TARGETING NON-MALARIAL COMA ETIOLOGIES IN CHILDREN WITH RETINOPATHY NEGATIVE CEREBRAL MALARIA AND ACUTE APARASITEMIC COMA: THE ROLE OF ADAPTIVE DESIGNS IN CLINICAL TRIALS

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ABSTRACT

TARGETING NON-MALARIAL COMA ETIOLOGIES IN CHILDREN WITH RETINOPATHY NEGATIVE CEREBRAL MALARIA AND ACUTE APARASITEMIC COMA: THE ROLE OF ADAPTIVE DESIGNS IN CLINICAL TRIALS

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Cerebral malaria is clinically diagnosed when a patient with an otherwise unexplained coma has a positive blood test for malaria parasites. Children diagnosed with cerebral malaria may be subdivided as to the presence of absence of a malaria specific retinopathy. It is unclear whether retinopathy negative cerebral malaria is due to an acute malarial illness with host modifying disease expression (e.g. mortality rates, retinopathy status) or due to a non-malarial etiology of coma with an asymptomatic parasitemia. Although autopsy studies support the latter possibility, disease in those who survive may be different than those who succumb.

Studies are conflicting as to whether retinopathy negative cerebral malaria is solely due to acute malarial infection or has an acute etiology of illness that is non-malarial. This grant application looks for three treatable etiologies of coma in children with retinopathy negative cerebral malaria: viral central nervous system infection, bacteremia, and non-convulsive seizures. It compares the proportion of these etiologies and pathogen identities to children with acute aparasitemic coma.

Using the results of this K23 Research Plan, we anticipate performance of clinical trials of adjunctive therapies. Adaptive trial designs may be useful in these studies, particularly adaptive randomization, combining Phase IIb and Phase III studies, and shifting target populations.

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KEY TO ABBREVIATIONS

µL= microliter

AAC= acute aparasitemic coma

BCS= Blantyre coma score

BMP= Blantyre Malaria Project

CI= confidence interval

CM= cerebral malaria

CMV= cytomegalovirus

CNS= central nervous system

CSF= cerebrospinal fluid

EBV= Epstein-Barr virus

EEG= electroencephalogram

ELISA= enzyme linked immunosorbent assay

HHV6= human herpesvirus 6

HIV= human immunodeficiency virus

HRP2= histidine rich protein 2

HSV= herpes simplex virus

IQR= interquartile range

L= liter

Log= logarithm

LP= lumbar puncture

Mmoles= millimoles

MP= malaria parasite

MRI= magnetic resonance imaging

n or N= number

PCR= polymerase chain reaction

PCV= packed cell volume= hematocrit

PfEMP1= Plasmodium falciparum Erythrocyte Membrane Protein 1

PRW= pediatric research ward

QECH= Queen Elizabeth Central Hospital

qPCR= quantitative polymerase chain reaction

Ret neg CM= retinopathy negative cerebral malaria

UCSF= University of California San Francisco

US= United States

VZV= varicella zoster virus

WBC= white blood cell

CHAPTER I: INTRODUCTION

This thesis is divided into three sections. Chapter I is an introduction to clinical malaria. Chapter II discusses the work performed between 2010 and 2015 to describe and better understand a common medical condition in sub-Saharan Africa, retinopathy negative cerebral malaria. To try to understand disease pathophysiology I performed the following four secondary analyses of data gathered on children with retinopathy negative cerebral malaria:

- A case series and formulation of four possible pathophysiological hypotheses
- A retrospective comparison of admission laboratory parameters and seasonality
- A retrospective cohort analysis assessing neurological outcomes in retinopathy negative cerebral malaria survivors
- A retrospective comparison of MRI characteristics in children with retinopathy negative *vs.* positive cerebral malaria

The results of these studies were somewhat conflicting and yielded few insights into the underlying mechanisms of disease. Their findings suggest that the etiologies of retinopathy negative cerebral malaria (ret neg CM) were likely due to a complex combination of host and parasite factors.

I therefore applied for a K23 (Patient-oriented research award) from the US National Institutes of Health to directly study pathophysiology of this disease. The Research Plan from this application is Chapter III of this thesis.

Chapter IV is a description of next steps. I anticipate that clinical trials of interventions aimed at underlying disease pathophysiology in retinopathy negative cerebral malaria will need to be formulated. I explore adaptive clinical trial design, methods to modify the performance of clinical trials using results from previously enrolled patients. I discuss in depth three adaptive clinical trials methods that might be used in studies of interventions aimed at the underlying pathophysiology of retinopathy negative cerebral malaria, or in any other clinical trial.

Before embarking on this discussion, I provide a background into clinical malaria and the diagnosis of cerebral malaria.

Epidemiology of malaria

Half of the world's population is at risk of contracting malaria. With 250 million clinical cases and over 800,000 deaths every year, it is the most important parasitic disease of humankind[1]. Ninety-percent of all malaria deaths occur in Africa and 90% of African malaria deaths are in children 5 years of age and younger[2]. Though there are five *Plasmodium* species infective for man, *Plasmodium falciparum* is responsible for most cases of severe disease, especially in Africa.

Humans are infected by the bite of an infective *Anopheles* mosquito. Malaria sporozoites are injected into the human as the mosquito probes for a blood meal. Sporozoites rapidly travel to the human liver and invade hepatocytes within 8 hours of host infection. Within the liver cell they undergo a first cycle of asexual reproduction. Upon hepatocyte rupture (termed hepatic *schizogony*) daughter merozoites are

released into the host's bloodstream. Merozoites have an apical complex that they use to invade erythrocytes.

Once inside the erythrocytes the parasite degrades red blood cell proteins (including hemoglobin) and uses the constituent amino acids for its own gene products. Some of these parasite encoded protein products are expressed on the red cell surface producing knob like prominences. The most important of these parasite encoded membrane proteins is likely PfEMP1 (Plasmodium falciparum Erythrocyte Membrane Protein 1)[3]. PfEMP1 has affinity to several human endothelial antigens, the most important of which may be ICAM1[4, 5]. Interaction between parasitized erythrocytes and vascular endothelium leads to irreversible binding of the two. Erythrocytes are removed from the effective circulation, a process termed *sequestration*. Sequestered red cells rupture (termed *erythrocyte schizogony*), producing ghost membranes and freeing daughter merozoites to continue the cycle of erythrocyte invasion and sequestration. The life cycle is completed when, in response to a number of different stimuli, some parasites divert from intra-erythrocytic multiplication and become male or female gametocytes. Gametocytes are taken up by mosquitoes during a blood meal, and in the mosquito stomach wall undergo a phase of sexual reproduction. The result is oocysts which upon rupture release sporozoites which eventually migrate to mosquito salivary glands. When the infective mosquito bites again, the cycle is repeated.

Clinical differentiation of uncomplicated and complicated malaria

Red blood cell sequestration and lysis are responsible for many of the clinical signs and symptoms of malaria, but cytokine abnormalities and changes in blood-brain barrier permeability may also contribute to clinical illness[6]. In general, malarial disease is divided into two broad categories: uncomplicated and complicated. Uncomplicated disease produces high spiking fevers, shaking chills, abdominal pain, back pain, and other signs of systemic illness, but is not lethal. Complicated malaria has several forms including cerebral malaria, severe malarial anemia, and respiratory distress. Complicated malaria may lead to death. Cerebral malaria is the most common form of complicated malaria[2]. While the case fatality rate of all forms of malaria is 0.02%, children with cerebral malaria have a 15% risk of death, even in centers with the best clinical care[2].

Clinical diagnosis and treatment of cerebral malaria

Cerebral malaria is defined as an otherwise unexplained coma in a patient with *Plasmodium* parasitemia[2]. Although human infection with any of three of the *Plasmodium* species may be associated with cerebral symptoms, the vast majority of cases in African children are with *P. falciparum*. Infection with *P. vivax* and *P. knowlesi* may be associated with coma, but will not be considered further here. Coma associated with *P. falciparum* infection carries high rates of both mortality and neurologic morbidity in survivors [7, 8].

In Africa, depth of coma in childhood is graded using the Blantyre coma score (BCS), a modification of the Glasgow coma scale. This BCS is a summary of voluntary eye movements, motor response and verbal response (Table 1)[9]; a score less than or equal to 2 is necessary for the diagnosis of cerebral malaria. Since the case definition for cerebral malarial specifies that the coma is otherwise unexplained, other non-malarial etiologies of coma must be ruled out before cerebral malaria can be diagnosed. This is challenging as in the areas where malaria is most prevalent, diagnostic resources to identify non-malarial etiologies of coma are often lacking.

Table 1: Comparison of the Blantyre and Glasgow coma scores

Coma Scales for Children and Adults				
	Blantyre Coma Scale		Glasgow Coma Scale	
Motor Response to painful stimulus	Localizes pain	2	Obeys commands	6
	Generalized withdrawal	1	Localizes pain	5
	No response	0	Flexion/withdrawal	4
			Decorticate posturing	3
			Decerebrate posturing	2
			No response	1
Verbal Response to speech or painful stimulus	Appropriate speech or normal cry	2	Oriented and converses normally	5
	Abnormal cry	1	Awake but disoriented	4
	No cry or sounds	0	Utters inappropriate words	3
			Incomprehensible sounds	2
			No sounds	1
Eye movements	Visually follows moving object or face	1	Opens eyes spontaneously	4
	Does not visually follow moving object or face	0	Opens eyes in response to voice	3
			Opens eyes in response to pain	2
			No eye opening	1

Difficulties with the clinical diagnosis of cerebral malaria

In geographic areas where malaria is common, asymptomatic parasitemia is present in a large proportion of the population[10]. When non-immune individuals are bitten by infectious mosquitoes, clinical disease usually develops. Eventually, after repeated infectious challenges, the bite of infective mosquitoes no longer produces clinical

illness. Humans develop a state of partial immunity termed *premunition (semi-immunity)*. In many individuals with premunition, malaria parasites may be detectable in the peripheral blood, yet no clinical symptoms of malaria disease are present. In areas of high malaria transmission, up to 60% of the population may have asymptomatic parasitemia[10].

Asymptomatic parasitemia confounds the clinical diagnosis of cerebral malaria[11]. Children with asymptomatic parasitemia who lapse into coma due to a non-malarial etiology (e.g. viral encephalitis, intoxication, or bacteremia) will often, after a positive malaria test, be diagnosed with cerebral malaria when, in fact, malaria is not responsible for their acute illness.

Retinal evaluation in children with cerebral malaria

This "false positive" scenario is not uncommon. In one autopsy study, 23% of children dying with clinically defined cerebral malaria lacked the pathological hallmark of this condition which is the sequestration of parasitized erythrocytes in post-capillary cerebral venules[12]. In these children, non-malarial etiologies of death (e.g. pneumonia, Reye syndrome) were seen. In children with post-capillary venular sequestration, non-malarial etiologies of coma were not found. Determining in life which children with clinically defined cerebral malaria who die and will be found to have a malarial *vs.* non-malarial etiologies of coma on autopsy was not possible until malarial retinopathy was described[13, 14]. Malarial retinopathy is evaluated by indirect ophthalmoscopy and has three clinical features: retinal whitening, white centered hemorrhages, and vessel color

change (orange and/or white)[14-16]. If seen in conjunction with papilledema, prognosis (mortality and neurologic morbidity in survivors) is worsened, compared to patients with malarial retinopathy but without papilledema[17]. Early reports detailed that two-thirds of children with clinically defined cerebral malaria had retinopathy. Autopsy studies confirmed that the presence of malarial retinopathy is 95% sensitive and 100% specific for the pre-morbid identification of children with erythrocyte sequestration in cerebral post-capillary venules at autopsy, the classically described pathology finding of children dying with cerebral malaria[12]. Therefore, children fulfilling World Health Organization case criteria for cerebral malaria but lacking malarial retinopathy (retinopathy negative cerebral malaria) who go onto autopsy are more likely to have a non-malarial etiology for their coma and illness, compared to patients with retinopathy positive cerebral malaria. While this is true for patients with fatal outcomes who come to autopsy, it is unclear whether these same associations hold true in those who survive.

Investigating the pathophysiology of retinopathy negative CM

Clinical work with cerebral malaria patients suggests that the interpretations based on autopsy findings may need to be reconsidered in children with non-fatal illness. In Africa, children with both retinopathy positive and negative cerebral malaria are treated with intensive supportive care and anti-malarials. The vast majority of these children survive. Without specific treatment of non-malarial coma etiologies (e.g. viral or bacterial co-infection), the low mortality rate (10%) of children with retinopathy negative cerebral malaria seems unlikely. To investigate whether the high sensitivity and

specificity of malarial retinopathy for differentiating “true” cerebral malaria from “false” cerebral malaria on autopsy held in children who survived, I performed a number of epidemiological and clinical comparisons between children with retinopathy positive and negative cerebral malaria. These are presented below.

CHAPTER II: BACKGROUND PUBLISHED WORK

Case series and formulation of four pathophysiological hypotheses

In 2010 we began our study of children with clinical cerebral malaria but without malarial retinopathy (retinopathy negative cerebral malaria) by considering two potential pathophysiological hypotheses to explain this condition[11]. We postulated that children with retinopathy negative cerebral malaria might have:

- Hypothesis 1: An asymptomatic incidental parasitemia with a second exposure (infectious or non-infectious) uniquely responsible for the acute coma and illness
- Hypothesis 2: An acute *P falciparum* infection modified by innate host factors (genetics or partial immunity) are changing disease expression from retinopathy positive to negative

The first hypothesis is supported by the previously mentioned autopsy studies, the second by clinical experience. It is also possible that both of these pathophysiological hypotheses are responsible for a proportion of the children with the clinical syndrome of retinopathy negative cerebral malaria[11].

Admission laboratory parameters and seasonality comparisons

Comparing laboratory parameters between children with retinopathy negative *vs.* positive cerebral malaria reveals clear differences between groups (Table 2), but most of these differences could be present whether or not the coma in retinopathy negative

cerebral malaria was due to a non-malarial etiology or simply a less severe (or different) malarial illness[18].

Table 2: Comparison of chronological age and acute laboratory parameters in retinopathy positive *vs.* retinopathy negative cerebral malaria patients

	Retinopathy Positive		Retinopathy Negative		
	N	Median (IQR)	N	Median (IQR)	P value*
Age (months)	1147	35.00 (25, 34)	577	36.00 (22,56)	0.99
Glucose (mmoles/L)	1140	5.8 (4.3, 7.6)	575	6.1 (4.2,8.3)	0.12
Lactate (mmoles/L)	747	6.4 (3.4,11.1)	181	5.4 (3.3,8.9)	0.01
Log (parasite density)	1107	11.0307 (8.2273,12.5274)	563	10.6874 (7.3920,12.2987)	0.01
Blood WBC count/1000 (per µL)	1053	10.5 (7.3,15.4)	524	10.8 (7.8,15.2)	0.90
Hematocrit (%)	1148	20 (15,26)	578	28 (21,32)	<0.0001
Platelet count/1000 (per µL)	968	68 (40, 119)	446	139.5 (57,226)	<0.0001

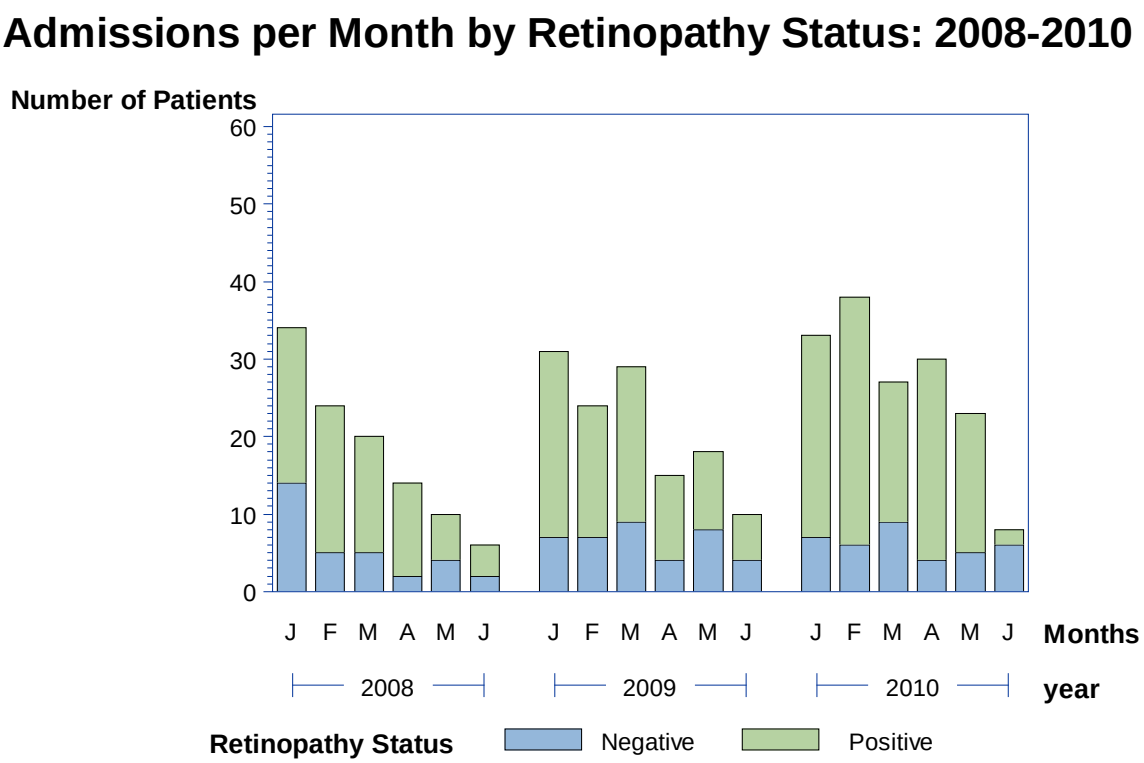
The similarities in parasite densities (measured logarithmically) between the two groups is interesting. Although peripheral parasite density is statistically significantly different in patients of differing retinopathy status, the differences in densities are not clinically significant. In general, patients with more severe malarial illness have higher peripheral parasite densities. (Patients with asymptomatic parasitemia have lower densities compared to those with uncomplicated malaria. Both of these groups have lower

parasite densities compared to children with complicated malaria disease.) Assuming that retinopathy positive cerebral malaria is due to acute malarial illness, the similar parasite densities in children of different retinopathy statuses supports Hypothesis #2 above: since parasite densities are similar in children with different retinopathy status, one would infer that both retinopathy negative and retinopathy positive cerebral malaria have the same underlying etiology of disease--- acute malaria infection. Unfortunately, peripheral parasite density reflects only circulating parasites and is not an accurate biomarker of total body parasite burden, the combination of sequestered and circulating parasites. Even in patients heavily infected with malaria, removal of parasitized erythrocytes by sequestration may lead to underestimates of total body parasite load if peripheral parasite density is used as a sole measure.

Data in Table 2 contrast with results from studies comparing the concentration of histidine rich protein 2 (HRP2), a parasite encoded protein released at red blood cell schizogeny, and a putative biomarker of total body parasite burden. Children with retinopathy negative cerebral malaria have lower quantitative HRP2 compared to those with retinopathy positive disease[19]. However, these differences do not provide information as to whether the coma in retinopathy negative cerebral malaria is due to a malarial or non-malarial etiology because what is needed is a comparison of true negative (i.e. malaria is not causing illness and no retinopathy) to false negative cases (i.e. malaria is causing illness but no retinopathy). In both cases one would expect that quantitative HRP2 would be different in children with retinopathy negative cerebral malaria compared to those who are retinopathy positive.

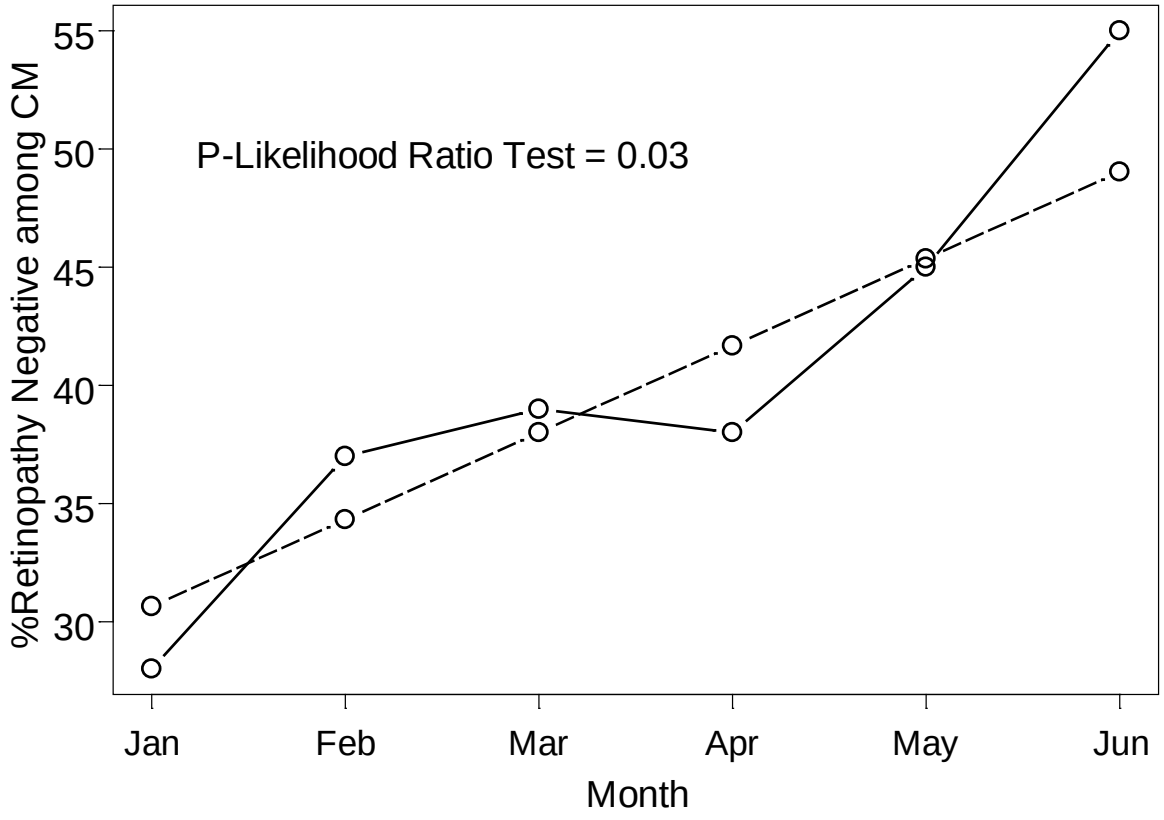
Comparing seasonality of these 2 syndromes may allow insight into whether acute malaria infection is responsible for the clinical syndrome of retinopathy negative cerebral malaria. Again assuming that retinopathy positive disease is due to acute malarial infection, if retinopathy negative disease is due to a non-malarial illness, the seasonality of the two conditions should differ. If both are due to acute malarial infection, the seasonality will be similar. Using data collected at the Blantyre Malaria Project (Queen Elizabeth Central Hospital) in Blantyre, Malawi, we determined the odds of being retinopathy positive *vs.* negative in January and June, from 1997 to 2010. In Malawi, January is the height of the rainy season and malarial illness is common. By late March the rains have usually abated and malarial disease is less common in June (compared to January). In some years (2009 and 2010) the proportion of cases that were retinopathy negative varied month to month, supporting the hypotheses that retinopathy negative and positive cerebral malaria have different underlying etiologies (one malarial, the other non-malarial) (Figure 1). In other years (2008) these month to month differences in proportions were less obvious (Figure 1). Data were not collected from July-December, limiting conclusions that can be drawn from this secondary data analysis.

Figure 1: Monthly variation in the number of retinopathy positive and retinopathy negative cerebral malaria patients admitted to Queen Elizabeth Central Hospital from January 2008 through June 2010



When data were combined across years (2004-2010), the proportion of children with retinopathy negative cerebral malaria (the complement of the proportion developing retinopathy positive cerebral malaria) significantly increased as one progressed throughout the malaria season, compared to the odds in January (Figure 2) [20].

Figure 2: Monthly proportion of children who were retinopathy negative out of the total number of patients admitted with CM in all years combined between 1997 and 2010. The proportion of children admitted with retinopathy negative CM significantly increased significantly in a linear fashion (p value of likelihood ratio test in crude analysis and analysis adjusting by month=0.03) from January to June. Dashed line represents the proportions estimated in the logistic regression and solid line represents the observed proportions



Therefore, seasonal variations between the two patient groups support the hypotheses that retinopathy positive and retinopathy negative cerebral malaria have differing underlying etiologies—if retinopathy positive cerebral malaria is due to acute malarial illness, retinopathy negative cerebral malaria could be due to an illness that is not malaria.

A cohort study comparing neurological outcomes in retinopathy negative cerebral malaria survivors

The incidence of adverse neurologic outcomes in cerebral malaria survivors has been determined by cohort studies[21, 22]. The incidence of neurologic sequelae in retinopathy positive cerebral malaria survivors is 30%. Deficits include cognition, motor function, epilepsy, and behavior. In this cohort study of neurologic outcomes in retinopathy negative cerebral malaria survivors, investigators recruited children with retinopathy negative cerebral malaria and concurrently hospitalized non-comatose controls to calculate the incidence of adverse neurologic outcomes in children surviving this illness. Children with uncomplicated malaria were eligible to be recruited as unexposed controls. A developmental and epilepsy screening questionnaire was administered to the carers of all enrolled subjects; children with pre-existing epilepsy were excluded. Enrolled subjects were followed for at least 18 months after hospital discharge. We compared the rates of adverse neurologic outcomes (motor, cognitive, developmental, behavioral) between children surviving retinopathy negative cerebral malaria and unexposed controls (Table 3). We also determined an odds ratio of adverse neurologic

outcomes comparing retinopathy negative *vs.* retinopathy positive cerebral malaria survivors.

Table 3: Outcomes in retinopathy negative cerebral malaria survivors, retinopathy positive cerebral malaria survivors, and controls

	Retinopathy negative CM survivors (N=35)	Retinopathy positive CM survivors (N=132)	Controls (includes children with uncomplicated malaria) (N=272)	Odds ratio (95%CI) comparing retinopathy negative survivors with controls	Odds ratio (95% CI) comparing retinopathy negative CM survivors with retinopathy positive CM survivors
Epilepsy	6/35 (17.1%)	12/132 (9%)	0/272 (0%)	Undefined	2.1 (0.7-6.0)
New neurodisabilities*	7/34 (20.6%)	28/131 (21.4%)	1/272 (0.4%)	70.3 (8.3-592.6)	0.9 (0.3-2.2)
Disruptive behavioral disorder	3/35 (8.6%)	14/132 (10.6%)	1/272 (0.4%)	25.4 (2.6-251.6)	0.8 (0.2-2.9)
Any adverse neurologic outcome	11/35 (31.4%)	42/132 (32%)	2/272 (0.7%)	61.9 (13.0-295.5)	1.0 (0.4-2.2)

Retinopathy positive and retinopathy negative cerebral malaria survivors have similar odds of adverse neurologic outcomes (Table 3)[22]. This similarity supports the hypothesis that retinopathy positive and retinopathy negative cerebral malaria have

similar underlying etiologies, acute malarial illness (Hypothesis #2 above). That two conditions of differing etiology (Hypothesis #1) could have similar odds of adverse neurologic outcomes in survivors would be clinically extremely unusual, though conclusions are tentative due to the low number of children with retinopathy negative CM who were studied. Certainly this finding could be by chance, but it raises the possibility that children surviving retinopathy positive and retinopathy negative cerebral malaria may have similar etiologies of coma. If one again assumes that retinopathy positive cerebral malaria is due to acute malaria infection, the similar odds of adverse outcome in the two conditions supports the hypothesis that the syndrome of retinopathy negative cerebral malaria is also due to acute malaria infection.

Epidemiological studies of children surviving cerebral malaria reveal that children who are retinopathy negative have a higher odds of a pre-illness developmental abnormality or of having a first degree relative with epilepsy, compared to children who are retinopathy positive[23, 24]. It is possible that in at least some children with retinopathy negative cerebral malaria, a pre-existing innate host factor (associated with a developmental abnormality or family history) is modifying disease expression. Instead of developing uncomplicated malaria during acute infection with the parasite, children with one of these epidemiological associations would more likely to lapse into coma. The intensity of infection necessary to produce coma in these children may not be high, explaining the lack of retinal signs.

A comparison of MRI characteristics in children with retinopathy negative *vs.* positive cerebral malaria

Neuroradiologic data of children with retinopathy negative cerebral malaria raises further questions concerning the underlying pathophysiology of the condition. We analyzed admission brain magnetic resonance imaging (MRI) studies on 44 Malawian children with retinopathy negative cerebral malaria. MRI variables found in children of different retinopathy status were compared. In retinopathy negative patients we also determined MRI factors associated with adverse outcome, defined as mortality and neurologic morbidity in survivors. Our goal for the latter analysis was to try to identify possible clues as to therapeutic targets associated with death and disability. Due to small patient numbers, several comparisons made in 2 by 2 contingency tables of MRI factors *vs.* adverse outcomes had cells that contained the numeral zero; this led to several odds ratios in univariate analysis of zero or infinity, both considered clinically unrealistic. As MRI data is frequently correlated (abnormalities in one brain area often have abnormalities in nearby areas) we selected the program Elastic Net to perform the multivariate analysis[25]. Elastic Net groups highly correlated data, bringing groups of covariates into or out of analysis simultaneously.

On regression analysis, no MRI variables were associated with mortality, likely due to the fact that only 3 subjects died. Eight MRI variables were selected by Elastic Net as having odds ratios associated with neurologic morbidity that were different than one. Since many of these variables had statistical separation (or near separation) on univariate analysis, Firth logistic regression was used to calculate 95% confidence intervals for these odds ratios. Likely due to the statistical separation (having a cell in a

2 by 2 contingency table containing the numeral zero) of many of our MRI variables *vs.* outcome data, all of these confidence intervals included one. Cortical (gray matter) abnormalities on admission MRI were associated with adverse neurological outcomes. The differential diagnosis of these abnormalities includes seizures and central nervous system viral infection. The differences in MRI findings between children of different retinopathy status do not help differentiate whether the coma in retinopathy negative cerebral malaria is due to a non-malarial or malarial etiology, though these findings are limited by the small sample size.

Summary of epidemiological and clinical studies: trying to understand the underlying pathophysiology of retinopathy negative cerebral malaria

In summary, data analyzed thus far comparing children with retinopathy negative and positive cerebral malaria are somewhat conflicting. Some analyses- such as seasonality data- support the pathophysiological explanation that children with retinopathy negative cerebral malaria have an asymptomatic parasitemia with a non-malarial etiology of illness; others support the hypotheses that retinopathy negative cerebral malaria is caused by acute malaria infection. Several other comparisons are equivocal (Table 5). None of these analyses were prospective and the parent study was not designed to test hypotheses concerning the underlying pathophysiology of this condition.

Table 4: Summary of studies supporting differing hypotheses of the underlying pathogenesis of retinopathy negative cerebral malaria

Comparison	Supports hypothesis that retinopathy negative cerebral malaria is due to a non-malarial etiology of coma	Supports hypothesis that retinopathy negative cerebral malaria is due to acute malaria infection	Equivocally supports both hypotheses
Lab differences other than parasite density			X
Parasite density		X	
Quantitative HRP2			X
Seasonality	X		
Neurological Outcomes		X	
MRI			X

Clearly another approach is necessary to resolve this pathophysiological puzzle.

Direct determination of non-malarial contributors to coma in children with retinopathy negative cerebral malaria

One clear way to determine if at least some children surviving retinopathy negative cerebral malaria have a non-malarial etiology of coma is to directly search for non-malarial coma etiologies in acutely ill patients, many of whom will not die. These etiologies are most commonly infectious, usually viral encephalitis, bacterial meningitis, or bacteremia. Seizures (both convulsive and non-convulsive) may also lead to coma and can be associated with these infectious illnesses. Acute bacterial meningitis is confirmed or ruled out at the time of admission for a child with clinical cerebral malaria,

as spinal fluid is analyzed for the presence of cells and with Gram stain. Central nervous system viral illness, bacteremia, and non-convulsive seizures are three treatable causes of coma seen in both the developed and developing world. To assess for these non-malarial coma contributors, we prepared and submitted a K23 application to the US National Institutes of Health. The overall purpose of the Research Plan of this K23 application is to determine the proportion of children with retinopathy negative cerebral malaria with each of these associated conditions, and identify the infectious pathogens causing illness. These identities and proportions will be compared to children in a control group, those with acute febrile coma but without malaria parasitemia, termed *acute aparasitemic coma.*

CHAPTER III: THE K23 APPLICATION

Introduction

I am currently funded by the US National Institutes of Health to investigate the role of CNS viral co-infection in children with retinopathy negative CM. This study's aims are to identify co-infecting viral pathogens, see if there are clinical characteristics associated with viral co-infection, and determine if viral co-infection changes the risk of mortality and morbidity at discharge. Recognizing the need to expand the search for non-malarial coma etiologies to other patient groups and to increase the number of non-malarial coma etiologies assessed, I formulated a K23 application to assess viral or bacterial co-infections and non-convulsive seizures in children with either retinopathy negative CM and acute aparasitemic coma.

Significance

For clinicians working in sub-Saharan Africa, the treatment of coma in children is problematic. Diagnostically a malaria test and lumbar puncture are usually first performed. If the malaria test is positive, clinicians diagnose cerebral malaria (CM) and begin antimalarials. If the lumbar puncture (LP) is abnormal, they tentatively diagnose acute bacterial meningitis. But if the malaria test is negative and the LP normal, clinicians usually have no other diagnostic modalities available to aid treatment decisions. Even a positive malaria test in a comatose child does not mean that malaria is necessarily responsible for illness. Children with a non-malarial etiology of illness but an asymptomatic malaria parasitemia (reflecting residence in an area of high

transmission[11]) will be diagnosed with CM when malaria is not the cause of their acute illness. A clinical-autopsy correlation study revealed that 23% of children dying of CM lacked evidence of sequestration of parasitized erythrocytes in cerebral vasculature[12]. This is the pathological hallmark of CM, and is necessary to conclude that acute infection with *P falciparum* was responsible for the patient's coma and illness. In this autopsy study, patients who lacked evidence of parasite sequestration had other causes of death identified at autopsy. Differentiating the group without central nervous system (CNS) sequestration from the total CM population during life was not possible until malarial retinopathy was recognized[13, 14]. This clinical finding is 95% sensitive and 100% specific for the premorbid identification of patients with CNS parasite sequestration at autopsy[12]. About 1/3 of children with clinically defined CM are retinopathy negative and are presumed to have an underlying non-malarial cause of illness. The types of non-malarial illnesses in both aparasitemic children (diagnosed with acute aparasitemic coma or AAC) and children with retinopathy negative CM are currently unknown.

This problem is common. There are approximately 447 million children living in Africa[1]. The annual incidence rate of coma in this population is 44 per 100,000 children[26]. Approximately 59% of comatose children have malaria parasitemia (and qualify for a diagnosis of CM), 4.2% have acute bacterial meningitis, and the remaining 36.1% are aparasitemic and the etiology of their coma is unknown[26]. Thus there are approximately 71,000 aparasitemic comatose African children hospitalized annually. In the vast majority their underlying coma etiology is never known. Since about 40,000 children annually have retinopathy negative CM[26], this means the annual number of non-malarial

coma cases in African children is approximately 110,000 per year. Children with CM or aparasitemic coma who do not reach the hospital are not included in these estimates. We are currently conducting an assessment of CNS viral co-infection in children with retinopathy negative CM (1R21HD078471-01). In the study proposed here we will expand the types of patients studied to include children with AAC. We will expand the number of non-malarial illnesses assessed (and their interactions with one another) from our current research. We will gather clinical data and laboratory specimens to identify viral and bacterial co-infecting pathogens in children with retinopathy negative CM and AAC (Specific Aim 1). We will assess if there are interactions between viral and bacterial CNS infection, bacteremia, and seizures (Specific Aim 2). We will address knowledge gaps by evaluating whether clinical characteristics can be used to characterize children with retinopathy negative CM or AAC who have a viral or bacterial co-infection, or non-convulsive seizures (Specific Aim 3). We will establish whether a viral or bacterial co-infection changes rates of mortality and morbidity in children with retinopathy negative CM or AAC (Specific Aim 3). Once we have filled these knowledge gaps we will use this information to design a clinical trial of adjunctive antiviral, antibiotic, and/or anticonvulsant therapy in children with aparasitemic coma at highest risk for these treatable co-morbidities.

Our findings will be significant because they represent the first step in a continuum of research that is expected to lead to development of effective adjunctive antiviral, antibiotic, and/ or anticonvulsant, therapies for African children with non-malarial comas. If such therapies are successful, the rates of mortality and neurologic morbidity in these children are likely to decrease.

Innovation

This is the first study to address the prevalence of and interactions between three treatable etiologies (bacterial CNS infection and bacteremia, viral CNS infections, seizures) of non-malarial coma, a common and clinically challenging neurologic problem in sub-Saharan Africa. This research will assay for a wider spectrum of viral and bacterial co-infecting pathogens (see Table 7, below) than has been tested for in any previous study and will assess the interactions between viral co-infection, bacterial co-infection, seizures, and adverse outcome. In addition to the public health impact of our study's results, our findings may be useful to researchers interested in CNS viral or bacterial infection, bacteremia, or non-convulsive seizures in African children.

This project is innovative in that it combines advanced microbiological methods (quantitative (q) PCR and ELISA) and a comprehensive database of patient demographic, laboratory, and outcome data to determine if there are demographic or laboratory characteristics that increase the likelihood of a detectable CNS viral or bacterial infection, bacteremia, or non-convulsive seizures in a comatose child. In addition this study will search for novel pathogens using next generation sequencing. It lays the groundwork for the performance of clinical trials of adjunctive antiviral, antibiotic, and/ or anticonvulsant therapy in children with retinopathy negative CM or AAC. Aparasitemic comatose African children have been the subjects of few interventional clinical trials and although there have been many trials of adjunctive therapies in CM (all negative), antiviral agents or a combination of targeted interventions has never been studied for this disease.

Approach

Introduction to the Research Plan: Data Collection

Clinical data will be collected prospectively from Malawian children with retinopathy negative CM and AAC admitted to the Pediatric Research Ward (PRW) at the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi, where studies of CM pathogenesis have been ongoing since 1986. Care will be provided by a well trained staff of research nurses and clinicians. Informed consent will be obtained from the caregivers of all eligible children. All children enrolled will be between 6 months and 13 years of age, comatose with a Blantyre Coma Score (BCS) ≤ 2, malaria retinopathy negative, and without other explanation for coma (negative cerebrospinal fluid (CSF) gram stain, normoglycemic, > 1 hours since last clinical seizure). Children with malaria parasitemia (fulfilling diagnostic criteria for retinopathy negative CM) will receive intravenous antimalarials for 48 hours followed by a full course of lumefantrine-artemether. Intravenous fluids, antipyretics, antibiotics, and anticonvulsants will be administered to all enrollees as clinically indicated. Each patient will be characterized in detail (Table 6). Children who awaken within 12 hours of admission will continue to be cared for but will be excluded from data analysis, as they likely are post-ictal. At the time of discharge, surviving children will be examined by a neurologist to establish whether or not neurologic sequelae are present. All survivors will be requested to return for a one month follow-up visit comprised of a parent/child interview and physical and neurological exams by a pediatric neurologist.

Table 5: Clinical observations and investigations

Time after Admission	0h	6h	12h	18h	24h	30h	36h	42h	48h	daily	Data Type
Physical exam	x		x		x		x		x	xx	Categorical, continuous
Vital signs	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	q2h	Continuous, ordinal
Eye exam (malaria retinopathy)[2]	x		x								
Routine Labs	x	x	x	x	x	x	x	x	x	q6h	Continuous
Study Labs	x										Categorical, continuous
Lumbar puncture	x										Continuous
EEG	x				x						Categorical

We plan to use this clinical data and results of CSF and blood analyses to test our central hypotheses. We will accomplish the objectives of this application by pursuing the following three **specific aims**:

Aim 1. In children with retinopathy negative CM or acute aparasitemic coma, identify and compare viral and bacterial co-infecting pathogens

Introduction

The identities of viral and bacterial co-infecting pathogens from children with retinopathy negative CM and AAC are currently unknown. The *objective of this aim* is to identify and compare viral and bacterial co-infecting pathogens from children with these clinical syndromes. To attain the objective of this aim, we will test the *working hypothesis* that in children with retinopathy negative CM or AAC these pathogens will be similar when compared between groups. We will test our working hypothesis by analyzing CSF and

plasma samples collected from children admitted with retinopathy negative CM or AAC and compare the identities of co-infecting pathogens between diagnosis groups. The *rationale* for this aim is that if the identities of co-infecting pathogens are similar between groups, a single formulation of antiviral agents or antibiotics can be tested in these groups together. If the identities of co-infecting pathogens are different between the 2 groups this will also be important, and may indicate that syndrome-specific adjunctive antiviral and/or antibiotic regimens may need to be tested. When the studies for Aim 1 have been completed, it is our *expectation* that rational design of a clinical trial of adjunctive antiviral or antibiotic therapy for children with retinopathy negative CM and AAC will be possible.

Justification and Feasibility

CSF was obtained and analyzed from 213 patients with acute fever with or without coma (BCS ≤2) and with or without malaria parasitemia. CSF specimens were prospectively gathered from a convenience sample of children with fever and either convulsion or change in mental status admitted to QECH in Blantyre, Malawi, between February 2002 and August 2004. Demographic and clinical data were obtained on all of these children. We performed a secondary analysis of this data (Table 7).

Table 6: Identities of viral pathogens detected in patients

Patient Diagnosis Group	n	Proportion with viral pathogen identified	Viral Pathogens identified in CSF	Mortality rate in those with viral co-infection	Mortality rate in those without viral co-infection	Morbidity rate in survivors with viral co-infection	Morbidity rate in survivors without viral co-infection
Retinopathy Negative CM (RET neg CM)	24	33% (8/24)	Adenovirus(3), mumps(2), HSV 1(2), EBV(1)	25% (2/8)	19% (3/16)	16% (1/6)	8% (1/13)
Aparasitemic coma without other explanation (AAC)	46	30% (14/46)	Adenovirus (4), mumps, HHV6, EBV, measles(1), CMV(2), HSV1 (2), parvovirus(1), rabies(2)	71%(10/14)	25% (8/32)	25% (1/4)	0% (0/24)

Preliminary data (Table 7) reveal that the identities of viral co-infecting pathogens vary between children with retinopathy negative CM and AAC. These data are limited, however, as patient numbers were small. During this study there was no nucleic acid based assessment for bacterial co-infection or pathogen discovery. This study was not performed using qPCR and therefore may have detected non-pathogenic viruses (e.g. adenovirus) present in CSF incidentally or in non-pathogenic numbers.

An additional case series of viral co-infections in patients with CM has been published[27]. In this study, malaria retinopathy status was not described. CSF of patients with a clinical diagnosis of either CM or AAC was analyzed with PCR for evidence of herpesviruses (HSV) or enteroviruses. Four of 49 (9%) children with CM had HSV1 DNA detected in their CSF. In patients with AAC, six of 47 (12%) had evidence of

HSV1 infection. Additionally, in the AAC group, acute infection with cytomegalovirus (CMV), varicella-zoster virus (VZV) and enterovirus, was found in one child for each pathogen (three total children).

The laboratory of Dr. Joe DeRisi at UCSF has performed novel pathogen discovery work on children with cerebral malaria (malarial retinopathy status unknown) admitted to Mulago Hospital in Kampala, Uganda. Ten percent of children admitted with CM had previously undetected bacterial co-infections in the CSF or blood (personal communication, Dr. Michael Wilson, co-investigator). The list of pathogens identified in the DeRisi lab will be used in our analysis of CSF and blood sampled in our study subjects (Table 8, below).

Research Design

The Blantyre Malaria Project (BMP) is located within the PRW of QECH in Blantyre, Malawi. We will enroll children for this research using the existing infrastructure of the BMP. Three milliliters of both blood and CSF will be collected for our analyses. Blood specimens will be centrifuged and the resulting plasma frozen at -80°C for later batch analysis. CSF obtained for our study will likewise be frozen at -80°C for future batch analysis to identify viral co-infecting pathogens. Routine EEG (30 minute recording time) will be performed at admission and 24 hours later.

We designed a panel for assessment of viral and bacterial co-infections based on our preliminary data (Table 7), knowledge of known causes of encephalitis in the developed world, preliminary data from UCSF, and a literature review[28]. This panel (Table 8) will be performed on CSF and plasma samples from children with retinopathy negative CM and

AAC. A core battery of tests will be performed on specimens obtained from each individual, testing for up to 18 (depending on HIV status- see Table 8) potential viral primary infections of CSF that may cause encephalitis and 8 bacterial pathogens in both blood and CSF. We will use standardized commercially available reagents for laboratory analyses. If there are limitations on sample volume (less than 3 ml available) we will initially perform analyses for the first 13 viruses and all the bacteria in Table 8. (The first 13 viral pathogens were those found in CSF in children in the preliminary data (Table 7)). This will require approximately 1.5 ml of CSF and plasma. For samples negative for the first 13 viral pathogens, if sufficient volume remains, analyses of the other 5 viral pathogens will then be done. As an individual patient could have 26 PCR analyses and 3 ELISAs done on CSF, and 8 PCRs and 6 ELISAs performed on plasma, sample volume should be adequate to perform all analyses in most patients.

Table 7: Analytic panel for acute bacterial and viral infections of the central nervous system in African children

Pathogen	Plasma serology (IgM)	Plasma qPCR	CSF serology (IgM)	CSF qPCR	Lab criteria for operational definition of acute CNS infection
Measles/ Rubeola			X	X	+CSF IgM and/or +CSF PCR
Mumps	X			X	+plasma IgM and +CSF PCR
Rubella	X			X	+plasma IgM and +CSF PCR
HSV1				X	+CSF PCR
HSV2				X	+CSF PCR
VZV			X	X	+ CSF IgM and/or +CSF PCR
HHV6	X (PCR)			X	+plasma IgM and +CSF PCR
Adenovirus				X	+CSF PCR
EBV	X (anti-VCA)			X	+plasma IgM and +CSF PCR
Parvovirus B19	X		X	X	+Plasma IgM and +CSF IgG or PCR
Enterovirus				X	+CSF PCR
Parechovirus				X	+CSF PCR
Rabies[=]				X	+CSF PCR
Mycoplasma pneumonia	X			X	+plasma IgM and + CSF PCR
Influenza A				X	+CSF PCR
JC virus (HIV pos only)				X	+CSF PCR
CMV (HIV pos only)				X	+CSF PCR
Toxo(HIV pos only)				X	+CSF PCR
Escherichia coli		X		X	+CSF PCR (+plasma PCR for bacteremia)
Strep. Pneumoniae		X		X	+CSF PCR (+plasma PCR for bacteremia)
N. meningitidis		X		X	+CSF PCR (+plasma PCR for bacteremia)
Staph aureus		X		X	+CSF PCR (+plasma PCR for bacteremia)

Analyses for the presence of IgM will be done by ELISA on specimens obtained at the time of admission. Analysis for the presence of viral DNA or RNA will be performed by qPCR on CSF obtained during admission. qPCR is the primary diagnostic technique for many viruses because of its utility in diagnosis of infection in immunocompromised patients whose antibody responses are delayed or absent.

To assay for novel pathogens, 300 µl of each CSF sample gathered will be exported to UCSF for next generation sequencing. We will also export enrollees' plasma to UCSF as a pilot study assaying transcriptome responses to CNS infection (see Future Directions, below). Universal precautions for blood-borne pathogens will be taken.

QA/QI will take place at each stage of laboratory set up and specimen analysis with use of specimens of known antibody titer (for ELISA) and viral load (for qPCR) to standardize equipment and procedures.

Data Analysis

We will list the most common co-infecting viral and bacterial pathogens in children with retinopathy negative CM and AAC. Pathogens found in greater than 10% of viral or bacterial co-infected children in both groups will be included in a list of common co-infecting pathogens. This list will be used to formulate the adjunctive antiviral and/or antibiotic therapy regimen to be tested in our anticipated clinical trial. If there are no common co-infecting pathogens in both retinopathy negative CM and AAC patients, syndrome specific trials of adjunctive antiviral and/or antibiotic therapy may be necessary.

Expected Outcomes

Aim 1 is expected to inform selection of antiviral or antibiotic agents to be used in adjunctive therapy trials in retinopathy negative CM and/ or AAC. We anticipate that approximately 1/3 of the children in both groups will be co-infected with viruses, and 10-15% with bacteria.

Potential Problems and Alternative Strategies: There is the possibility that standardizing the laboratory may be problematic. In our ongoing R21 project assessing CNS viral co-infection in retinopathy negative CM, we have established stringent lab QA/QI procedures before, during and after, lab analyses to aid standardization. We are utilizing both positive and negative controls for nucleic acid extraction and the qPCR itself. Co-investigators have a long experience with standardization and has worked diligently to assure the reliability of our lab's results. It is possible that commonly identified co-infecting viruses or bacteria may not be sensitive to currently available antiviral agents or antibiotics. We will select antiviral agents and antibiotics in future studies to cover as many commonly identified viruses and bacteria (respectively) as possible.

Aim 2: In children with retinopathy negative CM or acute aparasitemic coma, determine if viral and/or bacterial co-infections are associated with seizures

Introduction

Seizures, both convulsive and non-convulsive, are common in comatose children, including children with retinopathy negative CM. It is not known if viral or bacterial co-

infection changes the odds of clinical or subclinical seizures. The *objective of this aim* is to determine if convulsive and/or non-convulsive seizures are associated with non-malarial infection in children with retinopathy negative CM or AAC. To attain the objective of this aim, we will test the *working hypothesis* that in children with retinopathy negative CM or AAC, non-malarial infection increases the odds of convulsive or non-convulsive seizures. We will test our working hypothesis by using the *approach* of gathering information about clinical and subclinical seizures. We will analyze EEG studies obtained from children with these two syndromes at admission and 24 hours later. The *rationale* for this aim is that if seizures are associated with co-infection, clinical trials with more than 1 intervention (e.g. antibiotics + anticonvulsants) may be necessary to modify the odds of an adverse outcome. When the studies for Aim 2 have been completed, it is our *expectation* that we will know if an association between co-infection and seizures exists. This will inform design of our anticipated follow-up interventional study.

Justification and Feasibility

In the developed world, up at 36% of comatose children admitted to intensive care units have seizures without clinical symptoms[29]. Convulsive and non-convulsive seizures are also common in CM and are associated with poor outcome in both retinopathy positive and negative patients[7, 8]. In our R21 project, between April and June 2014, we enrolled eight children in Malawi with retinopathy negative CM. Seven of the eight children had convulsive seizures before or on admission. Two of these 7 children had non-convulsive seizures (a rhythmic repetitive spike-wave discharge with an electrical field

that evolved in frequency, morphology, or amplitude over at least 10 seconds) on admission EEG; one of these had non-convulsive *status epilepticus*.

Research Design

We will obtain 2 EEG studies on all enrolled subjects, one as soon as possible after admission and the second 24 hours later. Studies will be interpreted prior to analysis of CSF and serum, so the co-infection status of the subject will be unknown to the EEG interpreter. An electrographic seizure will be defined as a rhythmic discharge or spike and wave pattern with definite evolution in frequency, location, or morphology lasting at least 10 seconds.

Data Analysis

Chi square or Fisher exact test will be used to determine if the presence of co-infection is associated with clinical or subclinical seizures, or both. A subanalysis will be performed, stratifying by pathogen type (bacterial *vs.* viral) and identity.

Expected Outcomes

If non-convulsive seizures are associated with viral or bacterial co-infection, a combination of therapies (e.g. antivirals+anticonvulsants) may be needed to demonstrate reductions in mortality and morbidity. Aim 2 is expected to inform design of clinical trials targeting viral or bacterial co-infection and seizures in children with retinopathy negative CM or AAC.

Potential Problems and Alternative Strategies

EEG has been routinely used at the Blantyre Malaria Project for over 10 years. There are two trained EEG technicians who perform studies daily. We do not anticipate problems with obtaining EEG in enrolled children. Interpretation of EEGs will be performed by the study PI who has extensive experience with interpretation of EEGs recorded from comatose African children.

Aim 3: Compare the routinely available clinical characteristics, laboratory parameters, and outcomes of children with retinopathy negative CM and AAC with and without viral or bacterial co-infections, or non-convulsive seizures

Introduction

There are no published reports of clinical factors that increase the likelihood of viral or bacterial co-infection or non-convulsive seizures in children with CM or AAC. The *objective of this aim* is to identify clinical characteristics that change the odds of a viral or bacterial co-infection or non-convulsive seizures in these two groups, and to evaluate if co-infection changes the odds of mortality or morbidity. To attain the objective of this aim, we will test the *working hypotheses* that clinical characteristics will allow identification of children with retinopathy negative CM or AAC at higher risk of viral or bacterial co-infection or non-convulsive seizures. We postulate that children with a CNS viral or bacterial co-infection will be more likely to have higher CSF white blood cell counts compared to those without viral co-infections. We postulate that children with bacteremia will be more likely to have prolonged capillary refill, and a widened pulse pressure compared to those without bacterial co-infections. We postulate that patients

with non-convulsive seizures will have lower blood glucose and higher blood lactate, compared to children without seizures. We hypothesize that children with retinopathy negative CM or AAC with a viral or bacterial co-infection have higher rates of mortality and morbidity compared to those without a co-infection. We will test our working hypotheses by using the *approach* of gathering both clinical data (Table 6) and EEG studies (Aim 2) to identify clinical or laboratory characteristics that increase the odds of a viral or bacterial co-infection or non-convulsive seizures in children with these clinical syndromes. The *rationale* for this aim is that further pursuit of a clinical trial of adjunctive antiviral or antibiotic therapy is best supported by clear documentation that an acute viral or bacterial co-infection is a poor prognostic factor. If one or more clinical characteristics increase the odds of a viral or bacterial co-infection, or non-convulsive seizures, limiting clinical trial enrollment to subjects with these characteristics will increase study power. When the studies for Aim 3 are completed, it is our *expectation* that we will know whether or not a clinical trial of adjunctive antiviral, antibiotic, and/or anticonvulsant therapy in retinopathy negative CM or AAC is justified, and if so which children should be enrolled in this trial. If the presence of viral or bacterial co-infection (or an interaction between the two, or with seizures) does not influence rates of mortality or morbidity, clinical trials of these therapies may not be warranted.

Justification and Feasibility

Preliminary data (Table 7) show a trend toward increasing rates of mortality and neurologic morbidity when children with retinopathy negative CM or AAC have a viral co-infection, but numbers provide little power to test statistical significance. From these

data we cannot determine whether the virus, the malaria parasite (in retinopathy negative CM), or their combination is contributing to high rates of mortality and morbidity in these children. Children with CM who had viral co-infections were more likely to have had clinical seizures and higher CSF white blood cell counts than those without co-infections [30].

Research Design

Enrolled children's admission demographic and laboratory characteristics, clinical course (i.e. coma resolution time), and outcome at discharge (death, neurologically impaired, neurologically normal) are currently collected on all children admitted to the Pediatric Research Ward (Table 6). We will correlate these data with the presence or absence of viral or bacterial co-infection (Aim 1) and non-convulsive seizures (Aim 2).

Power Calculation

Using historic hospital admission rates, we assume that over a 48 month period we will enroll 168 children with retinopathy negative CM and 168 with AAC. If one third of these children has a viral co-infection (the proportion detected in Dr. Mallewa's study, Table 7), 64 samples from each group will be virus positive. We assume the mortality rate in children who are virus negative will be 10%, the historical mortality rate of children with retinopathy negative CM in the Blantyre Malaria Project. Assuming a two sided test with $\alpha=0.05$, in order to tell the difference between mortality and morbidity rates in the two groups:

Table 8: Study power calculation for varying level of mortality/morbidity in RET neg CM and AAC groups combined

Mortality rate in virus positive (n=112)	Mortality rate in virus negative (n=224)	Study power to differentiate study groups
29.5% (32/112)	9.8% (22/224)	0.99
24.1% (27/112)	9.8% (22/224)	0.92
19.6% (22/112)	9.8% (22/224)	0.69

Statistical Analysis

We will compare rates of mortality and neurologic morbidity in children with and without a co-infection or non-convulsive seizures using chi square or Fischer exact test, as appropriate. A sub-analysis will be performed, stratifying by HIV status. We will study the interaction effects of viral co-infection, bacterial co-infection, and seizures, on the odds of death and disability. In children with retinopathy negative CM we will assess the effect of co-infections and seizures, on the odds of death and disability. Continuous covariates (glucose, lactate, WBC count, platelet count) will be compared between patients with or without an identified co-infection or non-convulsive seizures using t-tests or Wilcoxon rank sum tests (as appropriate) and logistic regression. Categorical covariates (hypoglycemia, gender) will be compared between patients with or without an identified co-infection or non-convulsive seizures using chi square tests. We will also study the marginal effects of these covariates and their interaction effects on the odds of viral or bacterial co-infection or non-convulsive seizures using multivariable logistic regression. Using logistic regression, the predictive accuracy of a clinical or laboratory marker to identify patients with a viral or bacterial co-infection or non-convulsive seizures will be assessed using the area under the ROC curve (c statistic). A p value

less than 0.05 will be considered evidence of a statistically significant difference between groups.

Expected Outcomes

The work proposed in this aim is expected to determine whether children with viral or bacterial co-infection, or non-convulsive seizures can be identified on clinical grounds and whether the presence of co-infection influences outcome. This information will be important when designing our anticipated clinical trial.

Potential Problems and Alternative Strategies

There is the possibility that insufficient numbers of children will be recruited during the study period. To estimate the number of specimens we expect, we used very conservative historical data. If sample numbers are below expectations at the study halfway point, we will increase outreach to improve enrollment. If enrollment numbers stay below estimates for the entire duration of the study, archived (frozen) CSF and serum samples from children with retinopathy negative CM and AAC (admitted in the last 5 years) can be analyzed. These children have been clinically characterized by methods identical to those proposed here.

Table 9: Timeline

Activity	Year 1	Year 2	Year 3	Year 4	Year 5
IRBs	X				
Enroll and characterize 84 subjects per year	X	X	X	X	
EEG interpretations	X	X	X	X	
Laboratory analyses		X	X	X	X
Analysis and dissemination, R01 formulation				X	X

Future Directions

We anticipate that approximately 1/3 of the children in both groups will be co-infected with viruses, and 10-15% with bacteria. We expect that 20-30% of children will have non-convulsive seizures. We expect that a combination of interventions may be necessary to decrease rates of mortality and morbidity in these children. Results derived from this research will inform future clinical trials targeting co-infections and seizures in children with retinopathy negative CM and AAC.

This research includes studies of pathogen discovery and pilot analyses of host RNA profiling work in collaboration with UCSF. Pathogen discovery work may reveal additional microbes (e.g., viral, parasitic, bacterial or fungal) present in African children presenting with non-malarial coma that would require therapeutic targeting in future interventional studies[31]. Host transcriptome studies of peripheral blood mononuclear cells isolated from acute patient blood samples may lead to future evaluations of the contribution of host response to mortality and neurologic morbidity in children with non-malarial comas. Modification of these host responses (possibly in conjunction with antiviral, antibiotic, and/or anticonvulsant therapies) may modify disease outcomes in affected children.

CHAPTER IV: POTENTIAL ROLE OF ADAPTIVE DESIGNS FOR CLINICAL TRIALS OF ADJUNCTIVE ANTIVIRAL, ANTIBIOTIC, OR ANTICONVULSANT MEDICATIONS IN CHILDREN WITH RETINOPATHY NEGATIVE CEREBRAL MALARIA

Introduction

I believe it likely that the results of this K23 application will lead to clinical trials targeting non-malarial coma etiologies in children with acute aparasitemic coma and/ or retinopathy negative cerebral malaria. To prepare for this eventuality I researched adaptive clinical trial design.

In medical research the goal of most clinical trials is to evaluate the safety and efficacy of a new treatment compared to a control (placebo, standard of care, or active). When designing a clinical trial, investigators formulate a trial protocol, explicitly stating all of the policies and procedures to be performed during the study. Study protocols include objectives, enrollment criteria, randomization procedures, procedures to collect data and assess subject response to the intervention, and a statistical analysis plan. Adherence to the protocol is strict, as deviations to protocol procedures may introduce bias, potentially modifying study results and making hypothesis testing problematic.

The study target population is defined explicitly using inclusion and exclusion criteria. Study designs that are internally valid allow a fair and unbiased assessment of the treatment's effect on outcome within the target population. Randomization is the best way to balance both known and unknown cofounders between study arms. An unbiased assessment of response to the treatment is best accomplished by blinding the

evaluators of outcome to treatment assignment. The statistical analysis is pre-specified in the study protocol as well.

But not all clinical trial designs are inflexible. While a trial is in process, procedures may be modified, and made explicit in protocol amendments. These amendments may include changes in subject enrollment criteria (often a liberalization of criteria when enrollment numbers are less than anticipated), modifications of hypotheses (e.g. conversion from superiority to non-inferiority), changes in dosing or length of treatment in the intervention arm, changes in outcome assessment (e.g. using a biomarker instead of a clinical endpoint if the time to endpoint is longer than anticipated) or statistical procedures[32]. Changes in study protocol made on the basis of accrued data are known as *adaptive trial designs*. Changing trial procedures or data analysis after the trial has begun may change the statistical inferences that can be drawn from the data collected, and if changes are too great, the conclusions drawn may not be true of the original target population, compromising internal validity. In practice, adaptations after a trial is underway are often necessary.

Design adaptations may be prospective, concurrent, or retrospective. Prospective (also called changes *by design*) adaptations are the least flexible, as they are specified before the study begins. Examples include treatment-adaptive and covariate-adaptive randomization procedures, and drop-the-loser modifications of combined Phase IIb/Phase III clinical trials (see relevant sections, following). Concurrent (also called *on-going* or *ad hoc*) modifications occur as the trial is ongoing. A commonly used example is response-adaptive randomization, often called *play-the-winner*. Retrospective adaptations are made after the data are collected but before the data are unblinded.

Changes in study endpoints (e.g. superiority to non-inferiority) may be made retrospectively. Of the three types, *ad hoc* adaptations are the most commonly used and the most flexible[32]. They are discussed at length below.

Advantages and disadvantages of adaptive clinical trials

Diagnosis and treatment of patients is an adaptive process. Information learned from one patient is used to better diagnose and treat those who follow. If a therapy or treatment regimen is not successful in several consecutive patients, a clinician typically will no longer use it. Like all learning, medical care changes with time. Adaptive clinical trial designs therefore reflect standard medical care.

Performance of a traditional clinical trial is not adaptive. Investigators formulate null and alternative hypotheses and calculate an estimated sample size (given a type I error rate and study power) that will allow hypothesis testing. The study protocol lists inclusion and exclusion criteria for subject enrollment, randomization and blinding procedures, sampling and data collection, and details the anticipated statistical analysis. In traditional clinical trials, these policies and procedures are established in advance and are inflexible.

The development of adaptive trial designs was primarily motivated by medical ethics. Adaptive trials have three main advantages compared to non-adaptive designs[32]

1. By allowing more study subjects to receive therapies that previous enrollees proved to be less toxic and more efficacious, adaptation reflects standard clinical practice, the usual way that physicians treat patients
2. Adaptation in randomization or enrollment may lead to an assessment of efficacy and safety where fewer enrollees are exposed to non-efficacious or toxic therapies. This reflects patient benevolence, a cornerstone of medical ethics.
3. In early phase clinical development, adaptive designs may be more efficient. For example, to answer questions about drug dose optimization and efficacy, a combined Phase IIb/ Phase III trial requires fewer enrolled subjects compared to two separate studies.

The main disadvantage of adaptive clinical trial design is that in cases other than early phase clinical development (combined Phase IIb/Phase III studies), it is less efficient statistically. In most Phase III clinical trials designed to test a new versus standard therapy (or placebo), the minimum number of enrollees necessary to detect a given difference between study arms will occur if half of the subjects are enrolled in each arm. If one arm is more heavily weighted due to efficacy or safety concerns, a larger total number of study subjects will need to be enrolled to achieve comparable statistical power.

Statistical methods to correct for many kinds of adaptations are available in the literature, but not all types of adaptation may be compensated for statistically, making complicated (or repeated) adaptations risky with respect to maintenance of internal validity. Of the three types of study adaptations discussed in this thesis, adaptive randomization and combined Phase IIb/ Phase III studies are the better understood

(and compensated for) from a statistical standpoint. Of course, multiple modifications may complicate the analysis to the point where statistical inference is difficult if not impossible, compromising internal validity. If enrollment criteria are modified while the study is ongoing, these adaptations may lead to results for the total study population that are not necessarily true for the original target population. If the enrollment criteria are frequently modified (usually made more liberal), the result is termed a *moving target population*[33]. This may weaken causal inference linking exposure and disease, or therapy and outcome.

For a given study size, if modifications are made after the study has begun (often after a fixed number or proportion of subjects is enrolled) the resulting p values will, in general, be increased and confidence intervals widened. This is often due to increased variance in both fixed (demographic, clinical) and outcomes in the study population compared to the original target population. Although there are statistical methods to handle these adaptations, the greatest concern is that using adaptive methods in clinical trials may lead to a totally different study that is unable to answer the original questions posed. Adapting a clinical trial in progress should be done in consultation with a biostatistician, so that researchers may be assured that any modifications made will not compromise internal validity.

Three adaptive clinical trial designs could to be used in future studies of children with non-malarial coma

Although adaptive study methods include any change of trial or statistical procedure made after a trial has begun and using data already collected, this discussion focuses on three areas of adaptive design that may be used in a future clinical trial of adjunctive antiviral, antibiotic, and/ or anticonvulsant therapies in children with retinopathy negative cerebral malaria or acute aparasitemic coma. These areas are:

1. Adaptive randomization
2. Combining Phase IIb and Phase III clinical trials
3. Moving target populations

"Do no harm" is a cornerstone of patient care. Adaptive randomization allows clinician-scientists to minimize harm, shifting enrollment from less to more efficacious study arms, and away from more toxic interventions. Combining Phase IIb and Phase III studies of adjunctive therapies will allow the question of intervention superiority (or futility) to be answered with the minimum number of total subjects enrolled. Moving target populations are frequent in all clinical trials and may occur in our anticipated future studies.

The general aim of patient-oriented research is to improve the health of the target population. Carefully adapting an ongoing clinical trial may be a necessity or specified in advance for medical ethical or practical reasons. Although they are powerful procedures, adaptations that invalidate the goals or validity of research neither serve patients nor advance general medical knowledge.

Conventional and adaptive randomization

Randomization of enrolled study subjects minimizes bias by random allocation into study arms. This is the optimal way to balance both known and unknown confounders and covariates. Randomization is also the best way to assure that the subjects under study, who are a representative sample of the target population, provide an unbiased assessment of both the safety and efficacy of the test treatment. Statistical inference of the safety and clinical endpoints of the test treatment is based on the underlying probability distribution of the endpoints. If study arm assignment is not random, the distribution of outcomes in the study arms may not be valid. Subsequent causal inference derived from statistical analysis of these probability distributions may also not be valid, leading to conclusions that may be wrong.

Randomization procedures are based on the probability of assigning an enrollee to a study arm. Those that are commonly used in clinical trials are divided into four categories: conventional randomization, treatment-adaptive randomization, covariate-adaptive randomization, and response-adaptive randomization. We will discuss the first and last of these in depth.

Conventional randomization encompasses several techniques including simple (complete) randomization, stratified randomization, and cluster randomization. These are discussed in the following section.

Both conventional and adaptive randomization techniques may result in severe imbalances in assignment to study arms. In conventional randomization the likelihood of study arm assignment imbalance is greater in smaller studies. Imbalances in

assignment may reduce study power to the point where hypothesis testing becomes difficult if not impossible, compromising study internal validity.

Conventional randomization

Introduction

In all conventional randomization techniques the probability of randomization to an individual treatment arm is a fixed constant. Consequently, in all conventional randomization techniques the study arm assignment codes can be prepared before the study begins. Commonly used conventional randomization techniques include simple randomization, stratified randomization, cluster randomization, and block randomization.

- Simple randomization

Simple randomization is the most widely used randomization techniques in clinical trials. Assuming equal variances between treatment arms, it is most efficient and statistically powerful technique as well. Compared to other techniques of randomization, fewer total study subjects will need to be enrolled in order to detect a clinically meaningful difference in outcome between study arms. It is ethical in that all enrolled subjects have an equal probability of being exposed to possible toxicities and benefits[34-36]. A probability of assignment other than 0.5 (*a priori* decisions to assign more subjects to one study arm than another) can be made in advance.

Treatment imbalances (assignment of more subjects to one study arm than another) may occur by chance alone. The likelihood of imbalance increases as total sample size

decreases. The likelihood of treatment imbalances may be calculated for any sample size; details may be found in Rosenberger et al[37, 38].

- Stratified randomization

Simple randomization may not assure balance between treatment groups, an especially important problem when clinically meaningful covariates are not equally balanced between study arms. Stratified randomization is often used to reduce study arm imbalances in important covariates. In stratified randomization, the target population is divided into strata based on covariates thought (or known) to be important in outcome, e.g. gender, age, disease severity, study center. Within each formed stratum, a simple randomization is performed. As with simple randomization, imbalances in non-stratifying but possibly important characteristics may occur by chance alone. If there are a large number of strata, treatment balance (an equal number of subjects assigned to each study arm) may be challenging, especially if total sample size is small[39]. This imbalance will decrease the power of statistical analyses.

- Cluster randomization

In some clinical trials, the unit of randomization may be larger than the individual. Randomization of a group of individuals is known as *cluster randomization*. This technique is commonly used in community or school-based interventions where randomization of individuals is not possible. For example, when assessing educational interventions, all of the children in a single class will be randomized to the same cluster. Likewise, the efficacy of community health interventions (e.g. stop smoking or weight loss campaigns) is usually assessed at the community (cluster) level. As with the other

randomization techniques discussed above, chance imbalances in study arm assignment may occur. Calculation of the likelihood of random imbalances is similar to that in simple randomization[32]. The statistical assessment of data collected from cluster randomized studies differs substantially from studies with simple randomization, since both cluster and individual specific covariates must be taken into account during analysis. Intra-cluster variability often decreases statistical efficiency and complicates statistical analysis.

Adaptive randomization

Introduction

Adaptive randomization implies that the probability of an enrolled subject being assigned to a particular study arm varies over time. Covariate adaptive randomizations are used to minimize inequalities or imbalances in the ratio of known covariates in the study arms. In response-adaptive randomization and covariate-adaptive randomization, assignment algorithms are generated in real time. This is because the treatment arm assignment is based on the distribution of enrollee-specific covariates or on the responses up until the time at which randomization occurs. Response-adaptive randomization is primarily motivated by ethical considerations, as it allocates enrolled subjects to study arms with lower toxicity and higher efficacy.

Here we discuss four types of adaptive randomization in depth: co-variate adaptive randomization, and response-adaptive randomization which includes play-the-winner model, randomized play-the-winner model, and optimal randomized play-the-winner

model. Other techniques of response-adaptive randomization include Efron's Biased-Coin Model, Lachin Urn Model, and Friedman-Wei's Urn Model. For large sample sizes, the last two models approximate simple randomization. Details may be found in Chow and Chang pp. 48-51[32]. Most adaptive randomization techniques are used with dichotomous outcomes, but have been adapted to instances when more than 2 outcomes are possible (see below).

- Covariate-adaptive randomization

Covariate-adaptive randomization, also called adaptive stratification, is used to minimize imbalances in known covariates between study arms. The probability of allocation to a specific study arm changes over time, based on cumulative information about covariates in previous enrollees and their treatment assignment[32]. Commonly used models include Zelen's, Pocock-Simon, Wei's marginal urn, Atkinson optimal, and minimization[32]. The primary reason to use covariate-adaptive randomization is to increase the likelihood of balance of important covariates, especially in studies with small sample sizes.

- Response-adaptive randomization

In response-adaptive randomization, the likelihood of allocation to a study arm depends on the response (outcome) of previously enrolled subjects. Its purpose is to provide study enrollees with access to the more efficacious and/or less toxic treatment arm, based on accumulated knowledge. The cornerstone of medical ethics known as benevolence is best served by response-adaptive randomization. Commonly used response-adaptive randomization techniques include play-the-winner, randomized play-

the-winner, Rosenberger's optimization model, Bandit model, and the optimal model with finite population[32].

- Play-the-winner model

This model is most easily applied when there are two treatment arms with two possible outcomes (success or failure). The previously enrolled subjects' outcome must be known before the next patient is randomized. If an enrollee responds favorably to one treatment arm, the following enrollee is assigned to the same arm. If the outcome assessment of the previously enrolled patient is not available, the treatment assignment of the next enrollee is based on either the last known favorable outcome treatment assignment, or may be done randomly. This model obviously lacks randomness since treatment arm assignment is not done randomly.

- Randomized play-the-winner model

Randomized play-the-winner is also most easily applied when there are two treatment arms with two possible outcomes. Again, the previously enrolled subjects' outcome must be known before the next patient is randomized. Let the treatment arms be denoted as A and B. At the beginning of the trial, an urn containing equal numbers of A and B balls is created. At subject recruitment a ball is drawn, treatment is assigned to the arm of that letter, and the ball is replaced. When that study subject's outcome is known, the balls in the urn are updated. If the subject was assigned to treatment A and it was successful, additional A balls are added to the urn. Consequently, the proportion of balls with the letter of the more successful treatment arm is increased. Conversely, Randomized Play-the-Winner models can be used to minimize toxicity or other adverse

events. If a subject is recruited, assigned to treatment A, and this subject develops toxicity, a proportion of letter A balls may be withdrawn from the urn. The treatment arm assignment of the next patient is based on randomization but the probability of assignment changes as the contents of the urn are changed .

- Optimal randomized play-the-winner (Rosenberger's optimization model)

The basic concept of adaptive designs is to weight treatment arm allocations based on the response history of previously enrolled study subjects, giving a future subject a greater than 50% probability of receiving a more effective treatment. Optimal randomized play-the-winner models seek to minimize the number of treatment failures. In this technique, the proportion of successes and failures of each treatment arm are calculated after each subject's outcome is known. Using relative risk, odds ratio, or proportion difference statistics, an optimal assignment of the prospectively enrolled subject is calculated. Details may be found in Chow and Chang[40].

Adaptive models for non-dichotomous outcomes

Adaptive randomization techniques have been adapted for ordinal and continuous outcomes, including survival data[32]. The most commonly used technique for ordinal data is a modification of the Randomized Play-the-Winner model. For continuous outcomes with normal distribution, the most widely used method involves calculation of the difference in outcome from a population mean. Study arm assignment probability is based on allocating more subjects to the arm whose outcome difference is maximal (in a favorable direction).

Problems with adaptive randomization

- Accrual bias

With adaptive randomization, earlier enrolled subjects have a higher probability of being assigned to less efficacious or more toxic treatments. Knowledgeable subjects may wish to delay their enrollment until later in the study to maximize their chances of being assigned to a more effective or less toxic intervention.

- Selection bias

Selection bias occurs when allocation concealment is lost and an investigator is able to guess into which study arm a patient will be enrolled. Based on the assumed study arm assignment, the investigator may surreptitiously choose to enroll a specific study subject at a specific time on the assumption that the subject is best suited for the particular treatment in question. The amount of selection bias expected for various randomization models can be calculated; all equations contain values for the expected number of patient enrolled, block size, and the number of patients per block. [32]

- Additional statistical concerns with adaptive designs

Statistical inference is more challenging when analyzing data collected using non-randomized assignment procedures. In general, for a given sample size, statistical power is decreased for adaptive compared to simple randomized study designs. In general, non- simple randomized study assignment will require greater number of enrolled patients to reach an equivalent level of study power, compared to studies in which subjects are assigned to treatment arms completely randomly.

Combining Phase IIb and Phase III studies

A seamless Phase IIb/ III trial is a single clinical trial that seeks to simultaneously answer questions usually answered in separate Phase IIb and Phase III studies. Seamless trials are normally divided into two phases, the *learning phase* which is equivalent to the Phase II study and the *confirmatory phase*, whose objectives are similar to traditional Phase III studies. Seamless designs require fewer total patients to answer study questions compared to trials divided into separate Phase II and Phase III entities because subjects enrolled in the Phase IIb (learning phase) are included in the Phase III (confirmatory phase) studies. Compared to traditional separated Phase IIb and Phase III studies, seamless designs are more efficient and therefore more ethical, as fewer total subjects will need to be exposed to possible futile or toxic therapies during the study of treatment effect. Conversely, clear superiority of a treatment effect may lead to early stopping with fewer patients exposed to placebos or less effective therapies. Finally, the data collected during the learning and confirmatory phases are combined in statistical analysis. Statistical testing and power calculations differ compared to separated studies; details may be found in Chow and Chang p. 133-141. [32]

There are several types of seamless adaptive designs. The three used most frequently are studies with

- A fixed number of treatment arms. Study endpoints may include stopping early for futility, stopping early for futility informed by biomarkers, and stopping early for futility or efficacy with sample size re-estimation

- A flexible number of treatment arms (Drop-the-Loser, discussed below). This is often combined with response-adaptive randomization
- Population adaptations. The target population may be different in the learning and confirmatory phases. The patient groups are often correlated, i.e. they share clinical characteristics or genetic markers.

Combinations of the last two are common and are discussed in depth in the thesis chapter (below) entitled *Adaptive designs in clinical trials of children with retinopathy negative cerebral malaria*.

Drop-the-loser adaptive design

In practice, adaptive designs termed *drop-the-losers* are most often used when combining Phase IIb and Phase III studies into a single trial. This design has learning and confirmatory phases with a decision point where data collected in the learning phase informs the selection of interventions in the confirmatory phase.

In the learning phase, investigators administer an intervention (often a drug) typically at varying doses or in various combinations with other interventions. A control (placebo) is also administered during the learning phase. At the interim, unblinded data on patient responses (clinical, biomarker) are analyzed. The intervention group with the best outcome (often continuous, e.g. proportionate change in a biomarker) and the control group are retained. All other arms are dropped during the confirmatory phase. Details of hypothesis testing used in drop-the-loser adaptive designs may be found in Chow and Chang p. 139-142. [32]

Summary of seamless adaptive designs

Seamless adaptive designs are both economical and ethical, often shortening the time to bring effective therapies into clinical practice, as well as decreasing the number of study subjects exposed to non-efficacious or toxic therapies. Statistical modifications are necessary when combining phase IIb and Phase III studies.

An additional challenge of adaptive seamless designs (as with all study interventions) is the length of time that may be necessary for an enrollee to reach a study endpoint, and that biomarkers are often not equivalent to clinical effect. For example, anticonvulsants may be administered as adjunctive therapy in acutely ill children in an effort to decrease the rate of post-discharge epilepsy. As seizures may not appear for weeks, months, or years post-discharge, they cannot be efficiently used as a study endpoint in a Phase IIb study. Therefore the biomarker of acute decrease in epileptiform activity on electroencephalogram may be substituted for a long term patient outcome in the learning phase, as this Phase IIb study endpoint can be reached relatively quickly. But it may or may not be true that acute epileptiform on EEG during illness is causally related to the development of epilepsy.

Combining Phase IIb and Phase III intervention research is unwise if the data derived from the combined study is unable to answer the question: Is drug A better than drug B (or placebo) and what is its optimal dose?

Moving target populations

In any clinical trial, subject enrollment is constrained by inclusion and exclusion criteria. Study subjects who meet all of the inclusion criteria (and none of the exclusion criteria) are referred to as the *target population*. Inclusion and exclusion criteria can be used to enrich a study, enrolling subjects more likely to benefit from the intervention under evaluation.

Overall study hypotheses should relate to measured endpoints. Endpoints may be a clinical response, survival, lack of morbidity, or rates of complications of therapy. Study endpoints in the target population, whether continuous or dichotomous, can be denoted as a mean and standard deviation (μ and σ, respectively). Most clinical trials compare an intervention and a control (active, standard of care, placebo), and the *effect size* of the test treatment (adjusted for the standard deviation) is calculated as

$$\frac{\mu T - \mu C}{\sigma}$$

where μT is the population mean for the intervention arm and μC the population mean for the control arm [32]. Statistical inference is made by evaluating the effect size of the active treatment (compared with the control arm) per unit standard deviation (σ) within the target population. The effect of the intervention in the treatment arm is considered significantly different than that in the placebo arm if this fraction is greater than a pre-defined critical value. Given a fixed standard deviation of outcome means, a larger difference in mean outcome between study arms is more likely to show that the treatments are statistically significantly different from one another. Conversely, given a fixed difference in study arm outcome means, a smaller target population outcome

standard deviation (outcome variability) is more likely to show a statistically significant difference between treatments.

During the performance of a clinical trial, the inclusion and exclusion criteria are often modified after study enrollment begins. This is usually done for logistical reasons, when study enrollment numbers fall short of projections. In this case, enrollment criteria are often relaxed by liberalizing inclusion criteria, decreasing the number of exclusion criteria, or both simultaneously. Alternatively, new drug safety information may become available in the course of the trial, leading to more limiting inclusion criteria, so that study subject protection is maximized, though this makes enrollment more difficult. Modifications made after a trial has begun are known as *protocol amendments*. The post-amendment study population is referred to as the *actual* study population, and may vary substantially from the original study target population. If the differences between the actual and original target populations vary dramatically, a totally different trial may result. Multiple protocol amendments in inclusion and exclusion criteria lead to a *moving target population* which may lead to challenges in statistical and causal inference, often due to inflation of variability in outcome (σ) in the outcome *vs.* the target population.

Although appropriate statistical procedures can be used to control for sources of study population variation, the sources of variation in general must be known [40] (e.g. when a new laboratory test is substituted for an old laboratory test, reference standards can be analyzed to evaluate the differing variances of the two procedures. Population differences are usually more difficult to assess.). When enrollment criteria are changed, the way the new study population will react to the treatment *vs.* standard therapies is unknown and cannot readily be estimated. Changing inclusion criteria may lead to bias

and unexpected and uncontrolled sources of variation, referred to as *random error*.[32] Random error can lead to statistical inference and study conclusions derived from data collected from the actual population that may not hold within the original target population.

Liberalization of subject enrollment criteria often also introduces increased variation into the data collected in the trial. To show a statistically significant difference between study arms, a greater difference in population endpoint means (treatment *vs.* control) will be necessary, if variability (σ) in outcomes within the post-amendment study population has increased. The increased variability (σ) and change in outcome mean response (denoted as μ+ε, where ε refers to random error) can be compensated for by calculation of a sensitivity index, which estimates the change in effect size between the actual study population and the original study target population. Modifications have been made for both continuous and binary outcome data[41]. If ε is not or cannot be accurately estimated, the validity of statistical inference towards the original target population when drawn from calculations on data collected from the study population will be unknown. An example can be drawn from clinical trials in patients with malaria. Most investigators will use a positive malaria smear as necessary to diagnose active malaria, and necessary to enroll subjects in therapies targeting acute malarial illness. However, due to the limitations of microscopy (need for electricity, skilled microscopists), some centers may instead use a positive malaria rapid diagnostic test (RDT) for disease diagnosis. Malaria RDTs will be positive in patients with asymptomatic parasitemia and in subjects who have had malaria in the last 3 weeks, even in those who have been cured. Therefore, if a study begins by using a positive

smear as an enrollment criterion in the target population, but then due to slow enrollment or loss of expertise in malaria microscopy switches to a positive RDT as sufficient to enroll subjects (now in the study population), outcomes will likely have increased variability as the study population will be more variable than the original target population.

After modifications are made to enrollment criteria, the original study sample size may not be sufficient to detect a clinically significant difference in the treatment effect in the two study arms. Usually, study power is decreased when the difference in mean response is decreased or subject variability in response to the primary study endpoint is increased. Before changing enrollment criteria, a recalculation of sample size is necessary to be sure that the new study (evaluating the actual study population) will be feasible. An adjustment factor for sample size, R, can be calculated for both continuous and binary outcome data[40]. If the change in variability in outcome between the target and study populations is unknown, the new sample size may not be able to be accurately calculated.

Inflation of sample size estimates or decreases in study power may make a post-amendment study unfeasible. Amended studies that are not expected to be able to answer the clinical research question under evaluation are not ethical.

Potential uses of adaptive designs in clinical trials of children with retinopathy negative cerebral malaria

This K23 application assesses for viral and bacterial co-infection and non-convulsive (electrographic only) seizures in children in two clinical groups: retinopathy negative cerebral malaria, and acute coma without malaria parasitemia (acute aparasitemic coma (AAC)). It assesses whether there are admission clinical or laboratory features that can be used to identify patients at increased risk of one or more of these co-morbidities. We anticipate that the study results will lead to one or more clinical trials of adjunctive antiviral, antibiotic, and/ or anticonvulsant therapies in children with one or both of these clinical syndromes.

Adaptive study design is attractive to investigators as it allows flexibility and reflects normal clinical care. Ethical considerations also guide our attraction to adaptive designs, particularly adaptive randomization, though the disadvantages (when study arm size is unbalanced the need to recruit more patients to reach a given level of statistical significance, given a known treatment effect, compared to study designs where the number of subjects in each arm is balanced) would likely outweigh the advantages. Malaria incidence, in general, has been decreasing in Malawi and the need to optimize study power given a fixed number of possible enrollees is a crucial study design concern. As the adjunctive therapies in question have never been tested in these target populations, we expect that adaptive designs will be used in our upcoming trials, most likely combining Phase IIb and Phase III studies. Here we consider 3 types of adaptive designs and their possible impact on our upcoming research.

Adaptive randomization

We believe it likely that a clinical trial of adjunctive acyclovir plus artesunate will be performed in children with either retinopathy negative cerebral malaria or acute aparasitemic coma. Artemesinin based therapies, used to treat malaria, have weak broad spectrum antiviral effects. When combined with a stronger antiviral agent such as acyclovir, the two drugs may act synergistically.

Acyclovir has a low but non-zero risk of renal toxicity. The effect on kidney function of sequestration of parasitized erythrocytes in renal vasculature added to acyclovir is unknown. Therefore, this therapeutic combination has potential advantages (antiviral action) and disadvantages (renal toxicity). This results in clinical equipoise, making our planned intervention study arm more likely to be exposed to a more effective but potentially more toxic therapy, compared to those randomized to placebo. The adaptive study design most likely to be used in this scenario is a combination Phase IIB and Phase III study, as discussed in depth below. Nevertheless, I present the following theoretical argument about adaptive randomization.

Assuming our trial's target population is children with retinopathy negative cerebral malaria at highest risk of viral co-infection (as determined by multivariate analysis of the data gathered in the R21 and K23), the two study arms would be artesunate alone versus artesunate + acyclovir. Renal function would be monitored periodically throughout admission. Spinal fluid would be sampled at admission and Day 3 and analyzed for viral load (see Combined Phase IIb and Phase III studies, below). The study's primary endpoint would be survival to hospital discharge and secondary

endpoints would be the presence of neurologic morbidity (cognitive, motor, epilepsy, developmental) at discharge and 6 months post-randomization.

I discuss the use of a randomized play the winner model, as it is a combination maximizing harm (or minimizing exposure to potential toxicity) while maintaining an element or randomization. Let the estimated sample size be N. We will place N/2 balls labeled Artesunate and N/2 balls labeled Artesumate+Acyclovir in an urn. Subjects would be randomized into one of the 2 study arms by random selection of a ball from the urn. The ball will be returned to the urn once selected. The primary outcome of mortality would be used to guide a randomized play-the-winner model. A study subject will be randomized to one of the 2 arms and followed until discharge. If the enrollee survives, N/10 additional balls from the same the treatment arm will be added to the urn. (Ten is the usual divisor in randomized play the winner models, but any divisor may be used.) If the subject has a severe adverse event (particularly related to renal toxicity) and the Data and Safety Monitoring Committee feels the trial can continue, N/10 balls from the same arm to which the patient with the adverse event was randomized will be withdrawn from the urn. The next enrollee will be randomized by random selection of a ball from the urn with replacement. This randomized play the winner model will increase the number of children who reach the primary endpoint (survival to discharge) while minimizing severe adverse events related to renal toxicity. This model preserves a measure of randomness while maximizing benefit and minimizing harm to study subjects. Nevertheless, due to difficulties in recruitment of children with cerebral malaria in recent years, it is unlikely it will be used in clinical trials of adjunctive medications in children with retinopathy negative cerebral malaria.

Moving target population

One of the Specific Aims of the K23 application is to identify any admission clinical or laboratory features in children with retinopathy negative cerebral malaria or acute aparasitemic coma who are at higher risk of viral or bacterial co-infection or non-convulsive seizures.

For the purposes of argument we assume the K23 will identify one or more clinical characteristics of a child with retinopathy negative cerebral malaria at higher risk of bacterial co-infection, and that co-infection was associated with increased mortality risk. A clinical trial of adjunctive broad spectrum antibiotics (likely ceftriaxone) would be logical in this target population. Enrollment inclusion and exclusion criteria could be used to enrich the target population, increasing the odds that children with bacterial co-infection would be enrolled. The primary endpoint is defined as mortality at hospital discharge. The two study treatment arms would be artesunate *vs.* artesunate+ceftriaxone.

If interim analysis performed after 50% of the estimated sample size was enrolled revealed a clear benefit to adjunctive antibiotics, enrollment criteria could be liberalized to children with retinopathy negative cerebral malaria who were excluded from the original study target population. For example, in current studies in place at the Blantyre Malaria Project a positive malaria smear must be present for study enrollment. If inclusion criteria offered the alternative of a positive malaria rapid diagnostic test (rather than a positive thick smear) this would likely increase heterogeneity, as rapid diagnostic tests may remain positive up to 2 weeks after successful treatment with antimalarials.

Differentiating recrudescent, cured, and reinfection is impossible using these tests. By using a rapid diagnostic test rather than a positive smear, this would likely increase outcome variability and require an increase in sample size to achieve comparable statistical power compared to the original study on the target population. A sensitivity index could be calculated[41], using extrapolation from studies of adjunctive antibiotic therapy in children in coma without malaria parasitemia whose bacteremia status is unknown. If the inflation in sample size or decrease in power were too great, the enrollment criteria could not be liberalized, on the ethical grounds that the new study population could not be adequately evaluated with the post-protocol amendment study population.

Additional possible changes in enrollment criteria that would change the study population include using real time polymerase chain reaction to diagnose active malaria infection. If inclusion criteria were changed from a positive smear to a positive PCR, children with subpatent infections (negative smear, positive PCR) would be enrolled. This population that may react differently to the intervention, compared to those with a positive smear. This strategy is not applicable to the particular studies in question, and is for illustrative purposes.

Combining Phase IIb and Phase III clinical trials

As explained above, the combination of acyclovir and artesunate has not been previously tested in children with malaria. Though the effects of parasitized erythrocyte sequestration on renal function are usually minimal in children, acyclovir is eliminated by

renal filtration and excretion, and potential interactions exist that might increase renal toxicity.

A trial of adjunctive artesunate + intravenous acyclovir versus artesunate alone would best be accomplished by combining a Phase IIb and Phase III study, using children with retinopathy negative CM and a viral co-infection as our study target population. The learning phase of this study would evaluate for possible pharmacokinetic and pharmacodynamic interactions between the two drugs. Pharmacokinetics could be studied by drawing plasma level of acyclovir at known time points, comparing the elimination of the drug to historical controls without malaria. If spinal fluid were sampled at admission and 72 hours post-admission, a proportionate reduction in viral load could be used as the endpoint for the learning phase. Data concerning subject mortality and neurologic morbidity (at discharge and 6 months post-discharge) would be collected in the learning phase, and added to data gathered in the confirmation phase, where mortality and morbidity information would be the primary study endpoints. This would decrease the total number of subjects needed to be enrolled in this trial, compared to separate Phase IIb and Phase III trials of these therapies.

For example, if we perform a separate Phase IIb study and use percent reduction in viral load was our study endpoint, we would have very low power unless the effect size (proportionate reduction in viral load) were very large. We use historical controls as it is known that viral nucleic acid copy numbers do not decrease without specific antiviral interventions. For argument, we assume we use historical controls, allocating all patients to artesunate plus acyclovir in the Phase IIB study.

We assume (based on historical data) that we would enroll 30 children over 2 years. All children would receive acyclovir + artesunate. If the average baseline viral load of a study subject was 10,000 copies per microliter (SD= 5000 copies/microliter) at enrollment and historically there was no change in viral load after 72 hours, study power would be (assuming a 1 sample, 2 tailed test and $\alpha = 0.05$):

Table 10: Study power for theoretical Phase IIb study

Baseline viral load (copies per µL)(historical)	Intervention viral load at 72 hours (copies per µL)	Proportionate reduction in viral load	Effect size for difference[42]	Sample size (per arm)	Standard deviation (copies per µL)	Study Power
10000	9000	0.10	0.2	30	5000	0.19
10000	8000	0.20	0.4	30	5000	0.46
10000	7000	0.30	0.6	30	5000	0.75
10000	6000	0.40	0.8	30	5000	0.93

Therefore we would need to have a 40% reduction in viral load to have sufficient study power to detect this difference from historical controls; this proportionate reduction is not clinically likely. This very large effect size would be

If we use mortality data only derived from the confirmation phase (and do not include children enrolled during the learning phase, study power is low (Table 12). If we assume that we will enroll 15 children per year in each arm per year, during the 5 year phase III study we will enroll 75 children per study arm. Assuming mortality in the artesunate-only arm is 20% (the historical mortality rate of the Blantyre Malaria Project), with 2 sided test, $\alpha = 0.05$, study power is:

Table 11: Study power for theoretical Phase III study

Artesunate only mortality rate (%)	Artesunate plus acyclovir mortality rate (%)	Sample size (per arm)	Study power (%)
20	15	75	0.12
20	10	75	0.40
20	5	75	0.80
20	3	75	0.91

Therefore, given a fixed sample size we would therefore need a very large mortality rate difference (a 75% reduction) in order to have at least 80% chance of detecting it, if we separated Phase IIb and Phase III studies.

If, however, we planned ahead and randomized children in both the learning and confirmation phases to either artesunate or artesunate + acyclovir, used only

pharmacokinetic data from the learning phase, and combine outcome (mortality) data in the learning (Phase IIb) and confirmation (Phase III) phases, our study power changes. Assuming the learning + confirmation phases are 7 years and we enroll 15 children per year in each arm, we will enroll 105 children per study arm. Assuming mortality in the artesunate only arm is 20% (the historical mortality rate of the Blantyre Malaria Project), with a 2 sided test, $\alpha = 0.05$

Table 12: Study power for theoretical combined Phase IIb and Phase III study

Artesunate only mortality rate (%)	Acyclovir plus acyclovir mortality rate (%)	Sample size (per arm)	Study Power (%)
20	15	105	0.16
20	10	105	0.53
20	7	105	0.80
20	5	105	0.91

Though these increases are modest, this does prove that combining Phase IIb and Phase III studies improves study efficiency, increasing the total number of subjects able to be used in power estimates and therefore, statistical inference.

Conclusion concerning the future of adaptive clinical trial designs in the study of children with retinopathy negative cerebral malaria

Whether adaptive clinical trial designs are more ethical compared to traditional designs is debatable. Adaptive designs may lead to either gains or loss of trial efficiency, though in general modifications made after enrollment has begun lead to loss of efficiency, study power, and necessitate an increase in total study sample size. Of all the adaptive study designs discussed, combining Phase IIb and Phase III studies in clinical trials of adjunctive therapy in children with retinopathy negative cerebral malaria are the most likely.

THESIS CONCLUSION

Clinical and epidemiological retrospective studies performed thus far have provided limited insight as to whether the coma in children with cerebral malaria is due to acute malarial infection or a non-malarial etiology. This may be a reflection of fact that all these studies were the secondary data analyses; the parent study was not designed to test hypotheses concerning the underlying pathophysiology of retinopathy negative CM. Investigation of infectious pathogens associated with this condition through the funded R21 and proposed K23 mechanisms may provide greater insight into disease pathophysiology. It is likely that underlying disease etiologies will be better understood through these studies, rather than through clinical trials targeting viral co-infections. Adaptive clinical trial designs may need to be used in future intervention studies targeting non-malarial coma etiologies in children with retinopathy negative cerebral

malaria, though the most likely adaptive design to be used is a combined Phase IIb and Phase III study.

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