$, respectively).

Preliminary results demonstrated that sunitinib and pazopanib are both effective at rescuing endothelial integrity of VEGF-induced BBB dysfunction of HCMEC/D3 monolayers. Electrical resistance of endothelial monolayers increased 24 hours after the addition of sunitinib ($p<0.0001$, Figure 4.11 C) and pazopanib ($p<0.0001$, Figure 4.12 C) following a 24-hour challenge of the monolayers with VEGF. These data are from pooled results of only 2 experiments, and thus further replicates are required to ensure validity.

Decreases in electrical resistance following challenge of HCMEC/D3 monolayers with Pf-IRBCs were rescued 24 hours after the addition of sunitinib ($p<0.0001$, Figure 4.11 D) and pazopanib ($p<0.0001$, Figure 4.12 D). TEER remained significantly increased 48 hours after the addition of the pharmacological agents (sunitinib $p<0.0001$ and pazopanib $p=0.0045$).

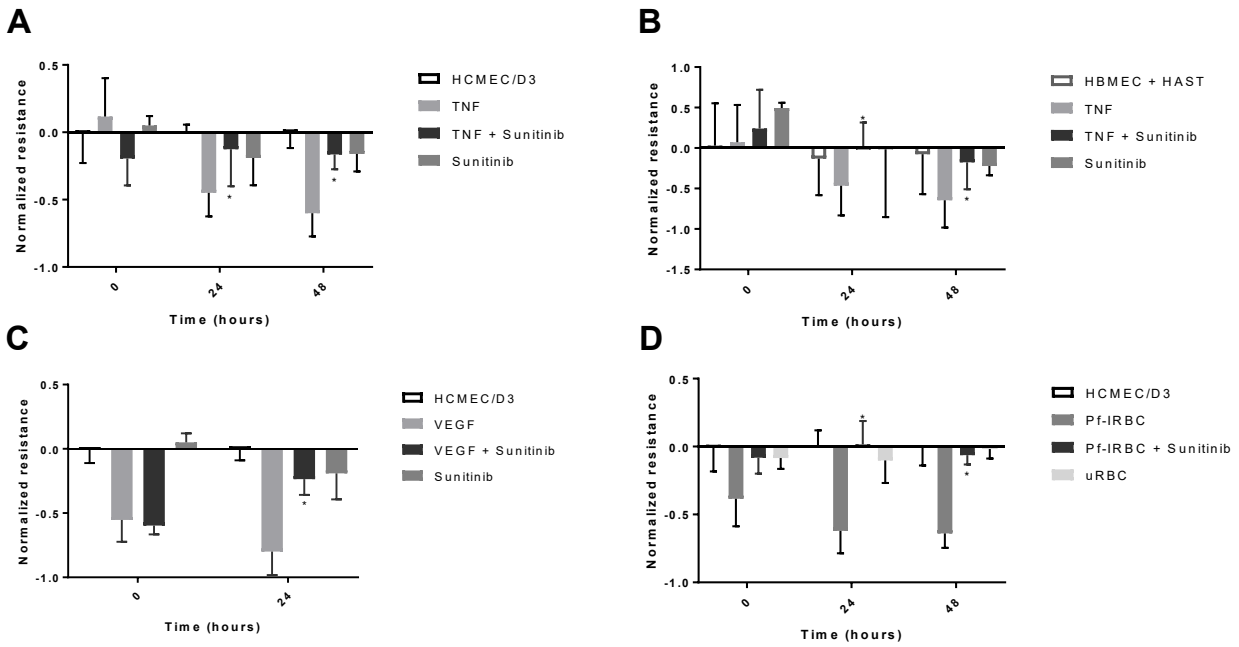

Figure 4.11 Sunitinib rescues *in vitro* blood brain barrier dysfunction. Twenty-four hours following cellular challenge by either tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF), or *P. falciparum*-infected red blood cells (Pf-IRBCs), sunitinib was added to confluent endothelial monolayers (time 0). When applied in A) our endothelial monoculture model following inflammatory challenge with TNF, sunitinib rescued endothelial integrity in a time and concentration dependent manner. Electrical resistance increased in B) our endothelial/astrocyte co-culture model following challenge with TNF. In response to C) VEGF, resistance of endothelial monolayers was increased by sunitinib. Following challenge with D) Pf-IRBCs, sunitinib again rescued endothelial integrity. Data shown represent pooled results from at least 3 independent experiments, with the exception of C) which includes only 2 independent experiments. Normalized resistance was calculated relative to control wells which contained only media and cells. uRBC, uninfected red blood cell. *$P < 0.005$ compared with respective control condition.

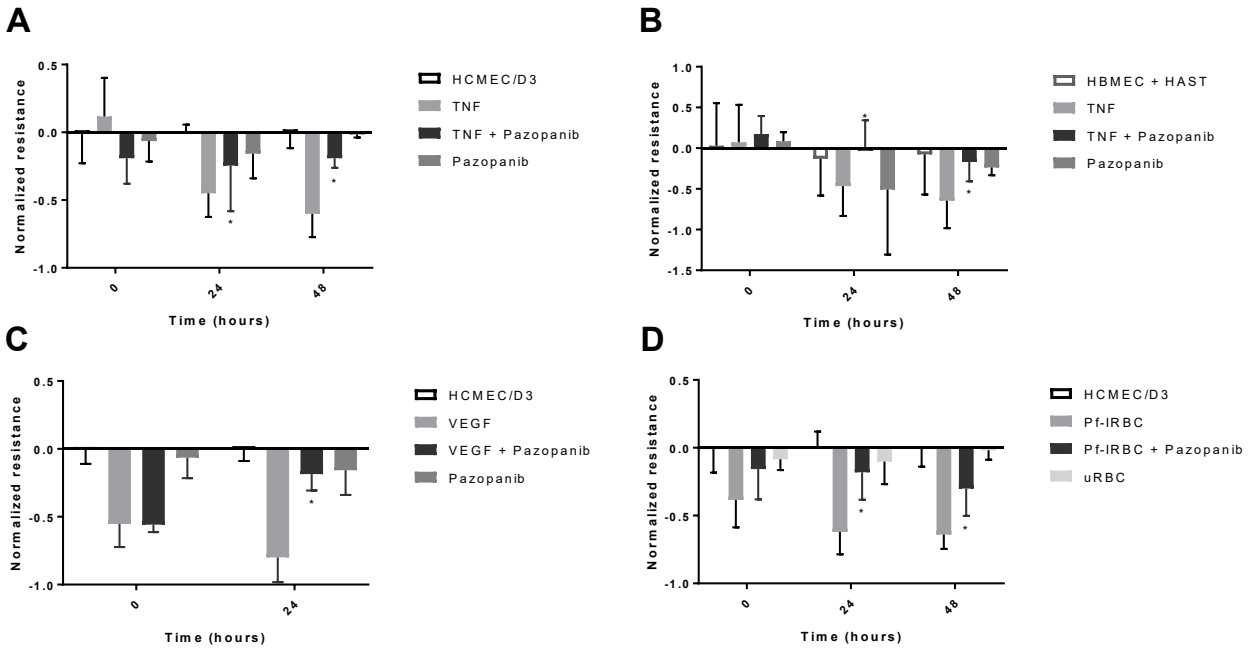

Figure 4.12 Pazopanib rescues *in vitro* blood brain barrier dysfunction. Challenges were applied to confluent monolayers for 24 hours, after which time pazopanib was added (time 0). Following challenge of HCMEC/D3 monolayers with A) tumour necrosis factor (TNF), pazopanib rescued endothelial integrity in a time and concentration dependent manner. When applied to B) confluent endothelial monolayers in co-culture with astrocytes pazopanib rescued TNF-induced BBB dysfunction. Following challenge with C) vascular endothelial growth factor (VEGF), pazopanib increased electrical resistance of HCMEC/D3 monolayers. Pazopanib also rescued endothelial integrity following challenge of monolayers with D) *P. falciparum*-infected red blood cells (Pf-IRBCs). Data shown represent pooled results from at least 3 independent experiments, with the exception of C) which includes only 2 independent experiments. Normalized resistance was calculated relative to control wells which contained only media and cells. uRBC, uninfected red blood cell. $*P < 0.005$ compared with respective control condition.

Licensed tyrosine kinase inhibitor imatinib did not rescue BBB function in vitro.

A third VEGFR2 antagonist, imatinib, was tested in our model system and did not significantly influence endothelial barrier stability following challenge with TNF or Pf-IRBCs. When tested on

endothelial monolayers 24 hours after the addition of TNF, imatinib did not statistically significantly increase TEER (p=0.056 after 24 hours and p=0.15 after 48 hours, Figure 4.13 A). In our endothelial/astrocyte co-culture model, imatinib was once again unsuccessful in rescuing TNF induced endothelial permeability after 24 hours (p=0.70) and was marginally successful after 48 hours (p=0.0082, Figure 4.13 B). In response to endothelial barrier challenge by VEGF, endothelial integrity was rescued 24 hours after the addition of imatinib (p=0.003, Figure 4.13 C) however results shown are preliminary and are pooled from only two experiments, thus further replicates are required to validate these findings. Lastly, although after 24 hours, differences were marginally statistically significant (p=0.042), imatinib did not rescue BBB dysfunction following cellular challenge with Pf-IRBCs after 48 hours (p=0.11, Figure 4.13 D).

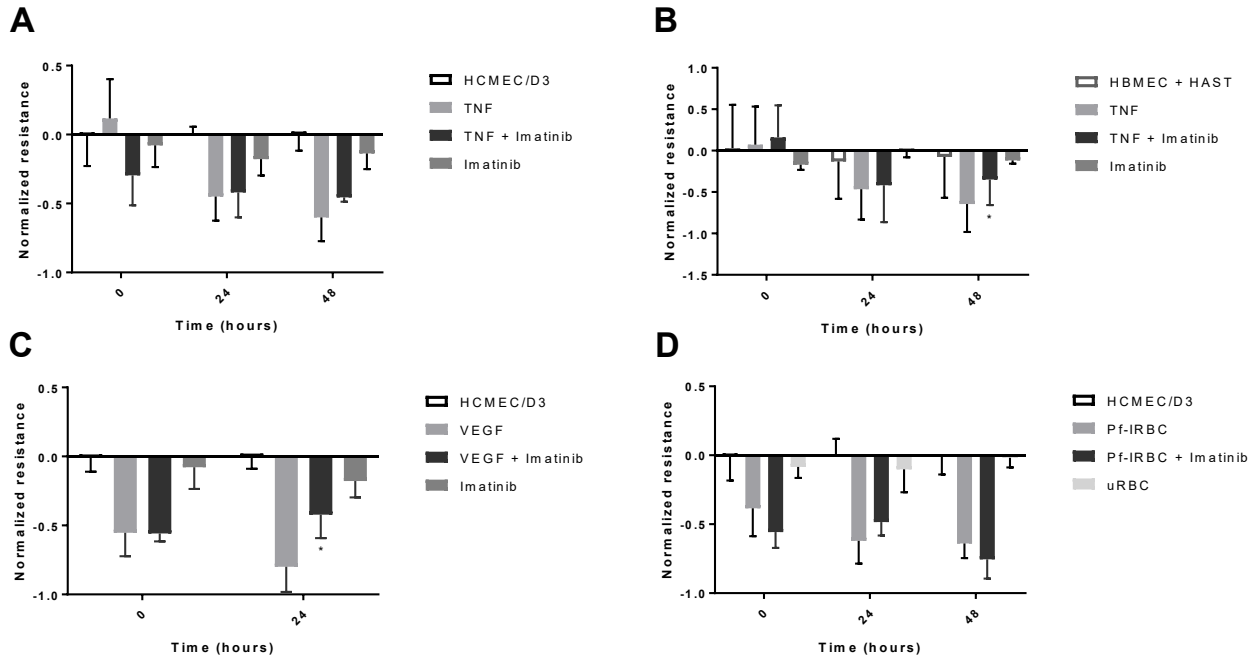

Figure 4.13 Imatinib does not rescue blood brain barrier (BBB) dysfunction in either monoculture or co-culture *in vitro* models. Cellular challenges were applied to confluent endothelial monolayers for 24 hours, after which time imatinib was added (time 0). Imatinib did not rescue BBB dysfunction following challenge with tumour necrosis factor (TNF) in either A) endothelial monoculture or B) endothelial/astrocyte co-culture models of the BBB. Following challenge of HCMEC/D3 monolayers with

C) vascular endothelial growth factor (VEGF), preliminary results show electrical resistance increased with the application of imatinib. Following challenge with D) *P. falciparum*-infected red blood cells (Pf-IRBCs), endothelial barrier integrity remained compromised despite the presence of imatinib. Data shown represent pooled results from at least 3 independent experiments, with the exception of C) which includes only 2 independent experiments. Normalized resistance was calculated relative to control wells which contained only media and cells. uRBC, uninfected red blood cell. *$P < 0.05$ compared with respective control condition.

4.6. DISCUSSION

Two successful *in vitro* BBB models were described herein using 1) the HCMEC/D3 cell line in monoculture, and 2) HBMEC primary cells in co-culture with primary HASTs. In both systems, decreases in electrical resistance were observed following the addition of TNF, an inflammatory cytokine that destabilizes endothelial cell junctions [214, 218]. In our monoculture model, inducers of vascular permeability VEGF and Pf-IRBCs decreased electrical resistance in a time and concentration dependent manner.

Our models were used to demonstrate rescue of BBB disruption by select pharmacological agents interacting with the S1P and VEGFR pathways. Fingolimod, a modulator of the S1P pathway, and tyrosine kinase inhibitors sunitinib and pazopanib, were all successful at rescuing BBB dysfunction induced by TNF, VEGF, or Pf-IRBCs. One drug, imatinib, was not fully successful in our *in vitro* models.

The partial failure of imatinib to rescue BBB dysfunction in our *in vitro* models is likely related to the molecule's mechanism of action. Unlike sunitinib and pazopanib, imatinib does not target all three VEGF receptors. Imatinib targets ABL1 kinase, which is induced by VEGFR-2 signalling [199] and has downstream effects on the Src/Rac pathway [202]. In previous studies, imatinib prevented parasite egress from RBCs [204], and decreased VEGF-induced endothelial barrier dysfunction both *in vitro* and *in vivo* [199]. Differences in endothelial cells lines and measures of endothelial resistance may account for our

findings, and more importantly, *in vivo* effects of imatinib are likely a result of the drugs effects on multiple cell types and complex cellular interactions. Although we did find statistically significant differences in TEER following VEGF-induced BBB dysfunction in our preliminary experiments, these results must be repeated.

In addition to comparing the effects of pharmacologic agents on endothelial cell permeability, we further demonstrated the *in vitro* properties of our model system by measuring Ang-2, sICAM-1, and sVEGFR1 levels in response to cellular challenge with TNF. Preliminary results using sandwich ELISAs demonstrated increased levels of sICAM-1 and sVEGFR1 in endothelial/astrocyte co-cultures 24 hours following challenge of cells with TNF, consistent with endothelial activation and exoenzymatic cleavage of cell surface receptors [133, 225].

Our study has several limitations. First, BBB disruption was measured every 24 hours using a simple resistance readout (TEER). More sophisticated methods to measure electrical resistance are available, such as electrical cell impedance sensing (ECIS), which measure electrical impedance continuously in real-time with limited mechanical disruption of monolayers. Tracer flux assays using fluorescently labeled markers, such as FITC-dextran, could also be used to measure permeability of monolayers. Monolayer formation could also have been verified using cell staining and light and/or fluorescent microscopy, to further confirm TEER measures. Microscopy would have enabled visualization of monolayers before and after challenges and drug addition to determine if changes in TJ and AJ formation were occurring, further validating alterations in endothelial barrier integrity as implicated by TEER measures. Lastly, *in vivo* studies are required to confirm our *in vitro* results. There are established murine models, including *P. berghei* ANKA in the C57BL/6 mouse, which can be used to test the hypothesis that novel adjunctive therapies will improve mortality and BBB leak in ECM.

Despite these limitations, we have demonstrated that newly licensed therapeutics in neurologic disease (e.g., S1P modulators) and cancer (e.g., VEGFR2 tyrosine kinase inhibitors) have activity on the endothelium, suggesting that they may be neuroprotective in CM. Although new drugs may be costly and

currently under patent, the typical course of severe malaria lasts only 3-5 days, during which time the patient either recovers or succumbs to his/her infection. Therefore, the cost of novel therapeutics would likely not be prohibitive and translation to low-income settings, where most malaria cases and deaths occur, may be feasible. As the agents are already FDA-approved, they have been well studied in human populations and adverse effects have been well documented. Additionally, the pharmacologic agents would not be required for extended periods of time, thus adverse effects would be minimal relative to those experienced by cancer or chronic disease patients who require daily perpetual doses of the pharmaceutical agents. The use of an oral pill rather than a vaccine or other forms of pharmaceuticals is desirable as the majority of CM treatment occurs in the tropics where resources and personnel for storage and administration of pharmaceuticals is limited. A single pill as an adjunct to first line treatments is appealing in these settings, however intravenous administration would likely be required in situations where the infected individual was already comatose. Pre-clinical and clinical data will be necessary to determine the most efficacious time point at which to begin administering the pharmacologic agents as adjuncts, whether it be at the onset of symptoms, to prevent progression of the disease, or once symptoms of CM have been established. Lastly, if shown effective, challenges in the implementation of new adjuncts for CM will need to be addressed, particularly in resource limited settings where they are most needed, in order to ensure widespread adoption of any new adjunctive therapy by frontline health care professionals.

4.7. CONCLUSIONS

In summary, the brain microvascular endothelium plays a central role in CM pathogenesis, and molecules targeting its regulatory pathways are promising candidates as adjuncts to anti-parasitic drugs in the treatment of CM. The drug repositioning approach outlined herein bypasses the prohibitive costs of traditional drug development processes by testing licensed neuroprotective agents used in the field of endothelial biology and cancer pharmacology, for their efficacy in CM model systems. Repurposing licensed agents as adjunctive therapies for CM is an attractive area of research, which could reduce the mortality and morbidity associated with severe malaria. We provide promising early pre-clinical data suggesting that licenced pharmacologic agents are active in the endothelium and rescue BBB dysfunction

in vitro. Further pre-clinical testing, including *in vivo* studies are planned to validate these potential anti-malarial therapeutics in order to accelerate their repurposing for children with CM. With the emerging global threat of drug-resistant parasites [1], molecules targeting host enzymes represent desirable therapeutic approaches that cannot be easily circumvented through selective mutations in *Plasmodium* parasites. Given the global burden of malaria in young children, new approaches to improve outcomes in CM could have widespread impact on child survival and neurocognitive morbidity.

CHAPTER 5 – CONCLUSIONS

5.1. SUMMARY OF RESEARCH

Reported in this thesis are three studies addressing malaria control and treatment among two key vulnerable populations: internally displaced persons (IDPs) and children under the age of five. Despite reductions in malaria associated morbidity and mortality, both IDPs and children under five continue to carry a large burden of disease.

Displaced populations are difficult to reach and lack stable health care infrastructure, compromising malaria control efforts. Displacement is often the result of complex humanitarian emergencies, and results in a myriad of challenges for both those displaced and those surrounding the newly displaced populations. Living conditions of IDPs are cramped and provide little access to employment, education, shelter, nutrition, clean water, sanitation and hygiene, and health care facilities. These characteristics of IDP camps further compound health concerns faced by IDPs, and create complex social systems where health interventions are difficult to implement and carry out. Furthermore, children make up more than 50% of displaced populations [15, 22]. Children under five are particularly vulnerable to infectious diseases, even without being displaced, and require prompt diagnostic and treatment services. Effective treatment of children requires that they seek medical attention when symptoms arise; however, in low and middle-income countries such as those of sub-Saharan Africa, where the burden of malaria is especially high, a large proportion of children live in rural and remote areas without access to health care facilities [11]. Even with malaria treatment, morbidity rates are high for children affected by severe malaria. The World Health Organization (WHO) has long focused on children under five and pregnant women, as two key vulnerable populations, however we encourage researchers not to forget about displaced populations, who in themselves have a high proportion of children and require targeted malaria control interventions.

Interventions with widespread coverage mainly focus on malaria prevention through vector control and treatment of confirmed cases with artemisinin combination therapy (ACT). The WHO has estimated that the use of insecticide treated bed nets have contributed to 68% of the approximately $900 million

USD saved between 2001 and 2014 [2]. In malaria endemic countries, free bed net distribution campaigns are widespread, and reported use of bed nets is high (82%) [2]. However, vulnerable populations with little to no data on bed net ownership and use are often left out of surveillance data, likely contributing to high estimates of bed net use. The second most cost-effective anti-malarial strategy is the treatment of confirmed cases with ACT, which is estimated to have contributed to 17% of the cost savings to health services over 13 years [2]. However, it was estimated that only 16% of confirmed cases received ACT in 2015 [2]. Without malaria prevention efforts such as bed nets, malaria morbidity would be exceedingly high; however, for those individuals with severe malaria infection, treatment is essential to survival. Therefore, studies aiming to improve malaria control and treatment efforts are essential for continued global reductions in malaria-associated morbidity and mortality.

In the first two studies of this thesis (Chapters 2 and 3) we described the burden of malaria in two IDP camps in Eastern DRC, where 58-60% of children under five were positive for malaria by rapid diagnostic test (RDT) and only 21-36% of individuals slept under a bed net. Barriers to bed net use identified by IDPs were primarily pragmatic and included difficulties installing and maintaining the nets within the cramped shelters of the IDP camp. Furthermore, competing nutritional needs took precedence over malaria prevention, even for families with children under five. Together, these data demonstrate the complexities and limitations of bed net use among IDP populations in the DRC, and call attention to the need for improved and tailored approaches to malaria control within this population.

Worldwide, both IDPs and children under five face health concerns warranting comprehensive and tailored solutions. Children under five are particularly susceptible to severe malaria, of which cerebral malaria (CM) remains especially challenging to treat. In our third study (Chapter 4) we described *in vitro* experiments undertaken to test putative neuroprotective agents against CM. Recently licensed pharmacologic agents targeting either the sphingosine-1-phosphate (S1P) pathway or the vascular endothelial growth factor receptor-2 (VEGFR2) pathway were tested in a cellular model of the blood brain barrier (BBB). Three pharmacologic agents, sunitinib, pazopanib, and fingolimod, were successful in our model of the BBB. These molecules rescued endothelial monolayer integrity following challenge with

tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF), and *P. falciparum*-infected red blood cells (Pf-IRBCs). One molecule, imatinib, was unsuccessful in our model. Although further pre-clinical studies and safety data are needed before the agents could be used in clinical practice, our findings suggest that some FDA-licenced agents, active at the endothelium, might be repurposed for use in CM.

5.1.2 Reflections on the methodologies employed

A wide range of research methods were conducted and reported within this thesis, adding to the richness of the data collected. Our first study was descriptive and observational, allowing us to address questions of population health for our chosen study population: IDPs. The systematic collection of epidemiological data generated statistics to illustrate the high burden of illness among IDPs and allowed for comparison of our study population with previously studied populations. However, with respect to risk factors for malaria, we were unable to infer causation based on our data due to the cross-sectional nature of the study.

In our second study, qualitative descriptions were a useful first step in our explorations of barriers to bed net use among IDPs. Quantitative descriptions of the burden of malaria and bed net use or disuse confirmed and extended our qualitative findings. This study was also observational in nature with a sequential exploratory design, whereby qualitative data was used to inform the development of our quantitative survey. FGDs were open-ended and enabled the collection of rich data that otherwise would not have been collected through quantitative methods. As with most research, our data was limited by our collection methods (FGDs and door-to-door surveys) and our study population, and therefore extrapolation of our findings should be done with caution.

Lastly, our third study employed cell culture methods to test novel, potentially neuroprotective agents for their ability to rescue BBB dysfunction as a result of *P. falciparum* infection. The *in vitro* methods used provided pre-clinical data to support the repurposing of licensed pharmacological agents for CM. This method avoids the need for testing on animals, thereby reducing the resources needed and potential ethical concerns. However, disadvantages of an *in vitro* system include its simplicity, whereby complex interactions within the NVU cannot be accounted for and thus data must not be over-interpreted to

humans. A series of *in vivo* experiments are necessary to validate our *in vitro* findings, prior to clinical studies. Nonetheless, taken together, the research methods employed in this thesis allowed for a comprehensive and in-depth exploration and study of malaria control and treatment efforts, and will inform future studies on the topic.

5.2. IMPLICATIONS OF RESEARCH AND FUTURE DIRECTIONS

The studies reported herein draw attention to the need for new and improved malaria control and treatment approaches among IDPs and children under five.

Within IDP populations, future studies examining the implementation and uptake of targeted vector control measures are necessary. We have described barriers to bed net ownership and utilization by IDPs, and have suggested areas for future studies including engineering improvements to current bed net design and the implementation of alternative vector control methods (indoor residual spraying, insecticide-treated tarpaulin, etc.). Successful implementation and uptake of malaria prevention intervention efforts among IDPs would be expected to improve the health of a large group of individuals, particularly in sub-Saharan Africa where the burden of malaria is highest. With over 40 million IDPs worldwide [14], this population should not be forgotten in anti-malarial efforts.

Secondly, our study of neuroprotective agents for CM was successful in an *in vitro* model and therefore subsequent studies are warranted to test the pharmacologic agents *in vivo* to determine if the agents could be translated to human clinical trials. If successful, these agents could be used as adjunctive therapies for CM to prevent severe neurocognitive deficits that occur in up to 30% of CM survivors [10].

With respect to knowledge translation and exchange, our results are noteworthy for several groups of knowledge users. Primarily, the findings reported herein are of interest to the communities of scientific and public health malaria researchers. Highlighting the burden of malaria among IDPs is essential for public health researchers to understand gaps in population wide anti-malarial interventions, and our findings demonstrate the need for targeted approaches to malaria control. Findings could also impact NGOs working with IDP populations in the DRC, and it is our aim to present our results to NGOs through face-to-face meetings. Our *in vitro* results will be of interest to the scientific community, and published

data are easily accessible. Pharmaceutical companies may also be interested in our findings as the implications of future related studies could lead to the repurposing of pharmacological agents for CM.

5.3. CONCLUDING REMARKS

Malaria is a preventable and treatable disease that remains a leading cause of childhood mortality. With approximately 3.2 billion individuals at risk of malaria infection [2], prevention and treatment efforts must constantly be adapted to respond to emerging global challenges in malaria control. Monumental reductions in malaria associated mortality and morbidity have been achieved, but progress must be maintained and accelerated to reach global targets in improving the wellbeing and livelihood of individuals worldwide. Gaps in coverage of malaria control and treatment interventions must be addressed within key vulnerable populations to prevent morbidity and mortality. High-burden populations such as displaced individuals and children under five represent key populations that immensely benefit from targeted anti-malarial strategies, and should not be forgotten in global efforts to reduce malaria morbidity and mortality. Finally, adjunctive therapies for the treatment of cerebral malaria are needed to prevent long term neurocognitive disability as a result of severe malarial infection in children under five.

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APPENDIX A – INDICATOR SURVEY

MALARIA INDICATOR SURVEY

DATE_____ INTERVIEWER_____

LOCATION_____ HOUSEHOLD REGISTRATION NUMBER:_____

HOUSEHOLD COMPOSITION

LINE NO.	HOUSEHOLD MEMBERS AND VISITORS	RELATION-SHIP TO HEAD OF HOUSE	SEX	AGE	RESIDENCE		BED NET	MALARIA		VIOLENCE
1	2	3	4	5	6	7	8	9	10	11
	Please provide the names of the individuals who normally live in this household and the visitors who stayed here last night, starting with the head of the household. AFTER LISTING THE NAMES, REGISTERING THE RELATIONSHIP TO THE HEAD OF HOUSEHOLD, AND THE SEX OF EACH PERSON, ASK QUESTIONS 2A-2C TO MAKE SURE THE LIST IS COMPLETE. NEXT, ASK QUESTIONS 5-11 FOR EACH PERSON LISTED.	What is the relationship of (NAME) to the head of the household? SEE CODES BELOW	Is (NAME) male or female?	How old is (NAME)? IN YEARS	Does (NAME) usually sleep in this tent?	Did (NAME) sleep in this tent last night?	Did (NAME) sleep under a bed net last night?	Has (NAME) had any of the following symptoms today?	RDT result?	Has (NAME) been the victim of one or more of the following since their displacement?
01			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐
02			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐
03			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐
04			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐

APPENDIX A – BED NET INDICATOR SURVEY

05			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐
06			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐
07			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐
08			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐
09			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐
10			M ☐ F ☐		YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	HEADACHE ☐ MYALGIA ☐ FEVER ☐	RDT +☐ RDT - ☐	THEFT ☐ AGRESSION ☐ SEXUAL AGRESSION/ RAPE ☐ STABBING ☐ SHOOTING ☐

2A) Just to be sure that I have a complete list: are there any other adults of children that we haven't listed? (If yes, add them to the table)
2B) Are there others who maybe aren't family members who are friends or friends of neighbors who normally live here? (If yes, add them to the table)
2C) Are there guests or temporary visitors or other people who slept here last night and who aren't yet listed? (If yes, add them to the table)

CODES FOR Q.3: RELATIONSHIP TO THE HEAD OF HOUSEHOLD

01 = HEAD OF HOUSEHOLD
02 = SPOUSE
03 = SON OR DAUGHTER
04 = SON OR DAUGHTER IN-LAW
05= STEP SON OR DAUGHTER
06 = MOTHER/FATHER
07 = PARENT IN-LAW
08 = BROTHER OR SISTER
09 = OTHER PARENT
10 = ADOPTED/ FOSTERED CHILD
11 = SPOUSE'S CHILDREN
12 = OTHER RELATIVE
13 = NO RELATIONSHIP
98 = DO NOT KNOW

APPENDIX A – BED NET INDICATOR SURVEY

CHILDREN UNDER FIVE

LINE NO.	NAMES OF CHILDREN UNDER 5 YEARS	FEVER		MEDICAL		MALARIA		
1	2	3	4	5	6	7	8	9
11	Please list the names of the children under 5 years of age and the line number from the household composition table.	Has the child had a fever in the past month?	If yes, did you seek medical advice or treatment at the medical centre?	Did the child have malaria?	If yes, was the diagnosis confirmed by RDT or microscopy?	Was antimalarial treatment administered?	What is the level of education of this child's mother?	Is this child's mother literate?
12	NAME_____ LINE NO. ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	NONE ☐ PRIMARY ☐ SECONDARY ☐ POST SECONDARY ☐	YES ☐ NO ☐
13	NAME_____ LINE NO. ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	NONE ☐ PRIMARY ☐ SECONDARY ☐ POST SECONDARY ☐	YES ☐ NO ☐
14	NAME_____ LINE NO. ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	NONE ☐ PRIMARY ☐ SECONDARY ☐ POST SECONDARY ☐	YES ☐ NO ☐
15	NAME_____ LINE NO. ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	NONE ☐ PRIMARY ☐ SECONDARY ☐ POST SECONDARY ☐	YES ☐ NO ☐
16	NAME_____ LINE NO. ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐	NONE ☐ PRIMARY ☐ SECONDARY ☐ POST SECONDARY ☐	YES ☐ NO ☐

APPENDIX A – BED NET INDICATOR SURVEY

BED NETS

LINE NO.		BED NET #1	BED NET #2	BED NET #3
17	ASK TO SEE ALL BED NETS IN THE HOUSEHOLD IF THERE ARE MORE THAN 3 NETS, USE MORE SURVEYS	OBSERVED ☐ NOT OBSERVED ☐	OBSERVED ☐ NOT OBSERVED ☐	OBSERVED ☐ NOT OBSERVED ☐
18	HOW LONG HAS YOUR HOUSEHOLD HAD THIS NET?	IN MONTHS ☐	IN MONTHS ☐	IN MONTHS ☐
19	DID YOU RECEIVE THE BED NET DURING A DISTRIBUTION CAMPAIGN WHEN YOU ARRIVED AT THE CAMP?	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐
20	DID YOU RECEIVE THE BED NET DURING A PRENATAL VISIT?	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐
21	WHERE DID YOU RECEIVE THE BED NET?	NGO DISTRIBUTION ☐ HOSPITAL/HEALTH CENTRE/PUBLIC HEALTH FACILITY ☐ PRIVATE HOSPITAL/CLINIC ☐ PHARMACY ☐ MARKET ☐ COMMUNITY HEALTH WORKER ☐ RELIGIOUS INSTITUTION ☐ SCHOOL ☐ OTHER (specify) ☐ ________________ DO NOT KNOW ☐	NGO DISTRIBUTION ☐ HOSPITAL/HEALTH CENTRE/PUBLIC HEALTH FACILITY ☐ PRIVATE HOSPITAL/CLINIC ☐ PHARMACY ☐ MARKET ☐ COMMUNITY HEALTH WORKER ☐ RELIGIOUS INSTITUTION ☐ SCHOOL ☐ OTHER (specify) ☐ ________________ DO NOT KNOW ☐	NGO DISTRIBUTION ☐ HOSPITAL/HEALTH CENTRE/PUBLIC HEALTH FACILITY ☐ PRIVATE HOSPITAL/CLINIC ☐ PHARMACY ☐ MARKET ☐ COMMUNITY HEALTH WORKER ☐ RELIGIOUS INSTITUTION ☐ SCHOOL ☐ OTHER (specify) ☐ ________________ DO NOT KNOW ☐
22	IS THE BED NET USED BY THE HOUSEHOLD? IF YES, GO TO Q24, IF NO, ASK Q23 AND PROCEED TO Q31.	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐

APPENDIX A – BED NET INDICATOR SURVEY

23	WHY DOESN'T YOUR HOUSEHOLD USE THE BED NET? SELECT ALL THAT WERE MENTIONED INSIST: ARE THERE ANY OTHER REASONS?	BED NET NOT EFFICACIOUS ☐ NEEDED MONEY ☐ SOME MEMBERS OF THE HOUSEHOLD DON'T LIKE IT ☐ SIZE IS UNSATISFACTORY ☐ INSTALLATION DIFFICULT ☐ CAN'T TUCK UNDER A MATTRESS ☐ SHAPE IS UNSATISFACTORY ☐ SMELLS BADLY ☐ CAUSES IRRITATIONS/COUGHING ☐ CAUSES SICKNESS ☐ CAUSES NAUSEA ☐ PRODUCES DANGEROUS CHEMICAL ☐ CAN SUFFOCATE/CAUSES DIFFICULTY BREATHING ☐ TOO HOT ☐ GETS DIRTY TOO QUICKLY ☐ GETS HOLES TOO QUICKLY ☐ OTHER (specify) ☐ ________________ DO NOT KNOW ☐	BED NET NOT EFFICACIOUS ☐ NEEDED MONEY ☐ SOME MEMBERS OF THE HOUSEHOLD DON'T LIKE IT ☐ SIZE IS UNSATISFACTORY ☐ INSTALLATION DIFFICULT ☐ CAN'T TUCK UNDER A MATTRESS ☐ SHAPE IS UNSATISFACTORY ☐ SMELLS BADLY ☐ CAUSES IRRITATIONS/COUGHING ☐ CAUSES SICKNESS ☐ CAUSES NAUSEA ☐ PRODUCES DANGEROUS CHEMICAL ☐ CAN SUFFOCATE/CAUSES DIFFICULTY BREATHING ☐ TOO HOT ☐ GETS DIRTY TOO QUICKLY ☐ GETS HOLES TOO QUICKLY ☐ OTHER (specify) ☐ ________________ DO NOT KNOW ☐	BED NET NOT EFFICACIOUS ☐ NEEDED MONEY ☐ SOME MEMBERS OF THE HOUSEHOLD DON'T LIKE IT ☐ SIZE IS UNSATISFACTORY ☐ INSTALLATION DIFFICULT ☐ CAN'T TUCK UNDER A MATTRESS ☐ SHAPE IS UNSATISFACTORY ☐ SMELLS BADLY ☐ CAUSES IRRITATIONS/COUGHING ☐ CAUSES SICKNESS ☐ CAUSES NAUSEA ☐ PRODUCES DANGEROUS CHEMICAL ☐ CAN SUFFOCATE/CAUSES DIFFICULTY BREATHING ☐ TOO HOT ☐ GETS DIRTY TOO QUICKLY ☐ GETS HOLES TOO QUICKLY ☐ OTHER (specify) ☐ ________________ DO NOT KNOW ☐
24	SINCE YOU HAVE HAD THE BED NET, HAS IT DEVELOPED ANY HOLES?	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐
25	IF YES, HOW MANY HOLES DOES IT HAVE?	MANY ☐ FEW ☐	MANY ☐ FEW ☐	MANY ☐ FEW ☐
26	DO YOU WASH THE BED NET?	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐
27	IF YES, HOW MANY DAYS HAS IT BEEN SINCE IT WAS LAST WASHED?	IN DAYS ☐	IN DAYS ☐	IN DAYS ☐
28	SINCE YOU HAVE HAD THE BED NET, HAS IT BEEN TREATED WITH	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐

APPENDIX A – BED NET INDICATOR SURVEY

	AN INSECTICIDE TO KILL OR DETER MOSQUITOES?			
29	LAST NIGHT, DID SOMEONE SLEEP UNDER THIS BED NET?	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐
30	IF YES, WHO SLEPT UNDER THIS BED NET LAST NIGHT? LIST ALL NAMES AND LINE NUMBERS FROM THE HOUSEHOLD COMPOSITION TABLE.	NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐	NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐	NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐ NAME_____ LINE NO. ☐

BED NETS RECEIVED SINCE DISPLACEMENT THAT YOU NO LONGER HAVE

LINE NO.	QUESTIONS	BED NET #1	BED NET #2
31	HOW LONG HAS YOUR HOUSEHOLD HAD THIS NET?	IN MONTHS ☐	IN MONTHS ☐
32	DID YOU RECEIVE THE BED NET DURING A DISTRIBUTION CAMPAIGN WHEN YOU ARRIVED AT THE CAMP?	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐
33	DID YOU RECEIVE THE BED NET DURING A PRENATAL VISIT?	YES ☐ NO ☐ DO NOT KNOW ☐	YES ☐ NO ☐ DO NOT KNOW ☐
34	WHERE DID YOU RECEIVE THE BED NET?	NGO DISTRIBUTION ☐ HOSPITAL/MEDICAL CENTRE/PUBLIC HEALTH FACILITY ☐ PRIVATE HOSPITAL/CLINIC ☐ PHARMACY ☐ MARKET ☐ COMMUNITY HEALTH WORKER ☐ RELIGIOUS INSTITUTION ☐ SCHOOL ☐	NGO DISTRIBUTION ☐ HOSPITAL/MEDICAL CENTRE/PUBLIC HEALTH FACILITY ☐ PRIVATE HOSPITAL/CLINIC ☐ PHARMACY ☐ MARKET ☐ COMMUNITY HEALTH WORKER ☐ RELIGIOUS INSTITUTION ☐ SCHOOL ☐

APPENDIX A – BED NET INDICATOR SURVEY

		OTHER (specify) ☐ _____ DO NOT KNOW ☐	OTHER (specify) ☐ _____ DO NOT KNOW ☐
35	WHAT HAPPENED TO THE BED NET?	SOLD ☐ EXCHANGED ☐ GAVE AWAY AS A GIFT ☐ THREW IT OUT ☐ USED IT FOR OTHER THINGS (eg: fish net, etc.) (specify) ☐ _____	SOLD ☐ EXCHANGED ☐ GAVE AWAY AS A GIFT ☐ THREW IT OUT ☐ USED IT FOR OTHER THINGS (eg: fish net, etc.) (specify) ☐ _____
36	WHEN DID YOU SELL/EXCHANGE/ GIVE AWAY THE BED NET?	IN MONTHS ☐	IN MONTHS ☐
37	WHY DID YOU SELL/EXCHANGE/ GIVE AWAY THE BED NET? CHECK ALL THAT ARE MENTIONED INSIST: ARE THERE ANY OTHER REASONS?	BED NET NOT EFFICACIOUS ☐ NEEDED MONEY ☐ SOME MEMBERS OF THE HOUSEHOLD DON'T LIKE IT ☐ SIZE IS UNSATISFACTORY ☐ INSTALLATION DIFFICULT ☐ CAN'T TUCK UNDER A MATTRESS ☐ SHAPE IS UNSATISFACTORY ☐ SMELLS BADLY ☐ CAUSES IRRITATIONS/COUGHING ☐ CAUSES SICKNESS ☐ CAUSES NAUSEA ☐ PRODUCES DANGEROUS CHEMICAL ☐ CAN SUFFOCATE/CAUSES DIFFICULTY BREATHING ☐ TOO HOT ☐ GETS DIRTY TOO QUICKLY ☐ GETS HOLES TOO QUICKLY ☐ OTHER (specify) ☐ _____ DO NOT KNOW ☐	BED NET NOT EFFICACIOUS ☐ NEEDED MONEY ☐ SOME MEMBERS OF THE HOUSEHOLD DON'T LIKE IT ☐ SIZE IS UNSATISFACTORY ☐ INSTALLATION DIFFICULT ☐ CAN'T TUCK UNDER A MATTRESS ☐ SHAPE IS UNSATISFACTORY ☐ SMELLS BADLY ☐ CAUSES IRRITATIONS/COUGHING ☐ CAUSES SICKNESS ☐ CAUSES NAUSEA ☐ PRODUCES DANGEROUS CHEMICAL ☐ CAN SUFFOCATE/CAUSES DIFFICULTY BREATHING ☐ TOO HOT ☐ GETS DIRTY TOO QUICKLY ☐ GETS HOLES TOO QUICKLY ☐ OTHER (specify) ☐ _____ DO NOT KNOW ☐

APPENDIX A – BED NET INDICATOR SURVEY

38	IF THE BED NET WAS SOLD/EXCHANGED, WHERE DID YOU SELL/EXCHANGE THE BED NET?	MARKET ☐ CAMP ☐	MARKET ☐ CAMP ☐
39	WHO BOUGHT/ACCEPTED THE NED NET?	VILLAGER ☐ DISPLACED PERSON ☐ NGO WORKER ☐ GOVERNMENT EMPLOYEE ☐	VILLAGER ☐ DISPLACED PERSON ☐ NGO WORKER ☐ GOVERNMENT EMPLOYEE ☐
40	IF THE BED NET WAS SOLD, WHAT PRICE DID YOU SELL IT FOR?	PRICE ☐	PRICE ☐

SOCIODEMOGRAPHICS

LINE NO.	QUESTIONS	ANSWERS
41	Does the household have any of the following? ELECTRICITY REFRIGERATOR TELEVISION RADIO	YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐
42	Does any member of the household have one of the following? MOBILE PHONE BICYCLE VEHICLE WATCH	YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐
43	Does the household own any of the following? POULTRY (Chickens/Ducks/etc) GOAT(S) COW(S)	YES ☐ NO ☐ YES ☐ NO ☐ YES ☐ NO ☐
44	HOW MANY TENTS IS THE HOUSEHOLD MADE UP OF	☐

APPENDIX A – BED NET INDICATOR SURVEY

45	OBSERVE THE MATERIAL OF THE TENT FLOOR. NOTE YOUR OBSERVATIONS.	NATURAL FLOORING EARTH/SAND ☐ MUD ☐ RUDIMENTARY FLOORING WOOD BOARDS ☐ TARPAULIN ☐ FINISHED FLOORING FINISHED WOOD ☐ CEMENT ☐ TILES ☐ OTHER ☐
46	OBSERVE THE MATERIAL OF THE ROOF. NOTE YOUR OBSERVATIONS.	NATURAL ROOFING PAS DE TOIT ☐ STRAW/PALM LEAVES ☐ RUDIMENTARY ROOFING RUG ☐ WOOD BOARDS ☐ TARPAULIN ☐ FINISHED ROOFING METAL ☐ WOOD ☐ CERAMIC ☐ CEMENT ☐ SHINGLES ☐ OTHER ☐
47	OBSERVE THE MATERIAL OF THE WALLS. NOTE YOUR OBSERVATIONS.	NATURAL NO WALLS ☐ EARTH ☐ RUDIMENTARY WALLS ROCKS AND MUD ☐ WOOD BOARDS ☐ CARDBOARD ☐ TARPAULIN ☐ PALMS/BRANCHES ☐ FINISHED WALLS BRICKS ☐ CEMENT BLOCKS ☐ CEMENT ☐ OTHER ☐

OTHER QUESTIONS:

48) HOW LONG HAS YOUR HOUSEHOLD BEEN DISPLACED (MONTHS)? _____

49) HOW LONG HAS YOUR HOSUEHOLD LIVED IN THIS CAMP (MONTHS)?_____

APPENDIX A – BED NET INDICATOR SURVEY

INTERVIEWER OBSERVATIONS

TO BE FILLED OUT AFTER SURVEY COMPLETION

COMMENTS ABOUT THE SURVEY:

COMMENTS ABOUT SPECIFIC QUESTIONS:

OTHER COMMENTS: