THE NEUROPATHOLOGY OF PEDIATRIC CEREBRAL MALARIA

by

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ABSTRACT

Cerebral Malaria (CM) results from a *Plasmodium falciparum* infection and is characterized by severe neurologic dysfunction, coma and death; it is responsible for the deaths of 1-2 million people annually and affects primarily sub-Saharan African children. Several theories have been proposed regarding the pathogenesis of pediatric CM, but a lack of basic information regarding the neuropathological features of this disease in children, and how these features relate to clinical findings and parasitological data, make it difficult to establish the relative role that any pathological mechanisms might play in CM. The purpose of this study was to determine the nature and extent of the axonal, myelin and endothelial damage and gliosis in pediatric CM by staining brain sections obtained post-mortem using immunohistochemistry (anti-β-Amyloid Precursor Protein, anti-Fibrinogen, anti-Glial Fibrillary Acidic Protein) and neurohistological stains (Hematoxylin and Eosin, Luxol Fast Blue). We also sought to determine if significant associations could be established between neuropathological changes and either retinal pathology or ocular fundus findings observed during life, potential indicators of disease severity and outcome in CM. A series of correlations between neuropathological findings and several clinical parameters were also carried out.

A common finding in the brains of CM patients (n=25) was the presence of parasite sequestration; anoxic neurons and Durck's granulomas were observed rarely or not at all in this group. CM patients differed significantly from malaria patients without neurologic dysfunction (severe malarial anemia, SMA, n=5) and patients dying with comas unrelated to malaria (comas of other causes, COC, n=19) with respect to both the presence and degree of perivascular ring hemorrhages (RH, 18/25 patients) and axonal damage (AD, 24/25 patients) ($p<0.05$). The distribution of these features varied across the CNS of CM patients, but both were greatest in the sub-cortical white matter. No correlation was observed between these features and general parasite sequestration, but positive correlations were observed between these features and the presence and degree of extra-erythrocytic pigment observed histologically (RH, $R=0.402$, $p<0.05$ and AD, $R=0.766$, $p<0.05$), suggesting a role for a parasite-stage specific involvement in the

production of neuropathological changes. The number of RH and extent of AD in the retinas of CM patients correlated with the number and severity of corresponding pathologies in CM brains ($R=0.415$, $p<0.05$ and $R=0.490$, $p<0.05$, respectively), and the patterns of hemorrhage and axonal damage in the brains of CM patients were reflected in their retinas. Together, these findings suggest that retinal pathology reflects brain pathology in CM. Other neuropathological features observed in this study, namely, blood-brain-barrier (BBB) dysfunction (as measured by fibrinogen leakage), and gliosis were common in CM, though both of these features were also common in SMA patients without neurologic dysfunction, suggesting that BBB dysfunction and gliosis may be involved in malaria pathogenesis more generally. As with AD and RH, BBB dysfunction and gliosis were observed in the retinas of CM patients, further suggesting that retinal pathology reflects brain pathology in this disease.

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LIST OF ABBREVIATIONS

AEC	3-Amino-9-Ethylcarbazole
AI	Axonal Injury
AIDS	Acquired Immune Deficiency Syndrome
AVM	Arteriole Venous Malformation
APP	Amyloid Precursor Protein
BBB	Blood Brain Barrier
BCS	Blantyre Coma Score
BRB	Blood Retinal Barrier
BS	Brainstem
CART	Classification and Regression Tree Analysis
CIDR	Cysteine-rich Interdomain Region
CD36	Collagen type-1 Receptor
CER	Cerebellum
CH	Cerebral Hemispheres and Hippocampus
CM	Cerebral Malaria
CNS	Central Nervous System
COC	Comas of other Causes
COCi	Comas of other Causes (infectious)
COCni	Comas of other Causes (non-infectious)
CSA	Chondroitin Sulfate A
CSF	Cerebrospinal Fluid
C+T	Caudate and Thalamus

DBL	Duffy Binding Like Domain
DIC	Disseminated Intravascular Coagulation
EC	Endothelial Cells
ECM	Experimental Cerebral Malaria
FMCM	Fatal Murine Cerebral Malaria
GFAP	Glial Fibrillary Acidic Protein
HA	Hemorrhage Associated
HAS	Heparan Sulfate A
H&E	Hematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HTLV-1	Human Lymphotropic Virus Type-1
HZ	Hemazoin
ICAM-1	Intracellular Adhesion Molecule - 1
IFN	Interferon
IgG	Immunoglobulin-G
IHC	Immunohistochemistry
IL	Interleukin
INOS	Inducible Nitric Oxide Synthase
LFB	Luxol Fast Blue
mAB	Monoclonal Antibody
MCP	Macrophage Chemoattractant Protein
MIP	Macrophage Inflammatory Protein
NCM	Non-Cerebral Malaria

NHA	Non-Hemorrhage Associated
NIH	National Institutes of Health
NO	Nitric Oxide
pAB	Polyclonal Antibody
PE	Parasitized Erythrocytes
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PFEMP-1	*Plasmodium falciparum* Erythrocyte Membrane Protein - 1
PI	Post Infection
PRBC	Parasitized Red Blood Cell
QECH	Queen Elizabeth Central Hospital
ROS	Reactive Oxygen Species
SE	Standard Error
SMA	Severe Malarial Anemia
TNF	Tumor Necrosis Factor
uPAR	Urokinase Plasminogen Activator Receptor
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular Endothelial Growth Factor
WCC	White Cell Count
WHO	World Health Organization
ZO-1	Zonula Occludens-1

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CHAPTER 1

INTRODUCTION

1.1. Malaria – Definition and Incidence

Malaria is an infectious disease caused by any of the four species of the human protozoan pathogen *Plasmodium* (*P.malariae, P.ovale, P.vivax and P.falciparum*), and remains a major health concern in many parts of the underdeveloped world. The World Health Organization (WHO) estimates that between 300 and 500 million people are infected by *Plasmodium* every year, making malaria the most important parasitic disease to affect humans (WHO, 1998). Approximately 90% of malarial infections occur in sub-Saharan Africa with the remainder of the cases concentrated in Southeast Asia, India, Brazil, Sri Lanka, Afghanistan and Columbia. Children are more susceptible to severe malarial disease than adults (see Sections 1.1.1 and 1.2.3.) and account for 90% of the 1.5 to 2.7 million malaria deaths recorded annually (Murphy and Breman, 2001). Despite many efforts at malaria eradication through insecticides (aimed at controlling the *Anophelene* vector), improved malaria prophylaxis, and vaccine development (Whitty et al., 2002), recent studies show that malaria infections and deaths attributable to malaria are on the rise (Snow et al., 2001).

1.1.1. Transmission to Humans

Malaria is primarily an intravascular disorder. As a result, *Plasmodium* can only be transmitted to humans through a few specific routes: intravenous drug abuse (Chau et al., 2002), blood transfusions (Choudhury and Phadke, 2001), congenitally (Ahmed et al., 1998), and by the bite of an infected mosquito (Fontenille and Lochouarn, 1999). The latter mode of transmission is the most common. In sub-Saharan Africa, five mosquito vectors of *Plasmodium* exist: *Anopheles gambiae, A.arabiensis, A.funestus, A.nili and A.moucheti* (Fontenille and Lochouarn, 1999), with females of the species *An.gambiae* being the primary vector of *P.falciparum*, the species of *Plasmodium* that results in fatal forms of the disease (Mbogo et al., 2003). The transmission intensity in a given region is

dependent on several factors including human factors such as farming (Ijumba and Lindsay, 2001; Ijumba et al., 2002; Somboon et al., 1998) and deforestation (Manga et al., 1995), and climatic and geographical factors such as altitude (Hay et al., 2002), temperature (Abeku et al., 2003; Bi et al., 2003), and rainfall (Hay et al., 2001; Koram et al., 2000; Vanderwal and Paulton, 2000), among many others. Interestingly, the transmission intensity of malaria affects the clinical spectrum of this disease. If the transmission intensity of malaria within an area is high, individuals are more likely to encounter the pathogen, develop partial clinical immunity, and be less symptomatic or have asymptomatic infections as they get older. Consequently, it appears that in areas where transmission intensity is high (i.e. sub-Saharan Africa) those at most risk of developing severe malaria are children who have had less opportunity to be exposed to *Plasmodium*, explaining why complicated forms of malaria such as cerebral malaria (CM) or severe malarial anemia (SMA) (see Sections 1.1.4. and 1.2.) usually only appear in children under the age of one year in malaria endemic zones (Snow and Marsh, 2002; Snow et al., 1997). In areas where transmission intensity is low (Southeast Asia) severe forms of the disease are more common in non-immune adults. Severe malarial disease is therefore a feature of either African children or Southeast Asian adults, and the majority of the clinical and pathological studies have focused on the disease features in these two groups. Interestingly, although the clinical presentation of malaria is similar between these two groups, there is a growing body of evidence suggesting that clinical signs and underlying pathogenetic mechanisms of severe forms of malaria such as CM are very different (see Section 1.2.3.).

1.1.2. Parasite Species

Of the four species of *Plasmodium*, *P.falciparum* and *P.vivax* are the only species that cause severe disease (Miller et al., 2002). *P.falciparum* is the more pathogenic of the two species, and is responsible for 90% of malaria mortalities (WHO, 2000). Both *P.vivax* and *P.falciparum* are able to produce severe malarial anemia (see Section 1.1.4.), but *P.falciparum* is also capable of producing other severe complications such as cerebral malaria, hypoglycemia, metabolic acidosis and respiratory distress (Weatherall et al.,

2002). The pathogenicity of *P.falciparum* is related to its success biologically. The lifecycle of *Plasmodium* is spent, for the most part, within erythrocytes where it can remain relatively hidden from a host immune response. *P.falciparum* and *P.vivax* differ in that *P.vivax* is limited to the types of erythrocytes that it is able to invade during the erythrocytic stage of its lifecycle (see Section 1.1.3), namely young reticulocytes that are Duffy group positive, while *P.falciparum* is not (Mons, 1990). This allows *P.falciparum* to infect more erythrocytes than *P.vivax*, resulting in a higher number of infected erythrocytes, and in most cases, more severe disease. In addition, *P.falciparum* is the only species of *Plasmodium* that has evolved to avoid splenic destruction by modifying the surface of infected erythrocytes for adherence to vascular endothelium within tissues (Bellamy et al., 1998; Chotivanich et al., 2002; Miller et al., 1994), a process termed microvascular sequestration, argued to be a starting point for some forms of severe disease such as cerebral malaria (see Section 1.3.1 and 1.3.1.1).

1.1.3. Lifecycle of *Plasmodium falciparum*

The life cycle of *P.falciparum* is complex (Figure 1). The initial parasite forms (termed sporozoites) first enter the body through the bite of a female mosquito of the *Anopheles* genus. Upon entering the circulation, the sporozoites selectively invade hepatocytes and undergo transformation into primary exoerythrocytic forms in which they remain for about a week. Merozoites, as they are termed following release from hepatocytes, then invade erythrocytes where they mature into ring and trophozoite stages and replicate asexually to form schizonts. Interestingly, erythrocytes infected with early-stage parasites can be found in the peripheral blood whereas late-stage parasites (trophozoites and schizonts) develop within the vasculature of tissues (see Section 1.3.1). After the schizont stage, merozoites burst from the erythrocytes and infect other red blood cells so that the erythrocytic cycle may continue (Hamer and Wyler, 1993).

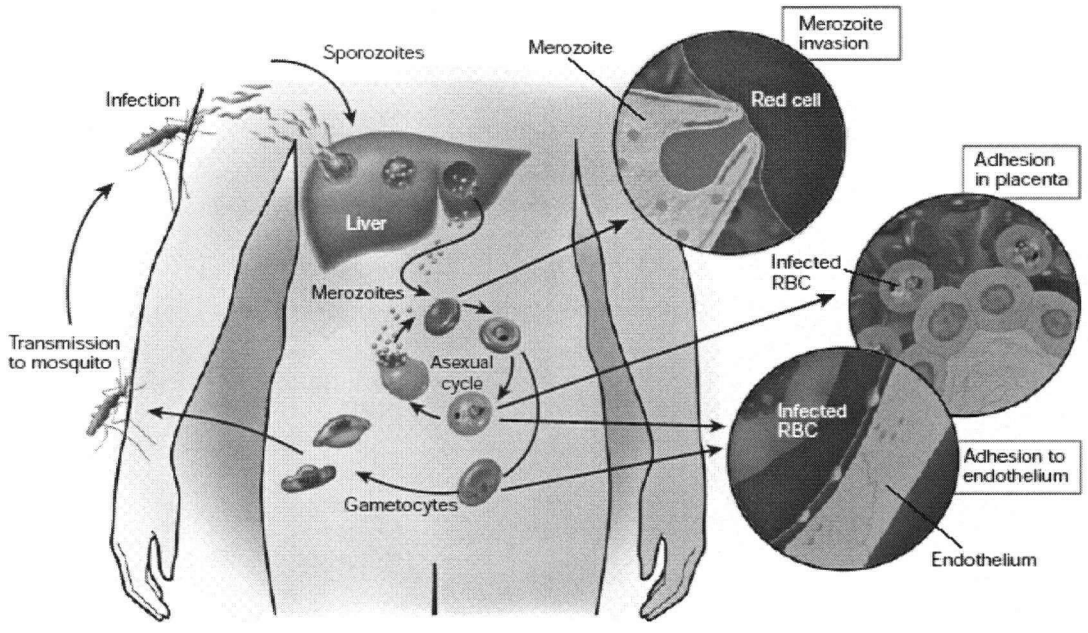

Figure 1. The lifecycle of *Plasmodium falciparum*. (Miller et al., 2002)

1.1.4. Clinical Features: From Mild to Severe Disease

Individual patients infected with *P.falciparum* may present with a diverse range of clinical symptoms ranging from asymptomatic infection to severe illness and death. Symptoms may appear on average 12 days (but occasionally six months or more) after inoculation of sporozoites into the bloodstream (Weatherall et al., 2002). Mild or uncomplicated malarial infections, which are observed more typically in immune-adults, produce symptoms such as fevers, rigors, nausea, headaches, vomiting, diarrhea and abdominal pain (Weatherall et al., 2002). These symptoms closely follow parasite development and the cyclic production of cytokines during parasite rupture (McGuire et al., 1998; Mordmuller et al., 1997). In some situations, and especially in children, human malaria may also progress to severe and life-threatening forms. To alert primary care physicians and researchers of the signs and symptoms associated with progression to fatal disease resulting from a *P.falciparum* infection, the WHO has selected 10 clinical manifestations and laboratory findings common to severe malaria (WHO, 2000). These parameters include: prostration, impaired consciousness, respiratory distress (acidotic breathing), multiple convulsions, circulatory collapse, pulmonary edema (radiological),

bleeding, jaundice, hemoglobinuria, and severe anemia. The relative prognostic significance and prevalence of these individual parameters varies. In a comprehensive study of 1844 Kenyan children diagnosed with malaria, Marsh *et al.* (1995) found that the most prevalent and important indicators of severe disease are coma, severe malarial anemia and respiratory distress, and that 84.4% of malaria deaths could be predicted on the basis of respiratory distress and impaired consciousness alone (Marsh et al., 1995). It is now understood that severe or complicated malaria in children encompasses three distinct but potentially overlapping clinical syndromes: malaria-associated hyperpnea (increased breathing rate and depth of breathing), severe malarial anemia and cerebral malaria (see Section 1.2.) (Newton et al., 1998).

Malaria-associated hyperpnea or respiratory distress is a common sign of severe disease (Crawley et al., 1998; Day et al., 2000; Genton et al., 1997; Maitland et al., 2003; Schellenberg et al., 1999; Varandas et al., 2000; Waller et al., 1995) and may be defined by the presence of any of the following signs: alar flaring, chest recession (intercostal or subcostal), the use of accessory muscles for respiration, or abnormally deep (acidotic) breathing (Marsh et al., 1995). The potential causes of respiratory distress include cardiac failure, direct sequestration of parasitized red blood cells in the lungs and increased central drive toward respiration due to cerebral malaria (Marsh et al., 1996). Evidence indicates, however, that the main cause of respiratory distress in severe malaria is metabolic acidosis. English *et al.* (1996) found respiratory distress to be present in 119 of 350 children with malaria and in 23 of the 30 deaths (relative risk = 6.5, 95% CI), and that deep breathing as an indicator of respiratory distress was sensitive (91%) and specific (83%) for the presence of severe metabolic acidosis (English et al., 1996). In a group of 145 Malawian children admitted to hospital with a diagnosis of malaria, 66 out of 145 (46%) were found to be profoundly acidaemic with a pH less than 7.3 and 72% of the patients who died were acidotic (Taylor et al., 1993). In this study, acidaemic patients had a slower mean respiratory rate and a higher incidence of respiratory rhythm abnormalities than other patients, suggesting that acidaemia is in part the result of inadequate respiratory compensation for metabolic acidosis.

Anemia is a relatively common occurrence in infectious diseases of all types and appears to be a particularly important complication in children infected with *P.falciparum*. The typical case of severe malarial anemia in children is a child presenting with fever, lethargy and decreased hemoglobin concentrations (Maitland and Marsh, 2004). Clinically stable anemia patients without respiratory distress or impaired consciousness have a mortality rate of 1-2%, but the mortality rate increases considerably (10-15%) when an anemic child presents with either coma or respiratory distress also, emphasizing the potential for overlap of clinical findings in cases of severe malaria (Maitland and Marsh, 2004). The underlying cause of anemia in malaria patients is likely to be multifactorial and may include hemolysis, decreased erythropoeitic activity resulting from an overabundance of inflammatory cytokines, erythrophagocytosis of nonparasitized red blood cells, increased splenic destruction of red blood cells (Ekvall, 2003; Nagel, 2002).

1.2. Cerebral Malaria – Definition and Incidence

Cerebral malaria (CM) is the most severe complication of human malaria and is an especially important complication in children. The estimated mortality rate of CM in sub-Saharan African children, where 90% of malarial mortalities originate (see Section 1.1.), is 1.2 and 0.5 cases per 1000 children aged < 5years and 5-9 years respectively (Murphy and Breman, 2001). Cerebral malaria is diagnosed when a child is unconscious and unable to localize pain or has a Blantyre Coma Score (BCS) < 2 (for definition see Section 2.1.) with an asexual parasitemia of any density and no other obvious cause of decreased level of consciousness (e.g. bacterial meningitis, HIV encephalitis) (Molyneux et al., 1989). Cerebral malaria is also restricted to those patients where altered consciousness could not be attributed to convulsions, hypoglycemia, hyperpyrexia, acidosis, severe anemia, or sedative drugs (WHO, 2000).

1.2.1 Clinical and Neurological Features

Cerebral malaria patients usually go into coma after a few days of symptoms, the earliest of which is fever, often occurring 2 hours to 7 days before admission to hospital, with rectal temperatures ranging from 36 to 41 degrees (Molyneux et al., 1989; WHO, 2000). Other initial symptoms before unconsciousness may include headache, malaise, anorexia and diarrhea (Phillips and Solomon, 1990). Jaundice, hepatosplenomegaly, hypoglycemia, and an associated respiratory distress reflective of acidosis may also be present (WHO, 2000). Neurologic signs in children are those of a diffuse encephalopathy with symmetrical upper motor neuron signs and brainstem disturbances including conjugate gaze abnormalities, hypertonic posturing, opisthotonus, and absent abdominal reflexes (Phillips and Solomon, 1990). Convergent spasms, also indicative of an upper brainstem lesion, are also occasionally present along with transient ocular changes, horizontal and vertical nystagmus and sixth nerve palsies (Newton and Warrell, 1998). Seizures are an important finding in CM occurring in 50% of patients. In most cases, seizures precede and precipitate coma and admission to hospital. Convulsions are non-febrile (Newton and Warrell, 1998) and are unassociated with hypoglycemia in strictly defined CM. Convulsions are most often focal motor or generalized tonic-clonic, and may be single or recurrent (WHO, 2000). In some CM patients seizure activity is sub-clinical and may be detectable only by electroencephalogram (Crawley et al., 1996). Mortality in CM patients is between < 5% and 20% dependent on whether this feature is present along with either anemia or respiratory distress (Maitland and Marsh, 2004). Therefore, most children with CM will survive and regain consciousness within 48 to 72 hours of beginning treatment (Newton and Warrell, 1998). It is also well accepted that patients that survive CM usually have a full recovery, with only 6.7% of patients developing neurologic sequelae (WHO, 2000). Neurologic sequelae of cerebral malaria in children include hemiplegia, cortical blindness, ataxia, recurrent seizures, dysphasia, hypotonia, spastic quadriplegia, psychosis, hearing loss, memory impairment and paresthesias (Hamer and Wyler, 1993). A number of studies have shown that the most common sequelae are ataxia (43%), hemiplegia (39%), speech disorders (39%) and blindness (30%) (WHO, 2000). At present, very little is known about why some children

with CM experience deep coma and neurologic dysfunction and go on to recover without apparent sequelae while other children do not.

1.2.2. Ocular Fundus Findings and Disease Outcome

Several ocular fundus abnormalities are observed using either direct or indirect ophthalmoscopy in CM patients that together may constitute a retinopathy unique to CM. These findings include retinal hemorrhages, 'cotton wool' spots, papilledema, retinal whitening and retinal vessel abnormalities (WHO, 2000).

Several studies have identified hemorrhage to be a key ocular fundus finding in CM patients. Retinal hemorrhages were found in 21 of 144 Thai adults (14.5%) with strictly defined cerebral malaria and were significantly associated with several indices of increased disease severity including high parasitemia, schizontaemia, and anemia, though retinal hemorrhages were not indicative of a poor prognosis (Looareesuwan et al., 1983). A study of fundoscopic features in a Malawi group of pediatric CM patients found hemorrhages to be present in 35-40% of children. Multiple hemorrhages (between 1 and 100) could be observed in each retina, and most hemorrhages were round and intraretinal with periretinal hemorrhages and flame-shaped hemorrhages occurring less frequently (Lewallen et al., 1999b). Although associated with severe disease, retinal hemorrhages were also not associated with outcome in this study. Similar findings related to retinal hemorrhage were found in a study of retinal findings in CM and non-cerebral malaria (NCM) pediatric patients in Mali (Schemann et al., 2002) and in a study of adult Indian patients with CM (Kochar et al., 2000). In the Mali study, hemorrhages were more typically a feature of CM (22.9% of CM patients) but were also found in NCM patients (11.8%). Hemorrhage was not associated with mortality in this study. Retinal hemorrhage was not indicative of poor prognosis in Indian adults and retinal hemorrhage was less frequent in CM patients in this group (11.68%). A study examining 735 patients in Malawi, Kenya and the Gambia by direct and indirect opthalmoscope Lewallen *et al.* (1999) defined several abnormal clinical features in the eye including retinal whitening, hemorrhages, unique vessel abnormalities, papilledema and cotton wool spots (Lewallen

et al., 1999a), some of which were significantly associated with a poor prognosis. Cotton wool spots were observed in approximately 5% of African children and papilledema in 8%. Discrete spots of retinal whitening appeared in approximately 30% of African patients. In a study of 141 children with strictly defined cerebral malaria it was found that the relative risk of death in patients with papilloedema was 6.7 times more likely than patients without papilledema and that extramacular retinal whitening was associated with a 2.9 fold increase in the relative risk of dying (Lewallen et al., 1996). Similar assessments of prognostic significance were found in the Mali study of ocular fundus findings in children, observing that exudates, papilledema, and the presence of cotton wool spots were associated with an increased risk of death (Schemann et al., 2002). Therefore, it appears that fundoscopic findings, which are common in CM, are helpful in indicating severity of disease or risk of death. Interestingly, observations have also been made to suggest that the events occurring in the retinas of CM patients, may be reflective of pathogenetic events occurring in the brains of CM patients, explaining why several fundoscopic features are prognostically significant or reflective of severe disease (see Section 1.5.)

1.2.3. Adult and Pediatric Cerebral Malaria

In areas of high transmission intensity (sub-Saharan Africa), CM occurs predominantly in children, while in areas of low transmission (Southeast Asia), the disease occurs more regularly in non-immune adults (see Section 1.1.1). Although some of the clinical features of CM are similar between children and adults (i.e. the presence of seizures and coma), several differences exist between CM in these groups, making it difficult to draw parallels between the pathogenetic mechanisms that may be common to them. In African children, for example, the time from the onset of fever to coma is typically only a few days while in adults the duration may be > 10 days (Phillips and Solomon, 1990). The duration of impaired consciousness to death in Southeast Asian adults is also greater than in African children (48 hours in adults compared to 18 hours in children), and the longer duration from onset of symptoms to coma and to death may account for the fact that adults also appear to have multi-system organ involvement and a

host of other complications that are rare in pediatric CM patients. These features include acute renal failure, jaundice, pulmonary edema, spontaneous bleeding, urinary tract infections, septicemia, and pneumonia (Looareesuwan et al., 1983; Phillips et al., 1986; Phillips and Solomon, 1990). At autopsy, pediatric CM patients typically do not show extra-central nervous system (CNS) pathology, something that is common to adult CM patients (Medana et al., 2001). It also appears that while adult patients are prone to several complications unrelated to malaria, pediatric CM patients are more likely to develop complicating factors reflective of severe malarial disease such as anemia, respiratory distress and hypoglycemia (Phillips and Solomon, 1990). Neurologic signs are also more common in children. Seizures, for example, appear in 82% of children with CM while only 40% of adults have this finding. Brainstem signs are also more common in pediatric patients compared to adults (34% compared to 12%). There are also significant differences in the frequency of some ocular fundus findings between these groups that may be suggestive of more pronounced disease. Retinal hemorrhages which are indicative of disease severity are more common in children than adults (see Section 1.2.2.), and papilledema, an indication of cerebral edema, is less common in adults then children (Davis et al., 1992; Lewallen et al., 1993; Looareesuwan et al., 1983). Lastly, it also appears that mortality and neurologic sequelae in CM are more common in children than adults (Medana et al., 2001).

1.3. Pathogenesis of Cerebral Malaria

Despite the large number of deaths attributed to CM (see Section 1.2.), relatively little is known about the pathogenesis of this disease. Several theories on CM pathogenesis have been proposed and include: mechanical or sequestration, toxin, cytokine, nitric oxide (NO), reactive oxygen species (ROS), permeability and immunological hypotheses (Medana et al., 2001). The key pathological feature of CM is the sequestration of erythrocytes containing mature forms of the parasite in the brain (see Sections 1.3.1. and 1.3.1.1.) and is a starting point in several of the dominant theories aimed at explaining the pathogenesis of CM (see Section 1.3.2).

1.3.1. Microvascular Sequestration of Parasitized Erythrocytes

Early studies on CM pathogenesis showed that during the course of the lifecycle of *P.falciparum* only early-stage parasites (sporozoites and merozoites) were found in the peripheral circulation and that late-stage parasites (trophozoites and ring forms) dropped out of circulation and developed within the deep vascular beds of vital organs including the brain, lung, gut and heart (Turner, 1997). Microvascular sequestration occurs as a part of the *P.*falciparum lifecycle so that the parasite can avoid the splenic destruction that normally occurs in erythrocytes with damaged or altered membranes, as would be the case in a *P.falciparum* infected cell (Kristensson et al., 2002). It is now understood that microvascular sequestration is the result of decreased deformability of the erythrocyte that occurs during parasite development (WHO, 2000) through a ligand-mediated mechanism (see Section 1.3.1.1.), and is a key neuropathological feature of CM (see Section 1.4.1.).

1.3.1.1. Parasite and Host Proteins Mediating Cytoadherence

Electron microscopic studies have shown that parasitized red blood cells (PRBCs) acquired irregularly shaped surface knobs that were not present in normal erythrocytes, and which formed adhesive contact points with endothelial cells. These erythrocytic knobs most often formed about 24 hours post-invasion, and range in diameter from 110-160nm with a base to peak height of 40nm or less (Aikawa et al., 1990; MacPherson et al., 1985; Sherman et al., 1992). The density of knobs on the surface of erythrocytes also seemed to be stage specific with trophozoite containing cells having relatively few knobs and schizonts having a greater number (Gruenberg et al., 1983). The stage-specific nature of the expression of erythrocytic knobs explains why sequestration is a feature of late-stage infected cells.

Biochemical studies indicate that sequestration in microvessels is mediated by a single parasite protein expressed on the surface of erythrocytic knobs, the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is a clonally variant

protein encoded by the large *var* gene family and is important in antigenic variation and immune evasion in addition to sequestration . Several var genes have been sequenced and five categories of Duffy binding-like (DBL) domains (α, β, γ, δ and ε) as well as three clusters of cysteine-rich interdomain regions (CIDR) (α, β, γ) are found on the external side of the erythrocyte while internally a conserved acidic terminal sequence is found (Weatherall et al., 2002). The DBL and CIDR domains from several *P.falciparum* isolates have been observed to bind to a number of host ligands including CD36, ICAM-1, PECAM/CD31, VCAM-1, heparan sulfate (HS), chondroitin sulfate A (CSA) (Flick and Chen, 2004; Miller et al., 2002; Turner et al., 1994). Several of these host-ligands have been found to be upregulated in the course of a malaria infection in response to circulating cytokines such as tumor necrosis factor-alpha (TNF-α) and have been found to bind PfEMP1 *in vitro* (Beeson and Brown, 2002; Turner et al., 1994).

1.3.2. Theories of Cerebral Malaria Pathogenesis

Several theories have been proposed to explain the pathogenesis of CM (Medana et al., 2001). The major theories of CM pathogenesis include the mechanical or sequestration theory (see Section 1.3.2.1), the immunological or cytokine hypothesis (see Section 1.3.2.2.) and the blood-brain-barrier permeability hypothesis (see Section 1.3.2.3.).

1.3.2.1. Mechanical or Sequestration Hypothesis

The more traditional theory known as the 'mechanical theory' or 'sequestration hypothesis' suggests that many of the complications of cerebral malaria result from direct effects of sequestering parasitized red blood cells (PRBCs) in the brain microvessels of patients with CM (Berendt et al., 1994). It is thought that sequestration causes abnormalities in cerebral blood flow or 'sludging' resulting in local hypoxia, acidosis, hypoglycemia and other metabolic derangements that may affect brain function (MacPherson et al., 1985; Medana et al., 2001). Evidence exists to support this theory. In a study of adult patients with CM, cerebral oxygen consumption was shown to be low

in >90% of comatose patients (Warrell et al., 1988) and increased cerebrospinal fluid lactate levels were found to be a sensitive prognostic index in humans (White et al., 1985). Metabolic evidence of ischemia was also observed in an experimental murine model of cerebral malaria (Sanni et al., 2001). Despite these observations, this theory fails to address some of the clinical features of CM in either children or adults. In particular, it fails to explain the apparent rapid reversibility of coma in some of the patients who develop CM and who do not have any residual neurological sequelae (Warrell, 1987). The sequestration hypothesis is also unable to address the finding that although parasite sequestration is a common feature of CM, cerebral sequestration occurs in non-cerebral malaria patients, and that some patients who die of severe edema and hemorrhage may have a virtual absence of PRBCs in their cerebral vessels (Boonpucknavig et al., 1990; Toro and Roman, 1978). So although parasite sequestration is a key neuropathological finding in this disease and is important in the development of this neurological syndrome, it does not seem sufficient to lead to irreversible brain damage and death.

1.3.2.2. Immunological or Cytokine Hypothesis

The second major theory known as the 'cytokine' or 'immunological' hypothesis purports that cerebral malaria is the result of an over aggressive immune response to the antigenic challenge of a *P.falciparum* infection (Medana et al., 2001; Toro and Roman, 1978). Several lines of evidence for this theory exist, and much of the evidence is derived from the observation that the pathophysiology of CM is similar in some ways to sepsis (Clark et al., 1991). In the cytokine theory, it is argued that parasite products cause an immune reaction resulting in a local or systemic production of inflammatory mediators such as TNF-α, which may directly or indirectly affect neuronal function (Clark et al., 1997). Histopathological evidence exists for the presence of several pro-inflammatory cytokines in the brains of CM patients including the interleukins (IL) IL-1β, IL-10, TNF-α and interferon-gamma (IFN-γ) (Maneerat et al., 1999). The local production of inducible nitric oxide synthase (iNOS), important in the production of NO, a known modulator of neuronal function has also been found to be elevated in CM patients in

response to cytokines (Clark and Awburn, 2002; Clark et al., 2003b; Maneerat et al., 2000). TNF-α is probably the most important of these inflammatory mediators (Odeh, 2001). *In-vitro* it was shown to be inducible by parasite products (Ghigo et al., 1995; Tachado et al., 1996), and to increase with disease severity (Grau et al., 1989; Kwiatkowski, 1990; Kwiatkowski et al., 1990; Maneerat et al., 1999). Evidence also shows that TNF-α may impair the BBB, causing edema or more subtle dysfunction (Sharief et al., 1992; Sharief and Thompson, 1992) (see Section 1.3.2.3.), and cause the upregulation of ICAM-1 (Turner et al., 1994), an adhesion molecule known to be important in microvascular sequestration (see Section 1.3.1.1). TNF-α, along with IFN-γ, may also exacerbate inflammation locally within the CNS (Maneerat et al., 1999) by activating CNS parenchymal cells, microglia in particular, to rapidly express major-histocompatibility molecules (MHC) Class II molecules (Vass and Lassmann, 1990). This may allow them to act as efficient antigen presenting cells to CD4+ T cells *in vitro* resulting in T cell activation. In studies of mice with fatal cerebral malaria and findings similar to those in CM, perivascular cells and microglia were found to express MHC Class II molecules, indicating the potential for antigen presentation and T cell activation (Medana et al., 2001). MHC Class II expression was found to be focally distributed in areas of BBB breakdown where astrocyte ensheathment of vessels was lost (Medana et al., 1996). Several studies have shown the potential for a lymphocytic infiltrate in CM and the presence of lymphocytic cuffs surrounding vessels in CM, indicating the potential of a local inflammatory process (Hearn et al., 2000; Patnaik et al., 1994).

Despite the strength of the theory, some questions as to the role of cytokines in CM still exist. Recent studies in children and in the experimental model of CM using neutralizing antibodies were shown either to not prevent CM or to increase mortality (Hermsen et al., 1997; van Hensbroek et al., 1996). Also, the immunological or cytokine theory fails to acknowledge the important role that sequestration (see Section 1.3.2.1) may be playing in this disease (Berendt et al., 1994; Medana et al., 2001).

1.3.2.3. Blood-Brain-Barrier Permeability Hypothesis

It is now well established that the exclusive nature of the blood-brain-barrier is due to the unique cellular characteristics of the endothelial cells (EC) lining the microvessels: tight junctions that result in a high electrical resistance along the EC lining, a lack of fenestrations, reduced transcytotic vesicular transport and a relatively low number of intracytoplasmic vesicles (Neuwelt, 2004). Disruption of the BBB has been observed in a number of neurologic disorders (Dobbie et al., 1999) and recent proposals include a role for BBB dysfunction in CM (Adams et al., 2002). The major initial findings to support this theory came from observations that cerebral edema is present at autopsy in some cases of human CM (SenGupta and Naraqi, 1992; WHO, 2000) and that increases in BBB permeability to albumin and water soluble dyes were observed in experimental mouse models (Migasena and Maegraith, 1968a; Migasena and Maegraith, 1968b; Schelp et al., 1977; Thumwood et al., 1988). Several recent studies into BBB dysfunction in CM showed disruption of tight junctions in the EC (see Section 1.4.7.). Despite these observations, it is currently accepted that if disruption of the BBB is important in this disease, it is likely that it has a subtle role, and that irreversible coma and death in most cases of CM is not the result of BBB dysfunction resulting in widespread cerebral edema (WHO, 2000). This realization comes from the fact that computed tomography and magnetic resonance imaging studies of the brain rarely show evidence of edema (Looareesuwan et al., 1995; Newton et al., 1994), and that many of the post-mortem observations of cerebral edema are probably the result of agonal events (WHO, 2000). Recent studies in the experimental mouse model of CM also showed that increased permeability of the BBB was not sufficient to cause neurologic dysfunction without the involvement of the immune response (Hermsen et al., 1998; Medana et al., 2000). BBB dysfunction is therefore important but not sufficient to explain the genesis of CM.

1.3.2.4. Overlapping Pathogenetic Mechanisms in CM

A large number of studies focusing on the host and parasite factors are beginning to suggest that neurological complications arising from *P.falciparum* infection are unlikely to be the result of a single pathophysiological process. Instead, it seems that several distinct but overlapping pathological mechanisms are occurring in individual patients, culminating in the 'final common syndrome' known as cerebral malaria (Marsh et al., 1995). The potential for multiple overlapping pathological mechanisms must be taken into account if effective therapies are to be developed. If different pathogenetic mechanisms are involved in different patients, or if various mechanisms are occurring in the same patient, then interventions targeted at a single specific process are unlikely to be effective. This may explain the apparent failure of various adjuvant therapies for cerebral malaria (Taylor et al., 1992). Unfortunately the relative contributions of the pathological mechanisms thought to be important in CM are unknown, due largely to the lack of detailed clinicopathological correlations for individual patients.

1.3.3. Experimental Murine Model of Cerebral Malaria

Several animals models have been developed to study the neuropathology and pathogenetic mechanisms involved in CM that include models in monkeys, rats and mice (de Souza and Riley, 2002; Turner, 1997). The most important experimental cerebral malaria model involves *Plasmodium* berghei ANKA strain infection of CBA mice. This results in fatal murine cerebral malaria (FMCM), and the model that shares many similarities with CM in humans including edema, erythrocyte sequestration, hemorrhage, gliosis and astrocyte activation, cytokine expression, and a host of other neurological complications (Lou et al., 2001; Medana et al., 2001). Several important differences exist between the FMCM model and human CM, however. For example, while more then 95% of the mice in FMCM die with neurological complications, very few CM patients die following cerebral complications. In the remainder, coma is reversed and patients survive without sequelae. Another important difference is that the microvasculature in the FMCM model is sequestered with host leukocytes rather then parasitized

erythrocytes. The latter difference is especially important, given the potential for a more localized immune response in the murine model, casting serious doubt on the relevance of this model to human CM (Turner, 1997).

1.4. Neuropathological Findings in Cerebral Malaria

Historically, integrating autopsy findings with clinical information gathered during life generates basic information regarding disease pathogenesis. Unfortunately, the full nature and extent of histopathological features of human pediatric cerebral malaria have yet to be determined. The lack of neuropathological data is due to a number of factors including the wide geographical variation in the clinical presentation of the disease, geographical strain variations in parasite virulence and host immunity, and a relative lack of tissue availability due to religious and cultural objections to autopsy that exist in Africa (Turner, 1997). Therefore, most of the neuropathological observations published to date focus on Southeast Asian adults or on the FMCM model. In both cases, the nature of the disease is different from that in children (see Sections 1.2.3 and 1.3.3.).

1.4.1. Sequestration

Several hallmark studies have found a positive association between sequestration in the brain and the development of cerebral malaria. Macpherson *et al.* (1985), focusing on a small clinical cohort of adult patients found parasite sequestration to be a major feature at the histological level in the brain microvessels of CM patients and that both the percentage of vessels with sequestration and the degree of sequestration was greater in CM patients compared to patients without coma (MacPherson et al., 1985). Other studies on Southeast Asian adults found the percentage of sequestration in the brains of CM patients to be higher than in patients without neurologic dysfunction and that the severity of malaria in CM patients is dependent on PRBC sequestration in the brain (Pongponratn et al., 1991). A recent clinicopathological study of 65 patients dying of severe malaria in Thailand and Vietnam reported that sequestration was significantly greater in the brains of CM patients compared to patients without coma over all brain regions examined

(cerebrum, cerebellum and medulla oblongata). Furthermore, within individual patients a hierarchy of sequestration by region was observed, with more sequestration in the cerebrum and cerebellum compared to the brainstem (Pongponratn et al., 2003). A similar hierarchy of sequestration was found in both simian and human studies by Sein *et al.* who observed the most sequestration in the cerebellum of CM patients largely in relation to the degree of vascularization of the cerebellum as compared to the cerebrum (Sein et al., 1993a; Sein et al., 1993b). More recent studies have attempted to describe and quantify the parasite stages sequestered within the brains of CM patients. Forty-six of fifty Thai and Vietnamese adults with severe malaria were observed to have sequestration within their brains and there were significantly more ring forms in the brains of CM patients then would be expected from free mixing of peripheral circulation within the brains of CM patients (Silamut et al., 1999). Within the same brain different vessels had discrete but different populations of parasites, indicating that the adhesion characteristics of cerebrovascular endothelium change asynchronously during malaria and also that significant recirculation of parasitized erythrocytes following sequestration is unlikely. A very interesting study recently conducted by Taylor *et al.* (2004) found that CM patients could be distinguished from non-malarial encephalopathies based on the presence or absence of sequestration and that the presence of pigment, reflective of a late stage parasite, is a helpful predictor of intra and perivascular pathology and is useful for delimiting different pathogenetic mechanisms in CM (Taylor et al., 2004). Although sequestration is known to be a common and important feature in CM, very little is known about the relationship between sequestration and neuropathology in this disease.

1.4.2. Petechial and Ring Hemorrhages

There are two types of hemorrhages common to cerebral malaria, both restricted to the white matter (Patankar et al., 2002; Rigdon, 1944; SenGupta and Naraqi, 1992; White et al., 2001). Autopsy studies have revealed petechial hemorrhages which are also found in a number of other disorders such as terminal asphyxia and carbon monoxide poisoning. In contrast, perivascular ring hemorrhage appears to be a unique feature of cerebral malaria (SenGupta and Naraqi, 1992; Turner, 1997). The latter type of

hemorrhages is termed a 'ring hemorrhage' because histologically it appears as two concentric rings surrounding a central necrotic vessel. The outermost ring typically contains a mixture of parasitized erythrocytes and non-parasitized erythrocytes, free pigment and host monocytes while the inner layer consists of uninfected erythrocytes and gliosis, indicating that this lesion may be a reperfusion injury (Turner, 1997).

Only a few studies detail the pathology of hemorrhages in human CM (Patankar et al., 2002; Rigdon, 1944; SenGupta and Naraqi, 1992; White et al., 2001), and very little is still known about either the distribution of perivascular ring hemorrhage across the CNS of CM patients or the relationship between hemorrhage and sequestration. One study found ring hemorrhages to be more common to the cerebellum then to the cerebrum, correlating closely with perivascular sequestration (Sein et al., 1993b), while another study found cerebral and cerebellar hemorrhages independent of sequestration (Boonpucknavig et al., 1990). A relationship may also exist between inflammatory mediators and the presence of hemorrhagic lesions in CM. A recent study in the mouse model of CM showed IL-10 knockout mice infected with *P.chaubaudii* to have greater sequestration, edema and hemorrhage than wild-type CM mice, and that anti-TNF-α treatment ameliorated CM complications (Sanni et al., 2004).

1.4.3. Durck's Granulomas

Durck's granulomas are lesions characterized by glial cell proliferation associated with ruptured vessels and necrotic areas caused by hemorrhage, representing an inflammatory defense process (Turner, 1997). Most studies describing Durck's granulomas are in adults though evidence of these lesions in children has been observed (Walker et al., 1992). Very little is known about the origin of these lesions, but some authors suggest that granulomas may be what remains after infected and uninfected red cells are cleared from a ring hemorrhage and an associated immune response following hemorrhage has been established (Turner, 1997). It is thought that once endothelial cell damage has occurred through hemorrhage, peripheral proteins, parasites and white blood cells enter the brain setting up a glial and immune reaction leading to the formation of a

granuloma. Immunohistochemical studies performed on the brains of adult CM patients with Durck's granulomas have found these lesions to be positive for vascular endothelial growth factor (VEGF) and proteins related to the VEGF signalling cascade (Deininger et al., 2003), known to occur secondary to hypoxia and transient ischemia, as well as the urokinase plasminogen activator receptor (uPAR) (Fauser et al., 2000). The latter protein was observed in astrocytes, endothelial cells and microglial cells in granulomas and around ring hemorrhages, and is thought to contribute to BBB abnormalities and immune dysfunction in CM. The former, VEGF and associated proteins, suggest a role for angiogenic processes within the developing lesion. The role of these proteins, however, is unclear, given that other, antiangiogenic proteins have been found in these lesions as well (Deininger et al., 2002). Granulomatous lesions have also been found to be positive for a number of inflammatory mediators, suggesting that Durck's granulomas may be important in contributing to immune exacerbation in this disease (Clark et al., 2003a; Deininger et al., 2000a; Deininger et al., 2000b; Schluesener et al., 2001).

1.4.4. Neuronal Changes

The mechanical hypothesis emphasizing the potential for ischemia secondary to parasite sequestration predicts the presence of neuronal cell loss as a potential neuropathological correlate (Turner, 1997). Despite the popularity of this theory, very little evidence for neuronal cell damage in CM exists. The major studies which discuss neuronal cell loss are those of Rigdon *et al.*, who describes the degeneration of brain cells and the depletion of Purkinje cells in the cerebellum of CM patients (Rigdon, 1944). Observations of neuronal cell loss has been gathered from the mouse model of CM, where neuronal degeneration was observed to be present with other neuropathological changes including astrocyte changes, demyelination, and neurologic dysfunction consistent with experimental cerebral malaria (ECM) (Ma et al., 1997).

1.4.5. Axonal Injury

Axonal injury (AI) has been recognized to be a common feature and key predictor of outcome in a number of CNS diseases including head and spinal cord trauma, multiple sclerosis, ischemic damage, and infectious diseases such as HIV (Medana and Esiri, 2003). In a recent study, immunohistochemistry for beta-amyloid precursor protein (β-APP) was used on postmortem brain tissue in 54 Vietnamese adults with severe *P.falciparum* malaria to identify axonal damage (Medana et al., 2002). The frequency and extent of β-APP staining were more severe in patients with cerebral malaria than those without clinical involvement, making axonal injury the only finding with the exception of parasite sequestration to be positively associated with CM. There was a certain degree of heterogeneity in the distribution of axonal injury across the CNS in this group. Significant differences were found between CM and NCM patients over all of the areas examined (cortex, internal capsule, pons and cerebellum) with the exception of the cerebellum. In this series, axonal injury was found to be associated with hemorrhage and areas of demyelination but not the presence or absence of intravascular leukocytes. Axonal injury was also not associated with focal astrocyte responses. Clinicopathological correlations revealed that there was no association between AI and edema, systemic hypoglycemia or other evidence of vital organ failure such as renal failure, jaundice and shock, or increasing number of criteria of severity. Nothing is known about the presence of axonal injury or the neuropathological correlates of axonal injury in pediatric CM.

1.4.6. Myelin Damage

Demyelination or myelin loss has been noted in some of the earlier post-mortem studies of brains of CM patients (Janota and Doshi, 1979; Toro and Roman, 1978), but detailed analysis of these lesions and the frequency of this finding was not given. In a recent study of axonal injury in CM, demyelination was found to be a common feature in the brains of Southeast Asian adults. 19 of the 47 adult patients with CM showed patches of demyelination judged to be secondary to axonal damage (Medana et al., 2002).

Demyelination is also a feature of the FMCM model. In the FMCM model, mice fall into coma on day 7 post-infection (p.i.), at the same time that they develop breakdown of the blood-brain-barrier, increased gliosis and patchy axonal demyelination, suggesting a potential relationship between demyelination, neurologic dysfunction and other neuropathological features in this disease (Ma et al., 1997). Demyelination or myelin loss is expected in both CM and FMCM, given that CM is a neurologic disorder thought to involve TNF-α (Furlan et al., 2004; Heremans et al., 1989).

1.4.7. Blood Brain Barrier Abnormalities

More recent studies in both adults and children with CM show evidence of subtle BBB dysfunction. In two separate studies, Brown *et al.* showed subtle but measurable plasma protein leakage between the cerebrospinal fluid and circulating plasma in CM in addition to a reduction in the expression of the endothelial cell junction proteins zona occludens-1 (ZO-1), occludin and vinculin (Brown et al., 1999; Brown et al., 2001). Studies on BBB dysfunction in CM, however, appear to emphasize that the permeability observed is not likely to be widespread. Albumin and immunoglobulin-G (IgG) indices in a Vietnamese study showed only a minimal degree of BBB breakdown in comparison to various types of meningitis used as a control. This infers that CM may involve only subtle functional changes in BBB integrity with minimal intraparenchymal responses compared with other neurologic infections. This suggests that CM is likely to involve local events around the cerebral vasculature rather than widespread parenchymal disease (Brown et al., 2000).

1.4.8. Gliosis

Gliosis is a non-specific proliferative response of astrocytes to CNS injury and is a classic feature of virtually all diseases of the CNS. The role and presence of gliosis in CM are not well documented, and only a few reports describe gliosis in CM. In a recent study of axonal damage in severe malaria, 52% of patients were observed to have some form of generalized gliosis present in either the subpial regions, perivascular regions or

subventricular regions. Gliosis was not found to be associated with axonal damage in this study (Medana et al., 2002), and gliosis has not been attributed to any cause in CM, though gliosis has been described as a feature of necrotic regions in perivascular ring hemorrhage (Turner, 1997). The role of gliosis and astrocytes have been more fully studied in the murine model. Studies in the FMCM model found that retinal astrocytes, lose their even distribution at 3 days p.i., developing into gliosis by day 5 p.i., two days before the onset of symptoms. These features were not present in resolving and non-CM murine models and may be related to neurologic outcome. This study also showed a relationship between immune dysfunction and blood-retinal-barrier (BRB) breakdown and astrocyte changes (Chang-Ling et al., 1992; Medana et al., 1996).

1.4.9. Limitations of Current Neuropathological Observations

Although a number of studies have been carried out to understand the neuropathology, and several of the main neuropathological features of this disease have been described elsewhere, most of the data has been derived from either adult patients or the murine model where the characteristics of the disease are much different from the disease in children. Also, in many of these studies the neuropathological features have not been fully described, and little potential for clinicopathological correlation exists.

1.5. Similarities in Brain and Retinal Histopathology in Cerebral Malaria

The brain and retina are both neuroectodermally derived, and when fully developed, these tissues have several cell types in common, including: microglial cells, astrocytes, perivascular macrophages, pericytes, neurons, and the tight-junction forming endothelial cells of the BRB (blood-retinal-barrier), the retinal equivalent of the blood-brain-barrier. As a result of these similarities, ophthalmological exams are often useful when trying to understand the nature of a CNS disorder and the pathophysiological mechanisms involved (Freeman et al., 2004; Jabs, 1995; Lim et al., 1997; Williams and Johnson, 2004). Several studies have identified retinopathy unique to CM, and it is known that several fundoscopic findings are indicative of poor prognosis or increased

disease severity, suggesting a close relationship between ocular findings and brain findings in CM (see Section 1.2.2.). Despite this, little is still known about the direct histopathological relationship between the brain and retina in CM. A recent study found that retinal vessels exhibit late stage *P.falciparum* sequestration similar to that observed in the brains of CM patients and that sequestration corresponds to pronounced dehemoglobinization, contributing to the abnormal retinal vessel whitening observed in CM (Lewallen et al., 2000). In addition to sequestration, another study found that the histopathological observation of hemorrhage, a common finding in the brains of CM patients, correlated well with the number of retinal hemorrhages observed clinically and during post-mortem evaluation, and of cerebral and cerebellar hemorrhages, suggesting that retinal pathology may be reflective of brain pathology in CM (White et al., 2001). Despite some of these observations, very little is known about the ophthalmic pathology in CM and how the opthalmic pathology might be related to either neuropathology or ocular fundus findings.

1.6. Hypothesis and Thesis Objectives

The overall objective of this project is to characterize several aspects of the neuropathology of children who die of cerebral malaria. The proposed study attempts to test the following hypotheses:

(1) Pediatric cerebral malaria is associated with significant axonal, myelin and endothelial damage and gliosis.
(2) Ocular pathology significantly correlates with brain pathology in cerebral malaria.
(3) Clinicopathological and neuropathological correlations exist among the described features of pediatric cerebral malaria

To test these hypotheses, three specific experimental objectives were carried out:

(1) To determine whether pediatric cerebral malaria is associated with significant neuropathological changes we used immunohistochemical and neurohistological stains to characterize and quantify the nature and extent of the axonal, myelin and endothelial damage in the brains of pediatric CM patients and appropriate controls.

(2) To test if a relationship exists between ocular and brain pathology and ocular pathology and clinical fundus findings, the same specific staining techniques used on CM brains were used on retinal sections from pediatric CM patients and controls. Statistical correlations are used to confirm any positive associations

(3) To understand the relevance of the neuropathological features to the clinical presentation and outcome of pediatric cerebral malaria, detailed clinicopathological and neuropathological correlations were carried out.

CHAPTER 2

MATERIALS & METHODS

2.1. Patient Selection

All subjects (n=49) were children (6 months to 12 years) who had been admitted to the Malaria Research Project in the Department of Pediatrics, Queen Elizabeth Central Hospital (QECH), Blantyre, Malawi. Patients admitted to the ward first undergo a screening process by Malaria Project Nurses. The screening process consists of taking fingerprick samples for hematocrit (microhematocrit centrifuge) and malaria parasites (thick film), and assessing the Blantyre Coma Score. The coma score measures the level of consciousness of patients by the addition of individual scores obtained through observing the patient's verbal and motor responses to painful stimuli, and the visual responses to a moving object (Table 1.). Malaria Project clinicians are notified of any and all children with BCS equaling 2 or less (unable to localize a painful stimulus, abnormal cry, see Table 1.) and all children with hematocrit readings of <15%. Both parasitemic and aparasitemic children who are unconscious are admitted to the Malaria Project Ward. Once admitted, patients undergo a comprehensive clinical evaluation through clinical history and examination and by laboratory tests. Patients are treated as clinically indicated with intravenous fluids, glucose, antimalarial drugs, antibiotics, anticonvulsants, antipyretic drugs and blood transfusions.

Table 1. Definition of Blantyre Coma Score (WHO, 2000).

Observation	Score
Best Motor Response	
Localizes painful stimulus	2
Withdraws limb from pain	1
Non-specific or absent response	0
Verbal Response	
Appropriate cry	2
Moan or inappropriate cry	1
None	
Eye Movements	
Directed (e.g. follows mother's face)	1
Not directed	0

2.1.1. Definition of Clinical Groups

Three clinical groups were defined prospectively: cerebral malaria (CM, n=25), severe malarial anemia (SMA, n=5) and comas of other causes (COC, n=19). Patients were considered to have CM when they (a) died with coma (BCS 2/5 or less, lasting for at least 2 hours before death), (b) had *P.falciparum* asexual parasitemia, (c) where other causes of unconsciousness had been excluded (hypoglycemia, meningitis or other encephalopathy), and (d) where histopathological investigation was able to corroborate the diagnosis (either by the presence of parasite sequestration within brain microvessels or the presence of microhemorrhages, see Section 2.1.2). SMA patients had blood smears positive for *P.falciparum*, hematocrit readings < 15%, and died without coma (BCS > 2). Patients dying with comas of other causes had very low or no *P.falciparum* parasitemia, BCS < 2, and on clinical grounds were observed to be suffering from diseases other than malaria. These patients were further classified into infectious (COCinfectious, n=14) and non-infectious (COCni, n=5) causes of death based on blood and CSF cultures or histopathological evidence. Details of the blood and CSF cultures and final diagnosis of COC patients is found in Section 3.1.2. Controls also included brains from five age-matched children (2 years 8 months to 5 years) children dying of non-CNS causes at Children's and Women's Hospital of British Columbia. Two of the

five patients suffered from cardiac deaths, one died suddenly in a motor vehicle accident, one of CHARGE syndrome and one of an unknown non-CNS cause.

2.1.2. Histopathological Classification of Cerebral Malaria

Parasite sequestration within cerebral microvessels is common in CM and was quantified in individual patients during histological examination of the brain (Taylor et al., 2004). Following autopsy, blocks from several regions of the brain were obtained and processed as described in Section 2.3. After processing, 3-5μm sections from the parietal lobe (B2) and were stained with hematoxylin and eosin (see Section 2.4.1.). Under oil immersion at least 100 capillaries were identified randomly throughout the section. A capillary was defined as a transected blood vessel, circular or oval in profile, with a maximum-to-minimum diameter of $\leq$ 2:1, and having at most one visible endothelial cell nucleus in the wall. The content of each vessel was counted with respect to the various erythrocytic stages of the parasite lifecycle (unpigmented parasites, pigmented parasites, and extra-erythrocytic pigment globules). Parasites were counted as unpigmented if no pigment was visible in the infected red cell. The common feature in pigmented parasites was the presence of malarial pigment, varying in size from a small black dot to a less well-defined larger mass. Extra-erythrocytic pigment appeared as a mass of ill-defined black pigment within a completely clear background, free within the capillary lumen, or within white blood cells. The percentage of cross-sectioned vessels containing intact parasites or malarial pigment globules was also recorded. These counts were used to classify CM patients into one of two groups (Class I or Class II) based on the presence and degree of extra-erythrocytic pigment globules present in the vessel lumen by classification and regression tree analysis (CART) by Taylor *et al.* as previously described (Taylor et al., 2004). The CART procedure uses recursive binary partitioning to identify nodes (cut-off values) among the parasitic elements that best discriminate between the pathological classes. The process continues until subjects in each end node belong to the same class or until there are only a few subjects in each end node. In addition to the histopathological classification of CM, percent sequestration and the number of either unpigmented parasites, pigmented parasites, or extra-erythrocytic

pigment globules were correlated with the neuropathological findings to determine whether a significant association could be found between parasite sequestration and neuropathology.

2.2. Clinical and Biochemical Parameters Observed

This work is included as part of a National Institutes of Health (NIH) funded clinicopathological study (Grant # NIH R41154) that has generated an extensive clinical data set. The clinical parameters investigated include: age, sex, history and duration of fever (hrs), history and duration of coma (hrs), history and duration of convulsions (hrs), time from admission to death (hrs), temperature, admitting and lowest Blantyre Coma Score (for definition see Section 2.1.), hematocrit (%), blood lactate, plasma pH, $pC0_2$, glycemic status on admission, blood glucose (mmol/L), white cell count in the blood and CSF, red cell count, platelet count, and CSF opening pressure. Parasitological data collected included initial and final parasitemia (blood).

2.2.1. Ophthalmological Exams and Grading of Ophthalmological Features

Cerebral malaria patients often show specific ocular signs as observed by direct or indirect ophthalmoscope that may include: hemorrhages, papilledema, retinal whitening and retinal vessel abnormalities. Some of these are reflective of disease progression and outcome (see Section 1.2.2.). To establish a relationship between the ocular fundus findings and the neuropathological and retinal pathology described in this study, most of patients included in the autopsy study were examined during life by an ophthalmologist (by indirect ophthalmoscope) using a standardized form to record the presence of retinal hemorrhages, vessel abnormalities and retinal whitening as previously described (Lewallen et al., 1999b). Retinal hemorrhages present on clinical exam were graded in each eye as 0, +1 (1-5 hemorrhages), +2 (5-20 hemorrhages), and +3 (20-50 hemorrhages). In cases where there was a difference between the two eyes, the grade of the worst eye was used for analysis. Retinal whitening consisted of discrete spots of retinal opacification which did not obscure retinal vessels. Retinal whitening was

classified as macular or extramacular as previously described (Lewallen et al., 1999b). Extramacular whitening was graded in four quadrants (superior, inferior, temporal and nasal). Occasional spots only were graded 1. More then occasional spots or patches of definite mosaic were graded 2. Grade 3 corresponded to widespread mosaic or large areas of confluence. Retinal vessel abnormalities were recorded as present or absent.

2.3. Brain, Whole Eye and Globe Specimens and Handling

Post-mortem examination was performed on all children dying with cerebral malaria after permission for an autopsy was granted. The post-mortem intervals ranged from between 2 to 14.5 hours. All samples were processed through alcohols and fixed in 10% neutral buffered formalin. Samples were collected from various regions of the brain including the cortex and sub-cortical white matter from the frontal (B1), parietal (B2), temporal (B3) and occipital (B4) lobes, as well as the hippocampus (B5), caudate (B6), thalamus (B7), midbrain (B8), pons (B9), medulla (B10), and cerebellum (B11 and B12) , and were embedded in paraffin. For purpose of comparison, samples were grouped anatomically into 4 regions: cerebral hemispheres and hippocampus (CH, B1-B5), caudate and thalamus (C+T, B6-B7), brainstem (BS, B9-B10), and cerebellum (Cer., B11-B12). 6μm and 3μm sections were obtained for neurohistology staining (see Section 2.4.) and immunohistochemistry (see Section 2.5.) respectively.

2.4. Neurohistological Staining

For routine histopathological examination and for the analysis of myelin loss and damage, Hematoxylin and Eosin (H&E) and combined Luxol Fast Blue (LFB)/Hematoxylin and Eosin (LFB/H&E) stains were employed. Both H&E and LFB/H&E were performed using 6μm tissue sections obtained from paraffin blocks (see Section 2.3.).

2.4.1. Hematoxylin and Eosin Staining (H&E)

Sections were dried in a 60 degree oven for 30-40 minutes, deparaffinized through four changes of xylene (C_8H_{10}) and rehydrated in 3 rounds of 99% ethanol (CH_3CH_2OH) and 1 round of 95% ethanol followed by three washing steps in running water. Sections were stained in Gill's II hematoxylin (VWR) for 5 minutes, differentiated in 0.5% hydrochloric acid (HCl) for 10 seconds followed by 3 more washing steps and a final differentiation in 1.5M sodium carbonate ($NaCO_3$). Sections were then stained in alcoholic eosin for 1 minute and were differentiated in 1 change of 95% ethanol and 3 changes of 99% ethanol. The slides were then quickly immersed several times in xylene and were cover slipped.

2.4.2. Luxol Fast Blue and Hematoxylin Eosin Staining (LFB/H&E)

Following fixation and paraffin embedding (see Section 2.3.) sections were cut at 6μm for luxol fast blue (LFB) staining. Sections were first heated in a 60 degree oven for 30-40 minutes and were deparaffinized and rehydrated as described previously (see Section 2.4.1.). Following deparraffinization sections were stained in 0.1% luxol fast blue solution (95% ethanol and 10% acetic acid [CH_3COOH]) overnight at 37 degrees. After staining, slides were immersed in 95% ethanol to remove excess stain, washed in distilled water and differentiated by quick immersions in 0.05% aqueous lithium carbonate ($LiCO_3$). Differentiation was continued in several changes of 70% ethanol until grey and white matter could be distinguished at 10X magnification, with care being taken to ensure that the slides were not over-differentiated. Sections were then washed thoroughly in distilled water. After LFB staining, the sections were stained with H&E as described above (see Section 2.4.1.) without the need to deparaffinize with xylene and ethanol changes.

2.5. Immunohistochemical Staining

Following fixation and paraffin embedding (see Section 2.3.) sections were cut at 3μm onto polylysine-coated slides (VWR) for immunohistochemistry (IHC). Sections were first deparaffinized by heating at 60 degrees for 30-40 minutes followed by 3 changes of xylene, 3 changes of 100% ethanol, and 1 change of 95% ethanol followed by immersion in several changes of water. Following deparraffinization microwave antigen retrieval was carried out in 10mM sodium citrate buffer (pH 6.0) (0.1M sodium citrate [$C_6H_8O_7Na_3$-$2H_2O$] and 0.1M citric acid [$C_6H_8O_7$]). Peroxidase quenching was carried out in 3% hydrogen peroxide (H_2O_2) in 100% methanol for 30 minutes under constant stirring. Slides were then washed twice in tris-tween (pH 7.6) (0.05M trizma base [Sigma, $C_4H_{11}NO_3$], 0.15M sodium chloride [NaCl], 3.25 mls 10N HCl, and 0.05% tween-20). Slides were incubated with primary antibody (see Section 2.5.1) for 1 hour at room temperature. Slides were then washed three times in Tris-Tween followed by incubation secondary antibody (see Section 2.5.1) for 1 hour. Slides were put through another series of Tris-Tween washes (3 x 5 minutes) and one change of 0.1M acetate buffer (0.1M sodium acetate trihydrate [CH_3COONa-$3H_2O$], 0.1% glacial acetic acid and 0.05% tween 20). Bound secondary antibody was detected by incubation in 3-Amino-9-Ethylcarbazole (AEC) for 5-10 minutes followed by 3 washes in water. Sections were counterstained in hematoxylin for 2-3 minutes followed by differentiation in 1.5% $NaCO_3$, dehydration in 1 change each of 95% ethanol, 100% ethanol and xylene and cover slipping. Negative controls were stained as above apart from the omission of primary antibodies.

2.5.1 Antibodies and Dilutions

Several of the neuropathological features were observed using immunohistochemistry, carried out as described above (see Section 2.5.). Axonal damage was found using a 1:500 dilution of primary murine monoclonal antibody (mAB) antibody to human β-APP (DAKO). Gliosis was visualized using a 1:1000 dilution of

polyclonal antibody (pAB) to rabbit anti-cow glial fibrillary acidic protein (GFAP). Blood-brain-barrier dysfunction was observed using 1:500 dilution of rabbit pAB anti-fibrinogen antibody. Goat anti-mouse and goat anti-rabbit secondary antibodies were used at 1:500 dilutions. Both brain and retinal sections were stained at the same dilution of primary antibody with the exception of β-APP and GFAP where the dilutions were both 1:100.

2.6. Quantification of Neuropathological Findings

Sections from each of the brain regions were stained and examined and the following neuropathological features were recorded and quantified: perivascular ring and petechial hemorrhages (H&E), axonal damage (β-APP), myelin loss (LFB/H&E), blood brain barrier dysfunction (fibrinogen) and reactive gliosis (GFAP). Hemorrhages, axonal damage and myelin loss are features of the white matter and were quantified over 5 randomly selected white matter fields at 100X magnification. Field size and area was limited by placing a 1mm X 1mm grid into the ocular eyepiece over the slide. For perivascular ring hemorrhages, the total number of hemorrhages over 5 white matter fields was counted in a given section. Ring hemorrhages are easily identifiable at 100X and were characterized by the presence of a central parasitized vessel surrounded by an area of necrosis and an outer ring of parasitized and unparasitized erythrocytes. The identity of the slides was masked before counting so that all counts were performed blindly.

Axonal damage and myelin loss were quantified by morphometric analysis using a modified version of a semi-quantitative method described elsewhere (Medana et al., 2002). To quantify axonal pathology in tissue sections following β-APP immunohistochemistry (see Section 2.5.), five white matter fields at 100X magnification were randomly selected and digitized. Regions of focal or diffuse axonal damage were then selected manually using the area selection tool of Image Pro-Plus (Version 5.0, Media Cybernetics) and axonal damage was expressed as micrometers^2 β-APP staining/5 fields at 10X (μm^2/5 fields at 10X). Once the area of axonal damage was determined, the

lesion was classified into one of two types: axonal pathology associated with hemorrhage (HA) and axonal pathology not associate with hemorrhage (NHA). Myelin damage was quantified using a similar procedure. In this case, however, the lesion area consisted of areas of myelin pallor and the results were expressed as μm^2 myelin loss/5 fields at 10X.

Gliosis and blood-brain-barrier dysfunction are features of both the grey and white matter as well as the mixed grey-white matter of the central regions of the brain (caudate + thalamus, brainstem). Consequently, the number of leaky vessels and the extent of gliosis were measured across 5 white matter fields and 5 grey matter fields of the cerebral hemispheres and cerebellum. In the caudate, thalamus, and brainstem, 5 randomly selected fields were chosen without discrimination between white and grey matter. Gliosis was quantified by counting the number of hypertrophied GFAP positive astrocytes in every randomly chosen field following GFAP immunohistochemistry. Results were expressed as the total number of reactive astrocytes/5 fields at 10x of either the white matter, grey matter or mixed grey-white matter, depending on the location. BBB dysfunction was quantified by measuring the number of vessels permeable to fibrinogen over 5 fields compared to the total number of vessels in the given region. BBB dysfunction was therefore expressed as the percentage of vessels permeable to fibrinogen/5 fields at 100X.

2.6.1. Quantification of Retinal Histopathology

Because of the small area of the retina in sections compared to brain sections, the entire length of the retina was examined when quantitating retinal pathology. Retinal hemorrhages were quantified by setting up a grading system (0-2). The area of β-APP staining was measured by morphometric analysis in the same way that it was measured in the brains of patients in this study (see Section 2.6.). Retinal fibrinogen leakage and gliosis were noted to be present or absent in a given patient. Gliosis is apparent by the presence of GFAP positive Muller cells extending through the length of the retina. Fibrinogen leakage, as evidence of blood-retinal-barrier dysfunction, was considered to

be present if the area surrounding vessels was observed to be positive by fibrinogen immunohistochemistry in a similar way to that observed in the brain.

2.7. Statistical Analysis

Statistical analyses were performed using Sigma Stat for Windows (Version 2.03, SPSS Inc.). Comparisons of clinical data with neuropathological data across the entire brain between clinical groups and between sub-groups of CM patients was carried out using parametric t-test with a $P<0.05$ being regarded as significant. To determine whether significant differences existed between given sites in a patient group, multiple comparisons of neuropathological findings between brain regions were carried out using ANOVA ($P<0.05$). Discontinuous data was compared using Chi-squared analysis ($P<0.05$) if necessary. Correlations between brain sites, clinical findings and neuropathological findings and ophthalmic findings were carried out using Pearson correlation for continuous data ($P<0.05$). Discontinuous data was correlated using a common correlation for Nominal Scale Data.

CHAPTER 3

RESULTS

3.1. Clinical and Laboratory Features

All of the study subjects (n=49) were children previously admitted to the Malaria Project Ward in the Department of Pediatrics at the Queen Elizabeth Central Hospital, Blantyre, Malawi. Once admitted to the ward, a series of clinical and laboratory parameters were measured and recorded in individual patients (see Section 2.2.). Table 2 summarizes the clinical and laboratory features obtained in this series.

Table 2 – Clinical and laboratory features of patients included in this autopsy series.

Clinical Parameter		CM	SMA	COCi	COCni
Number of Patients (n)		25	5	13	6
Age (months)		36.6±5.1	28.2±6.6	44.0±10.2	30.2±9.9
Sex (No.male, No.female)		14M, 11F	3M, 2F	5M, 9F	3M, 2F
Duration (hrs)[1]	Illness	76.88±9.39	89.80±34.57	91.13±33.29	53.28±17.75
	Fever	62.0±8.5	108±34.6	81.4±39.2	52.0±23.6
	Coma	**23.7±2.5***	4.5±2.5	31.9±8.5	14.4±5.6
	Conv.	**11.6±2.2***	0.0±0.0	20.6±9.8	3.5±0.5
	Adm.	**1.08±0.16***	3.00±1.05	1.21±0.41	0.80±0.37
BCS	Last	**1.00±0.22***	2.20±1.16	1.64±0.40	1.20±0.58
Asexual Parasitemia (10^3/µl)	Adm.	**289.49±64.26****	76.73±75.23	46.49±27.95	14.54±8.64
	Last	**540.43±373.08****	76.69±75.24	150.23±144.57	7.51±5.36
Hematocrit (%)	Adm.	**20.84±1.36+**	7.20±1.97	30.36±2.95	25.00±5.62
	Last	**20.56±1.26+**	8.80±2.39	32.07±3.02	24.80±4.95
Glucose (mmol/L)	Adm.	5.02±0.57	4.00±1.28	5.15±1.07	2.60±1.05
	Last	6.43±0.56	5.67±1.59	6.15±0.97	7.40±2.38
White Cell Count (WCC/µl)	Blood	16.92±3.59	22.97±9.76	19.58±4.77	29.36±8.26
	CSF	0.63±0.62	0.0±0.0	1.89±1.19	0.65±0.65
Platelet Count (10^3/µl)	Blood	**55.55±6.76++**	345.25±177.37	210.55±55.49	255.80±78.31
Positive Bacterial	Blood	2/24	0/4	3/10	0/5
Culture	CSF	0/14	0/1	3/10	0/2
HIV Positive		2/24	1/4	2/11	0/4

[1] – Duration of symptoms to death (hours). Illness = first sign of symptoms, Fever = onset of fever to death, Coma = onset of coma to death, Conv. = onset of convulsions to death.

* - Significant difference ($p<0.05$) between CM and SMA patients

** - Significant difference ($p<0.05$) between CM and both COCi and COCni

+ - Significant difference ($p<0.05$) between CM, SMA and COCi

++ - Significant difference ($p<0.05$) between CM, SMA, COCi and COCni

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3.1.1. Comparison of Clinical Features between CM and SMA patients

CM (n=25) and SMA (n=5) patients did not differ with respect to age, sex, duration of illness, fever duration, or measure of white cell count, blood glucose measurements, or asexual parasitemia. A significant difference was found between the duration of coma in CM and SMA patients ($P<0.05$) and duration of convulsion to death ($P<0.05$). None of the SMA patients experienced convulsions during their clinical course, though two SMA patients (MP7 and MP51) experienced brief periods of coma (MP7 – 2hrs, MP51 – 7hrs). In each case, the comas occurred > 6hrs before death and the patients did not die while in a comatose state. As expected, CM and SMA patients differed in their mean BCS ($P<0.05$, both admitting and last measured BCS). CM patients had mean admitting and final BCS of 1.08 ± 0.16 and 1.00 ± 0.22, respectively, while SMA patients had mean admitting and final BCS of 3.00 ± 1.05 and 2.20 ± 1.16. CM and SMA patients also differed in their admitting and last hematocrit measurements. CM patients had higher hematocrit measures then SMA patients (Table 2). CM patients also showed significantly lowered platelet counts ($P<0.05$). Clinically, therefore, CM and SMA patients differed with respect to the degree of neurological abnormality observed (with CM patients presenting with coma and convulsion), and relative hematocrit and platelet counts (with SMA patients having significantly lower hematocrit measurements and increased platelet counts). Blood and/or CSF cultures for the SMA patients were all negative. Only 2/23 (8.69%) CM patients had positive blood cultures - MP11 and MP27 (see Section 3.1.3.), and where lumbar punctures were performed, CSF cultures were also negative.

3.1.2. Comparison of Clinical Features in CM and COC Patients

CM patients and COC patients dying from either infectious (COCi, n=13) or non-infectious causes (COCni, n=6) did not differ significantly in age, sex, duration of illness, fever, coma or convulsions. They also did not differ in their BCS, or measures of glucose, white cell count in either the blood or CSF, or platelet count. These groups did, however, differ in some respects from CM patients. CM patients had significantly lower

hematocrit values at admission and before death than COCi patients ($P<0.05$). COCni patients had significantly lower hematocrit measures than COCi patients ($P<0.05$), but did not differ from CM patients to a significant degree. Both COCi and COCni patients had significantly lower counts of asexual parasitemia than CM patients (Table 2). Despite having lower parasitemias on the whole than the CM group, it is important to recognize that many individual COCi and COCni patients were observed to be positive for *P.falciparum*. Positive but low blood smears were observed in 7/13 (53.8%) of COCi patients and 4/6 (66.7%) of COCni patients throughout their clinical stay even though their cause of death was other than CM, suggesting that *P.falciparum* infections are common in African children presenting with coma, irrespective of cause of death (Taylor et al., 2004). Table 3 gives a summary of the bacterial culture findings and final diagnosis of individual COCi and COCni patients.

Table 3 – Blood and CSF culture findings and summary diagnosis of COCi and COCni patients.

Clinical Group	Case	Culture Findings		Summary Diagnosis
		Blood	CSF	
COCi	MP8	-	Neg.	Viral encephalitis
(n=13)	MP12	Pos.	Neg.	Sepsis, *Streptococcus enteriditis*
	MP14	Pos.	-	Sepsis, Gram negative septicemia
	MP18	Neg.	Pos.	*Haemophilus influenzae*
	MP20	Neg.	Neg.	Meningitis, Tuberculous
	MP24	-	Pos.	Meningitis, *Streptococcus pneumoniae*
	MP31	Neg.	-	Sepsis, *Streptococcus pneumoniae*
	MP33	Neg.	Neg.	Viral pneumoniae
	MP40	Neg.	Neg.	Sepsis
	MP45	Neg.	-	Sepsis, *Streptococcus pneumoniae*
	MP47	Neg.	Neg.	Viral pneumoniae
	MP50	-	Pos.	Sepsis, *Streptococcus pneumoniae*
	MP53	Pos.	Neg.	*Klebsiella oxytoca*, HIV+
COCni	MP10	-	-	Organophosphate toxicity
(n=6)	MP17	Neg.	-	Reye's syndrome
	MP22	Neg.	Neg.	Hepatic necrosis, not infectious
	MP43	Neg.	Neg.	Reye's syndrome
	MP49	Neg.	-	Ruptured AVM
	MP58	Neg.	-	Acute lymphocytic leukemia

3.1.3. Clinical Features of Individual CM Patients

Detailed clinical information was collected for individual CM patients and is found in Table 4. CM patients were classified into two groups based on histopathological observations (for explanation of classification scheme see Section 3.4). As observed in previous studies, a statistically significant difference was not observed between the two classes of CM patients with respect to any of the clinical parameters (Taylor et al., 2004). Two of the patients in this clinical cohort had positive blood bacterial culture findings (MP11 and MP27). In each case the patient was treated with intravenous antibiotics but the clinical and histopathological evidence suggests that they died of CM. Both patients had marked *P.falciparum* peripheral parasitemias (MP11 = 29.5% of erythrocytes, MP27 = 61% of erythrocytes) and brain microvessel sequestration (MP11 = 94.17% vessels, MP27 = 84.68% vessels, Table 6). Two other CM patients were found to be HIV positive (MP37 and MP42) by a Rapid HIV Test. In either case there was no evidence of acquired immune deficiency syndrome (AIDS) or an AIDS-related-encephalopathy, and the patients had marked peripheral parasitemia, vascular plugging, and a clinical course consistent with CM.

Table 4 – Clinical features of individual CM patients included in this autopsy series.

Class	MP	Age (Mo.)	Sex (M/F)	HIV (+/-)	CSF Culture (+/-)	Blood Culture (+/-)	Duration (hrs)				BCS (0-5)	
							Illness	Fever	Coma	Conv.	Adm.	Last
I	16	51	F	Neg.	Neg.	Neg.	30	24	28	22	1	1
(n=5)	21	25	F	Neg.	-	Neg.	30	18	14	2	2	0
	25	44	F	Neg.	Neg.	Neg.	35	24	12	-	2	2
	37	6	M	Pos.	-	Neg.	86	48	14	-	2	5
	38	84	F	Neg.	Neg.	Neg.	251	216	40	5	0	0
Mean		42±13.1	4F, 1M	1+, 4-	0+, 3-	0+, 5-	86.4±42.5	66.0±37.8	21.6±5.41	9.66±6.22	1.4±0.41	1.6±0.96
II	05	14	M	Neg.	Neg.	Neg.	90	72	18	12	1	1
(n=20)	06	17	F	Neg.	-	Neg.	73	72	20	19	0	0
	09	16	M	Neg.	Neg.	Neg.	143	120	34	11	1	1
	11	41	F	Neg.	Neg.	Pos.	101	72	58	28	1	1
	13	22	M	Neg.	-	Neg.	96	96	16	-	0	0
	15	8	F	Neg.	Neg.	Neg.	34	22	23	5	2	2
	23	30	M	-	-	Neg.	64	58	12	5	0	1
	26	30	M	Neg.	Neg.	Neg.	78	48	32	-	1	1
	27	20	M	Neg.	-	Pos.	26	24	3	-	0	0
	28	61	F	Neg.	Neg.	Neg.	120	120	12	14	2	2
	29	43	M	Neg.	-	Neg.	62	48	29	18	2	2
	32	18	F	Neg.	Neg.	Neg.	27	24	27	24	0	0
	34	70	M	Neg.	Neg.	Neg.	74	72	39	-	1	1
	35	114	M	Neg.	Neg.	Neg.	62	48	27	12	1	1
	36	21	F	Neg.	Neg.	Neg.	83	72	13	-	2	0
	39	18	M	Neg.	-	-	65	48	24	6	2	2
	42	37	F	Pos.	-	Neg.	52	48	-	1	0	0
	48	48	M	Neg.	-	Neg.	94	72	26	-	2	1
	52	24	M	Neg.	Neg.	Neg.	69	36	-	-	1	0
	55	52	M	Neg.	-	Neg.	71	48	26	1	1	1
Mean		35.2±5.59	7F, 13M	1+, 18-	0+, 11-	2+, 17-	74.2±6.47	61.0±6.24	24.4±2.89	12.0±2.35	1.0±0.18	0.85±0.17
P-value		0.603	0.133	0.380	1.000	1.000	0.613	0.208	0.657	0.687	0.335	0.683

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3.2. Ocular Fundus Findings

Eye exams were performed on CM, SMA and COC patients where possible and several characteristics were considered including the presence and degree of retinal hemorrhage, retinal whitening (macular, central foveal and peripheral), retinal vessel abnormalities (retinal vessel whitening or discoloration) and papilledema (see Section 2.2.1). Of the 19 CM patients examined for the presence of papilledema, 5 were positive (5/19, 26.3%). Papilledema was only present in one SMA patient (MP57), one COCi patient (MP45), and two COCni patients (MP22 and MP49). A significant difference could not be found in the frequency of papilledema between patient groups. Whereas papilledema was observed in every clinical group, retinal whitening appeared to be more specific to malaria patients, and particularly CM patients. This finding was not present in COCi or COCni patients and was found in only one SMA patient (MP51). By contrast, retinal whitening was frequently present in CM cases (19/21, 90.4%), resulting in a significant difference between CM patients and SMA patients ($P<0.05$) and COCi ($P<0.001$) and COCni patients ($P<0.001$) with respect to this feature. Retinal vessel delineation appeared to be unique to CM as well. None of the SMA or COC patients appeared to have this feature whereas it was commonly present in CM patients (12/21, 57.1%). A significant difference was observed between the frequency of this finding in CM patients and COCi and COCni patients ($P<0.01$ and $P<0.05$ respectively). A similar picture was observed when considering the presence of retinal hemorrhage in this series. Only one patient in both the SMA and COCi patient groups showed the presence of retinal hemorrhage (MP30 and MP12 respectively) and none of the COCni patients showed retinal hemorrhage. 20/22 (90.1%) of CM patients had observable retinal hemorrhages. There were significant difference between the frequency of retinal hemorrhage in CM patients compared to SMA ($P<0.05$) and COCi ($P<0.001$) and COCni patients ($P<0.001$). These results are summarized in Table 5.

Table 5 – Summary of ocular fundus findings in this series.

Clinical Group	Papilledema		Retinal Whitening		Retinal Vessel Delineation		Retinal Hemorrhage	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
CM	5	14	19	2	12	9	20	2
SMA	1	3	1	3	0	4	1	3
COCi	1	7	0	8	0	8	1	7
COCni	2	3	0	5	0	5	0	5

3.3. General Neuropathological Features of Cerebral Malaria

Routine histopathological examinations of every case were carried out using H&E. The most striking feature in CM was the presence of distended microvessels (venules and capillaries) containing intraluminal parasitized erythrocytes (PE) (Figure 2). Sequestration of parasitized erythrocytes was present in every case of CM (see Section 3.4). In smaller microvessels it was possible to find the entire vessel occluded with PE while in the larger vessels, and pial vessels, sequestration was found to be in close apposition to the endothelial cells of the vessel wall only, with PEs rarely filling up the entire lumen. In certain instances, parasite sequestration was associated with fibrin thrombi with no obvious evidence of hemorrhage, though hemorrhage and sequestration were seen. Parasite sequestration was present in the vessels of both the grey and white matter with no apparent predilection for a given region. Several stages of the erythrocytic lifecycle of *P.falciparum* could be observed and the quantification of the various stages in the microvessels of the brain were used to develop a classification scheme for CM based on histopathology (see Section 3.4.).

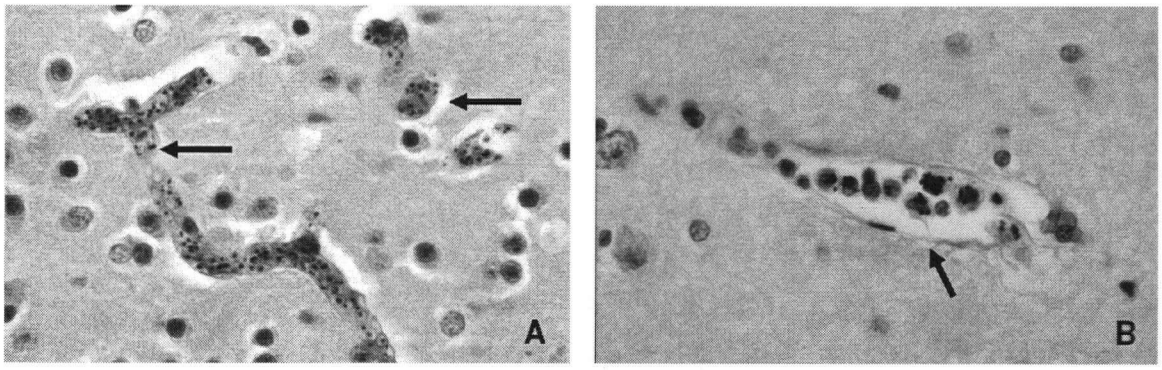

Figure 2 A-B: Sequestration of parasitized erythrocytes (PE). A: Small cortical vessels packed with PE. 40X. B: Cortical vein filled with PE. 40X.

Given the possibility of an anoxic or ischemic event in CM due to parasite sequestration throughout the brain (see Section 1.3.2.1.), attempts were made to identify anoxic neurons, which appear shrunken with eosinophilic cytoplasm and pyknotic nuclei. Although it was possible to identify these neurons in a few cases of CM (Figure 3), anoxic neurons were not a common feature in this group. An important and common finding in CM patients, and one that was most intimately associated with parasite sequestration, however, were perivascular ring hemorrhages, found primarily in the white matter (see Section 3.5.). Contrary to the observations in other studies of CM (see Section 1.4.3.), Durck's granulomas were not a feature of CM in this series.

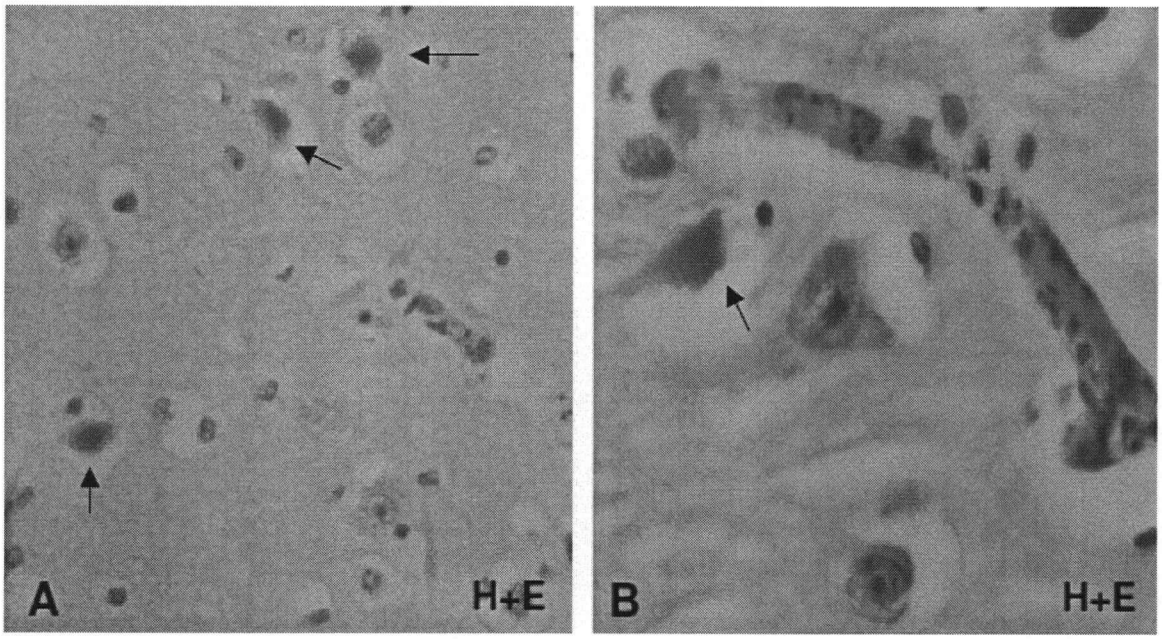

Figure 3 A-B: Anoxic neurons in CM. A: Several anoxic neurons scattered in the cortex from a CM patient. 40X. B: Highly eosinophilic anoxic neuron in close apposition to a microvessel with PE. 100X.

3.4. Classification of Cerebral Malaria by Parasite Sequestration

The sequestration of parasitized red blood cells in brain microvessels is recognized to be a major feature of CM, and a recent study by Taylor *et al.* (2004) showed that the presence of sequestration is useful for distinguishing CM patients from patients that developed coma of non-malarial etiology. This study also showed that the relative distribution of the various parasite stages sequestered in the brains of CM may be useful to determine whether different pathogenetic mechanisms are occurring in CM patients. To assess this hypothesis, different stages along the parasite lifecycle were counted by Taylor *et al.* (2004) as described (Taylor et al., 2004) (see Section 2.1.2.): unpigmented parasites, pigmented parasites, and pigment globules. These counts were then compared using CART analysis to establish whether individual groups could be distinguished in CM by the prevalence of a given parasitic element. From this analysis, two Classes of CM patients emerged: Class I (n=5) and Class II (n=20) which differed by the presence of the number of pigment globules measured. Neither the percentage of vessels parasitized, nor the number of pigmented or unpigmented parasites could

distinguish the two classes. Table 6 summarizes the parasite counts obtained in the individual Class I and Class II CM patients in this study.

Table 6 - Classification of CM by parasite sequestration.

CM Class	Case	Percentage of Vessels Parasitized	Number of Pigmented Parasites/vessel	Number of Unpigmented Parasites/vessel	Number of Pigment Globules/vessel
Class I	MP16	76.70	0.09	3.10	0.16
	MP21	96.15	0.00	6.97	0.26
	MP25	94.39	0.00	3.80	0.15
	MP37	26.21	0.00	0.05	0.44
	MP38	62.39	0.51	0.81	0.24
Mean±SE		71.17±12.83	0.12±0.10	2.95±1.22	**0.25+0.05 (P<0.05)**
Class II	MP05	89.32	0.04	5.84	1.24
	MP06	85.58	0.00	2.03	1.95
	MP09	23.81	0.00	0.06	0.42
	MP11	94.17	0.00	2.09	4.85
	MP13	96.23	0.24	3.38	0.98
	MP15	83.81	0.01	2.05	1.55
	MP23	67.96	0.09	0.63	1.13
	MP26	56.31	0.00	1.11	1.89
	MP27	84.68	0.20	2.50	1.01
	MP28	96.15	0.01	2.43	0.93
	MP29	86.36	0.03	2.74	1.16
	MP32	77.23	0.05	1.52	2.02
	MP34	91.51	0.01	3.60	1.26
	MP35	83.70	0.00	1.93	1.50
	MP36	45.05	0.04	0.35	1.63
	MP39	93.14	0.00	4.12	1.37
	MP42	95.19	0.00	2.88	0.67
	MP48	68.27	0.00	0.83	2.46
	MP52	89.32	0.04	5.84	1.24
	MP55	85.58	0.00	2.03	1.95
Mean±SE		79.67±4.24	0.04±0.02	2.40±0.36	**1.56+0.21 (P<0.05)**

3.5. Perivascular Ring Hemorrhages in Cerebral Malaria

Perivascular ring hemorrhages were a common finding in CM patients, but were not present in every instance of CM, appearing in 18 of the 25 CM cases examined (18/25, 72%). Perivascular hemorrhages were not present in SMA patients or age-matched controls without neurologic dysfunction but were present in a minority of COCi patients (MP24, 1/13, 7.69%) and COCni patients (MP10, 1/6, 16.6%). Malaria pathology in the form of perivascular ring hemorrhages was not expected in either of these cases. MP10 had an incidental parasitemia and MP24 had a negative blood smear. Despite little or no evidence of peripheral parasitemia in these patients, however, both showed evidence of parasitized erythrocyte sequestration within their brain microvessels, suggesting that malaria may have been a complicating feature in these patients despite the clinical picture. Of all the patient groups examined, only CM patients were significantly different from the age-matched-control patients based on the frequency of patients with hemorrhage ($P<0.01$, Table 7). In addition to the frequency, the average number of hemorrhages was calculated across the entire CNS in all groups. The CM group was the only one to show an appreciable number of hemorrhages and to differ significantly from controls ($P<0.05$). CM patients also differed from SMA and COC patients in the frequency of patients with hemorrhages and the overall number of hemorrhages ($P<0.01$, Table 7 and Figure 4).

Table 7 – Frequency of perivascular ring hemorrhages in CM and controls.

Patient Group	No. of Patients with Hemorrhage	No. of Patients without Hemorrhage	Fisher Exact Test (versus control)	Fisher Exact Test (versus CM)
CM (n=25)	18	7	**P<0.01**	-
SMA (n=5)	0	5	P=1.000	**P<0.01**
COCinfectious (n=13)	1	12	P=1.000	**P<0.01**
COCother (n=6)	1	5	P=1.000	**P<0.01**
Controls (n=5)	0	5	-	**P<0.01**

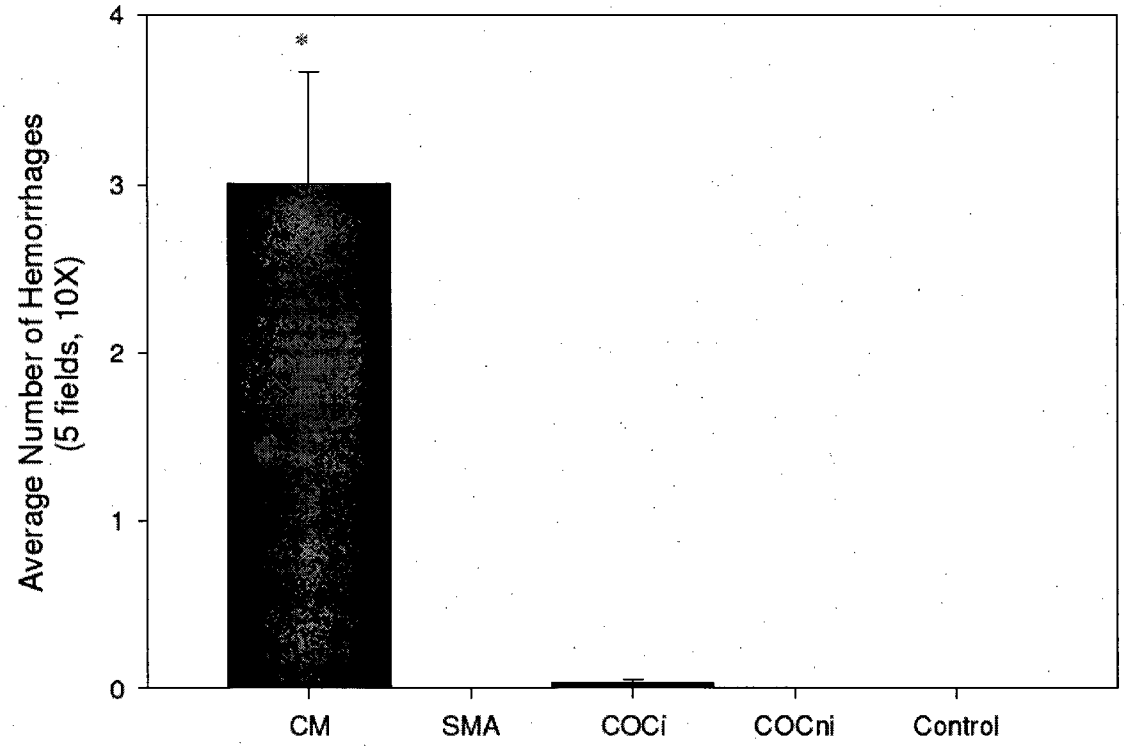

Figure 4: Comparison of the number of perivascular ring hemorrhages in CM patients and controls. Average number of hemorrhages observed across all brain regions. H&E. 10X.

Hemorrhages consisted of a central vessel containing parasitized red blood cells, an inner ring of myelin damage and axonal necrosis (see Sections 3.6. and 3.7.), and an outer ring of extravasated infected and uninfected red blood cells (Figure 5). Hemorrhages localized to the white matter and were only found up to the grey matter white matter junction in the cerebral hemispheres.

The distribution of hemorrhages across the CNS in CM patients was not uniform. Comparisons between the number of hemorrhages in individual brain regions revealed that there was a statistically smaller number of hemorrhages in the brainstem (BS) than in the other brain areas ($P<0.05$, Figure 6). There was no statistically significant difference between the number of hemorrhages observed in cerebral hemispheres (CH), caudate and thalamus (C+T) and cerebellum (Cer.). There was a positive correlation between the number of hemorrhages observed in individual brain sites, suggesting that when there

was an increased number of hemorrhages in a given region of the brain it is likely that hemorrhages will be increased across the entire CNS (Table 8), confirming what is observed histologically.

Table 8– Correlations between number of hemorrhages observed in various brain regions in CM.

Brain Region	CH	C+T	BS	Cer.
CH	1	**R=0.658, P<0.001**	**0.608, P<0.01**	**0.648, <0.001**
C+T		1	**0.563, P<0.01**	**0.492, P<0.05**
BS			1	0.216, P=0.216
Cer.				1

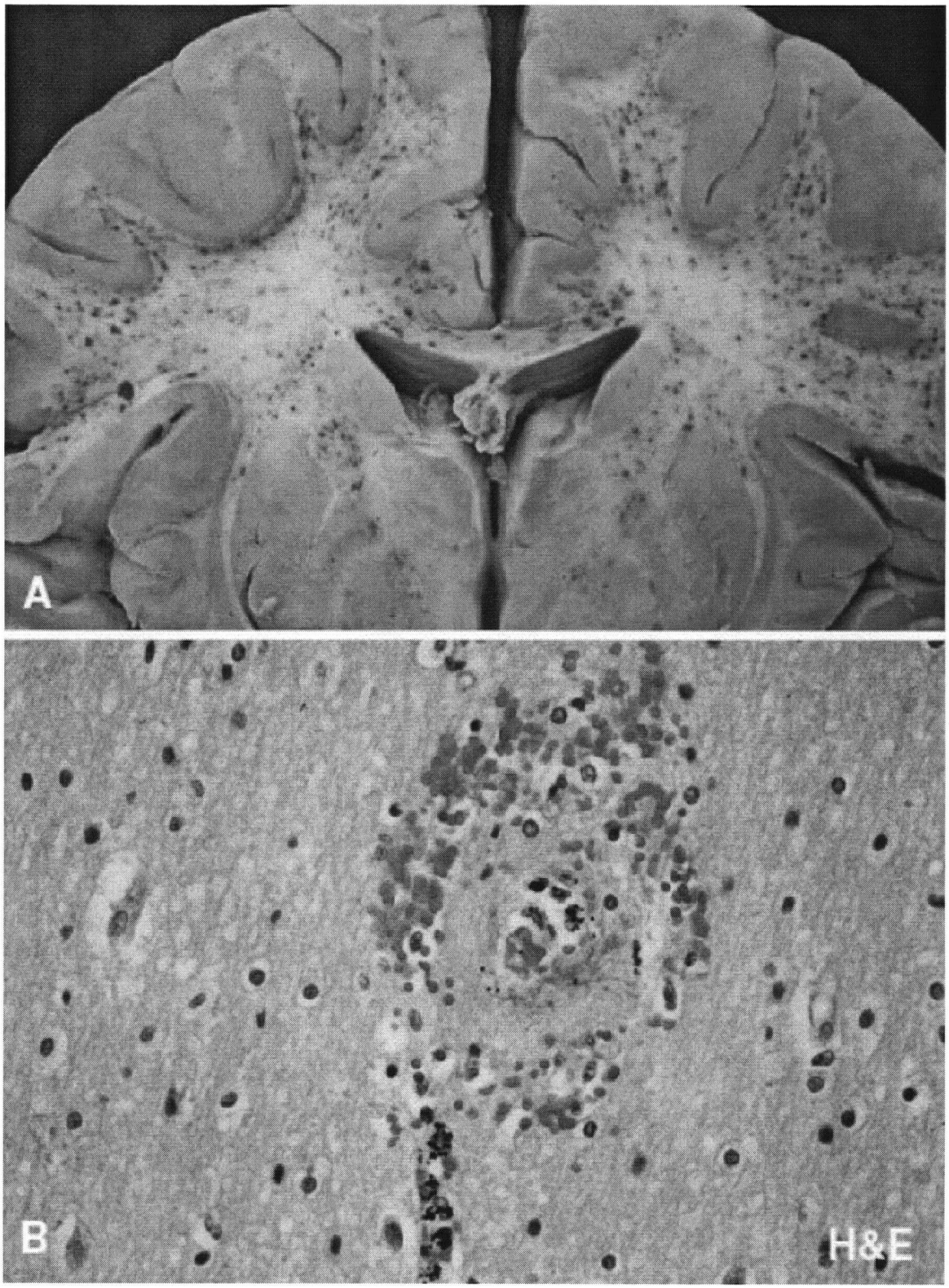

Figure 5 A-B: Hemorrhages in Cerebral Malaria. A: Gross-pathology showing petechial and ring hemorrhages restricted to the white matter. B: H&E showing morphology of ring hemorrhage. 40X

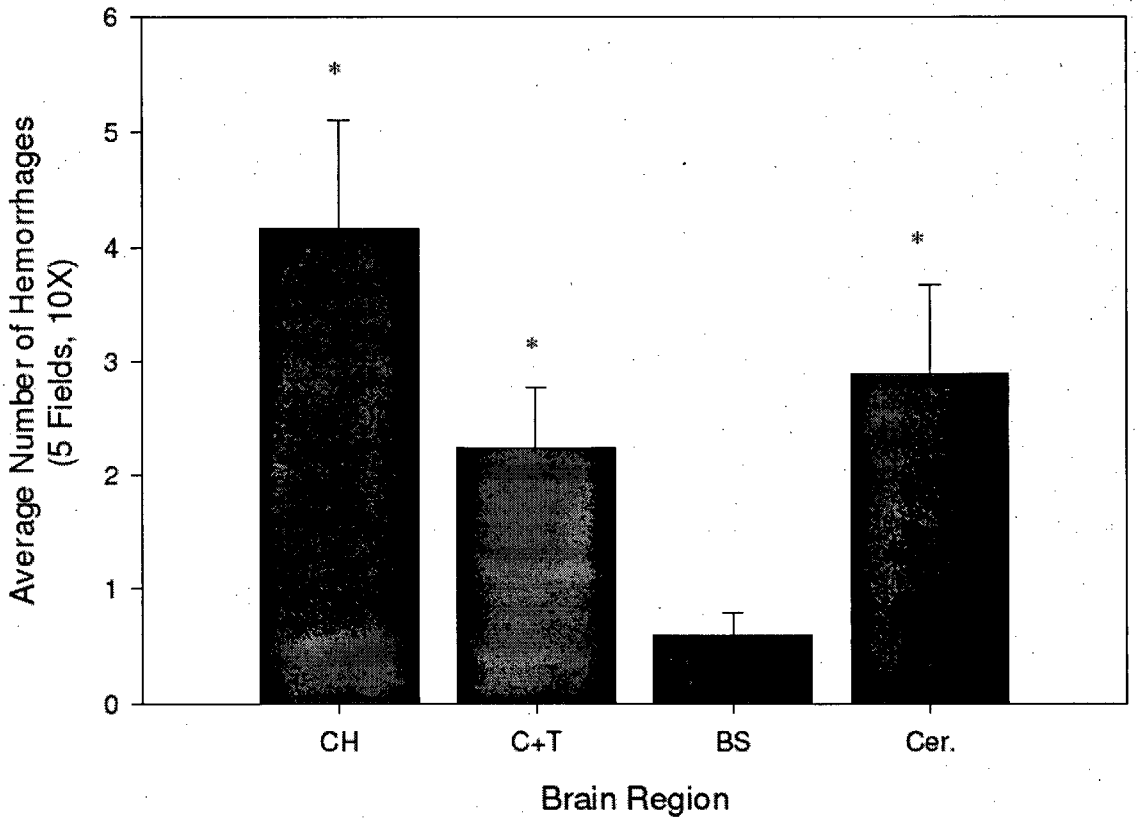

Figure 6: Distribution of perivascular ring hemorrhage in CM. (*, significant difference from SMA and COC patients ($p<0.05$).

3.5.1. Correlation of Ring Hemorrhages with Parasitological Data

The various measures of parasite sequestration were correlated with the number of cerebral hemorrhages observed in CM patients. Statistical analysis reveals that Class II CM patients had a greater number of hemorrhages than Class I CM patients ($P<0.05$, Figure 7). In a separate comparison, a positive correlation between the number of hemorrhages and the number of pigment globules observed in the parietal lobe of CM patients was observed ($R=0.402$, $P<0.05$, Figure 8). Similar results could not be found between the number of hemorrhages and other measures of sequestration (Table 9).

Table 9 – Pearson correlations coefficients between hemorrhage and different counts of parasite sequestration in CM patients.

Measure of Sequestration (Parietal Lobe)	Hemorrhage (R value, P-value)
Percentage of Sequestered Vessel	R=-0.166, P=0.448
#Unpigmented Parasites/Vessel	-0.288, P=0.103
#Pigmented Parasites/Vessel	-0.331, P=0.123
#Pigment Globules/Vessel	**0.402, P<0.05**

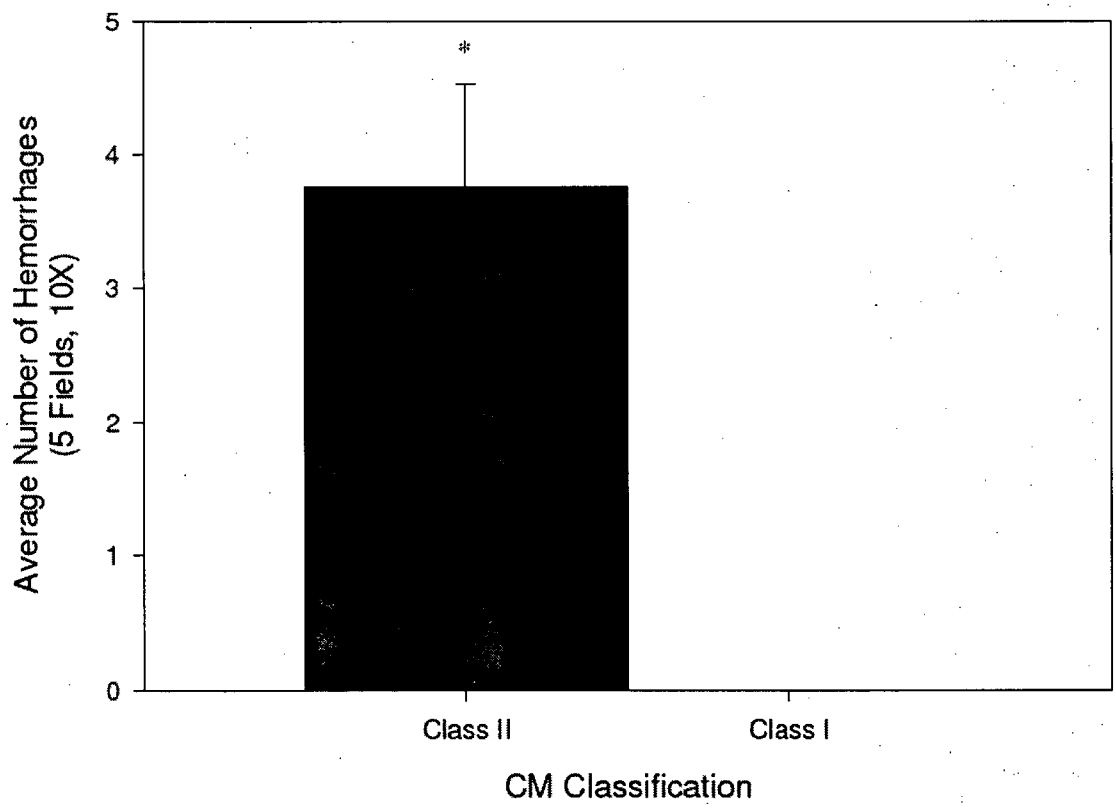

Figure 7: Perivascular ring hemorrhage in Class I and Class II patients. (*, $p<0.05$).

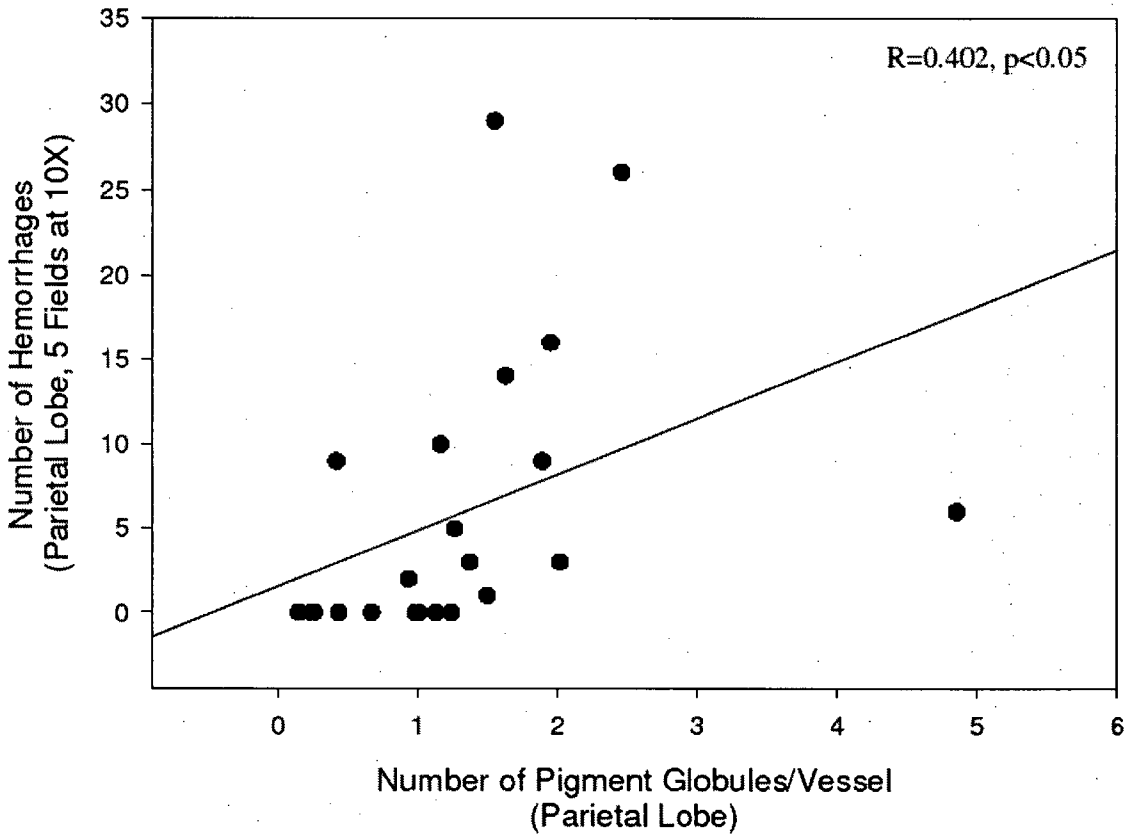

Figure 8. Correlation between total number of perivascular ring hemorrhages and number of pigment globules/vessel. (R=0.402, p<0.05)

3.5.2. Clinicopathological Correlation of Ring Hemorrhage

To understand the significance of hemorrhages in CM we carried out a detailed series of clinicopathological correlations between the average number of hemorrhages over all brain regions and several clinical and laboratory parameters. Of the parameters investigated, negative correlations were found between the average number of ring hemorrhages and the parasitemia and hematocrit measurements taken on admission (Table 10). Also, CM patients with documented seizures during admission had significantly more hemorrhages than patients without convulsions (6.91 ± 1.53 hems/5fields compared to 1.78 ± 1.08 hems/5fields, $P<0.05$). Furthermore, patients with an increased number of hemorrhages over all brain regions in CM had longer coma durations and lower parasitemias (Table 11).

Table 10 – Perivascular ring hemorrhages correlate negatively with parasitemia and hematocrit counts.

Clinical or Laboratory Parameter	R-value, P-value
Asexual Parasitemia (10^3 per µl blood, adm.)	**-0.428, P<0.05**
Hematocrit (%, adm.)	**-0.511, P<0.01**

Table 11 – Patients with increased number of hemorrhages at autopsy experienced longer duration of coma and lower peripheral parasitemias.

Clinical Parameter	High Hemorrhage (>3 Hems/5 Fields)	Low Hemorrhage (<3 Hems/5 Fields)	P-value
Duration of Coma (hrs)	**29.80+3.91**	**19.15+2.73**	**P<0.05**
Parasitemia on Admission (10^3/µl blood)	**134.04+49.90**	**414.78+96.76**	**P<0.05**

3.5.3. Relationship to Ophthalmic Pathology and Ocular Fundus Findings

Retinal hemorrhages observed by indirect ophthalmoscopy were a major clinical feature of CM and recent evidence suggests that there is a correlation between the number of brain hemorrhages observed at autopsy and the number of retinal hemorrhages observed clinically (see Section 1.2.2). To confirm the relationship between brain and retinal hemorrhages, comparisons were made between the average number of hemorrhages observed across the entire CNS and (1) the number of hemorrhages observed on gross examination of whole eyes obtained at autopsy and (2) the number of hemorrhages observed in the retina on histological exam.

Retinal hemorrhage was a common autopsy feature of CM patients, present in 17/19 (89.5%) eyes on gross pathological examination and 18/22 (81.8%) CM retinas examined histopathologically. Positive correlations were found between the number of hemorrhages observed in the brain in CM and the number of hemorrhages observed in the eye on gross examination ($R=0.544$, $P<0.05$) and on histopathological examination of the retina ($R=0.415$, $P<0.05$). A positive correlation or association was also found between the number of hemorrhages measured on clinical exam (by indirect ophthalmoscope) and

the number of hemorrhages observed across the CNS in CM (R=0.419, p<0.05) (Table 12). Despite a relationship between retinal hemorrhages and brain hemorrhages in CM, it is important to recognize that retinal hemorrhages are not a feature of CM patients only, and were observed infrequently in SMA and COC patients (infectious and non-infectious) (see Section 3.2.) despite the lack of brain hemorrhages in these groups.

Table 12 – Positive correlations are observed between brain and retinal hemorrhages in CM.

Case	Mean No. Brain Hemorrhage (5 fields, 10X)	Hemorrhage on Clinical Exam (Retina, 0-3)	Gross Pathology (Whole Eye, 0-2)	Histopathology (Retina, 0-2)
MP05	1.64	1	1	1
MP06	6.5	-	-	-
MP09	4.4	2	2	2
MP11	3.8	1	2	2
MP13	1.10	-	-	0
MP15	9.9	1	1	1
MP23	0	1	1	1
MP26	7	1	2	2
MP27	0	1	1	1
MP28	1	-	-	-
MP29	5	2	2	2
MP32	1.82	3	2	2
MP34	4.2	2	2	2
MP35	0.72	1	1	1
MP36	7.55	3	2	2
MP39	3	1	-	-
MP42	0.36	1	2	1
MP48	9.7	3		0
MP52	6.63	1	2	2
MP55	1.09	3	2	1
MP16	0	1	-	1
MP21	0	1	2	2
MP25	0	1	1	1
MP37	0	0	0	0
MP38	0	0	0	0
No. Positive Patients	18/25	20/22	17/19	18/22
Correlation with Brain	-	**R=0.419 P<0.05**	**R=0.544, P<0.05**	**R=0.415, P<0.05**

3.6. Axonal Damage in Cerebral Malaria

Axonal injury reduces neural function and leads to the abnormal distribution of neurosecretory granules, proteins and enzymes involved in neurotransmission (Medana et al., 2002). A key marker for axonal injury is the presence of β-APP (beta-amyloid precursor protein) by immunohistochemistry. β-APP is transported along the lengths of axons and is not identifiable by IHC in normal tissue. It is only detectable when axonal injury allows for the focal accumulation of β-APP at the injured site. In this way, β-APP staining allows for the identification of axons that have undergone biochemical or structural changes that might have otherwise been overlooked by routine neuropathological examination (Medana et al., 2002). In this series, axonal damage was found in CM patients (24/25, 96%), SMA patients (3/5, 60%) and patients dying from comas of other causes (7/19, 36.8%). Positive staining was not observed in the normal aged-matched controls (Table 13). All but one of the CM patients had APP staining (MP37). Interestingly, MP37 had relatively minor neuropathological changes in general, showing no hemorrhages and only minimal BBB dysfunction or gliosis.

Table 13 – Frequency of axonal damage in CM patients and controls.

Patient Group	No. of Patients with Axonal Damage	Total Number of Patients	Fisher Exact Test (versus cont.)	Fisher Exact Test (versus CM)
CM	24	25	**P<0.001**	-
SMA	3	5	P=0.167	P=0.064
COCinfectious	6	13	P=0.128	**P<0.001**
COCother	1	6	P=1.000	**P<0.001**
Age-Matched Controls	0	5	-	**P<0.001**

Of all the patient groups, only CM patients differed significantly from the age-matched controls in the frequency of axonal damage (P<0.001). Differences were also observed between CM patients and COC patients dying of infectious and other causes (P<0.001). A significant difference could not be found between the frequency of patients with axonal damage in CM versus the SMA group (Table 13). Although some axonal damage was found to some degree in each patient group, the severity of axonal damage

was minimal in the SMA and COC groups compared to that observed in CM. Measurements made of the average axonal damage over all brain regions in each patient group demonstrated that CM patients had significantly more APP areas than SMA and COC patients and controls (Figure 9, $P<0.01$).

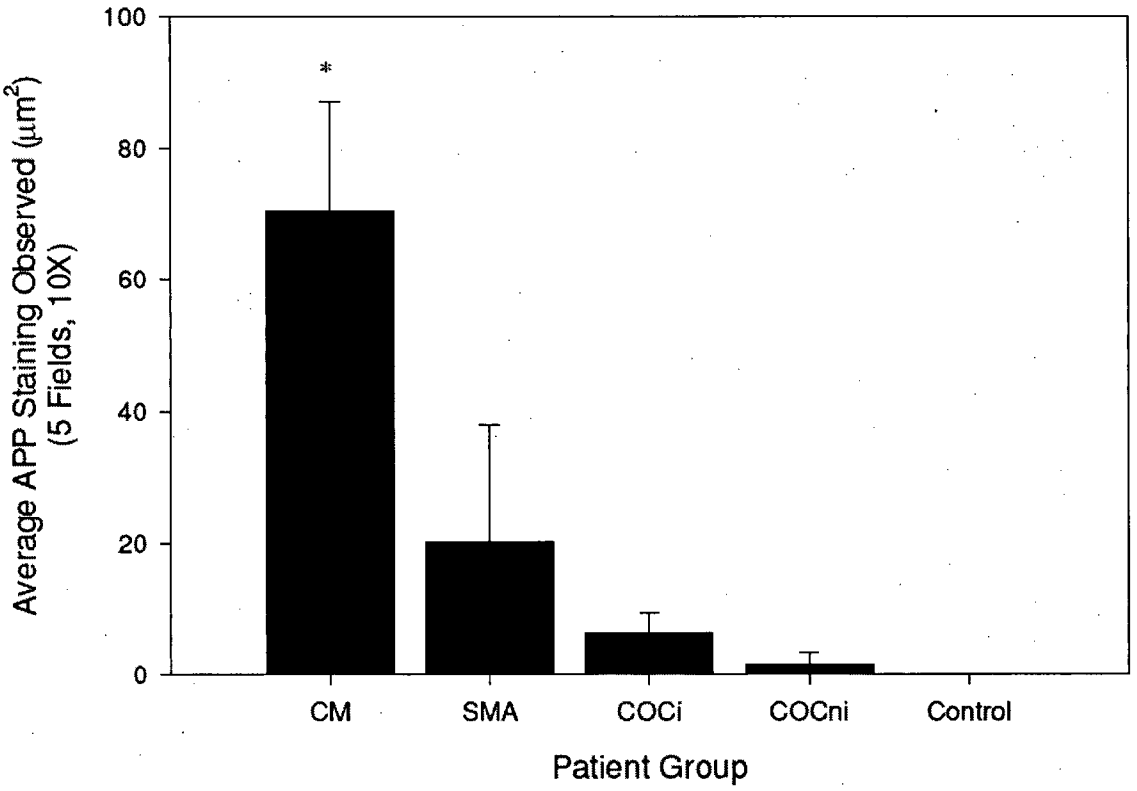

Figure 9. Axonal damage in CM patients and controls. (*, significant difference between CM and all groups, $p<0.001$).

The distribution of axonal damage was not uniform across the CNS of CM patients. Axonal damage appeared to be greatest in the CH and decreased caudally towards the cerebellum (Figure 10). Statistical analysis revealed that the only statistical differences between any regions in the CM group was between the CH and both the BS and Cer. There was a statistically significant difference between the amount of APP staining observed over all the regions when comparing CM patients to SMA and COC patients, except in the CH where a significant difference between the extent of APP staining between CM and SMA patients could not be found. SMA patients had a

significant amount of axonal damage in the CH, accounting for the lack of a significant difference between them and the CM patients.

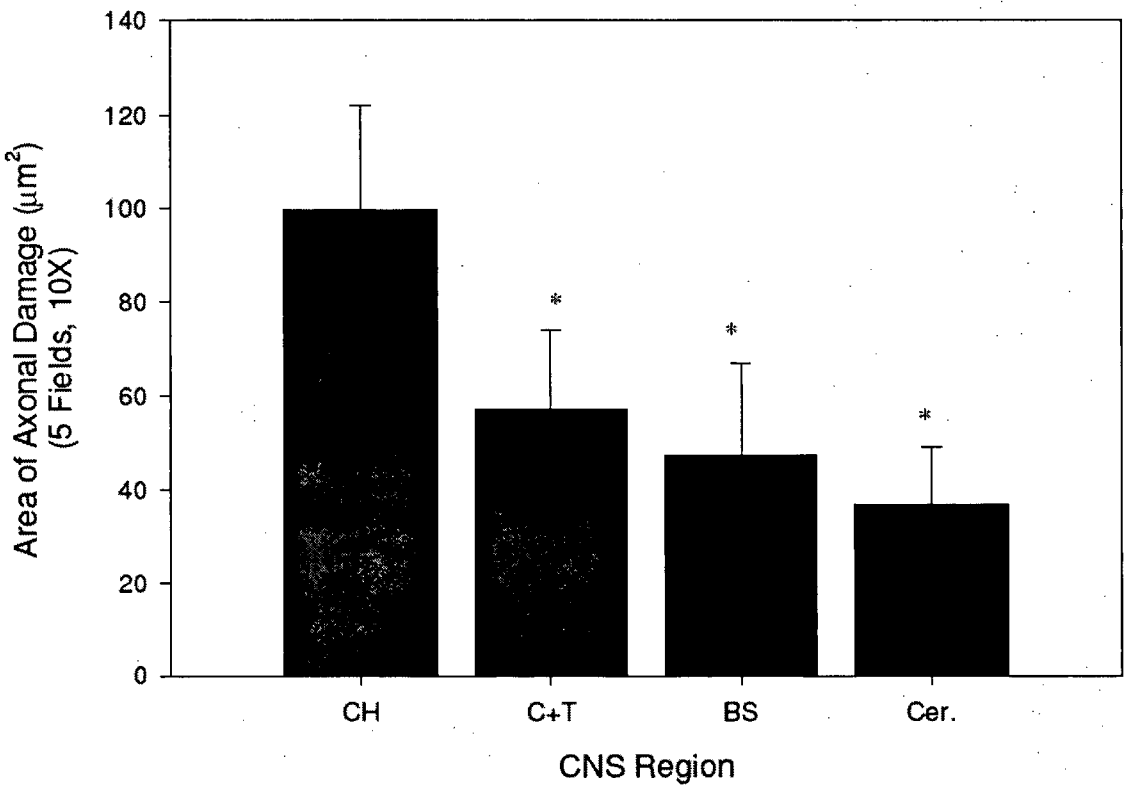

Figure 10. Distribution of axonal damage in CM (*, significant difference between CM and all control groups).

Despite the lack of uniformity in the amount of APP staining across the various regions of the CNS, there was a strong correlation between the APP staining observed in various regions (Table 14). This confirms what was observed histologically. If an individual patient appeared to have extensive axonal damage in one region of the CNS, it was likely that the patient would have an increased amount of APP staining in other regions of the CNS as well.

Table 14 – Correlation between the degree of axonal damage in the various regions of the brain in CM.

Brain Region	CH	C+T	BS	Cer.
Cerebral Hemispheres (CH)	1	**R=0.692**	**0.520**	**0.679**
Caudate and Thalamus (C+T)		1	**0.864**	**0.571**
Brainstem (BS)			1	**0.476, P<0.05**
Cerebellum (Cer.)				1

*P<0.001 unless otherwise noted

3.6.1 Patterns of Axonal Damage in Cerebral Malaria

Two distinct histological patterns emerged from the β-APP staining in CM cases: positive perivascular APP staining associated with hemorrhage (HA, hemorrhage associated), and diffuse APP staining not associated with hemorrhage (NHA, non-hemorrhage associated) (Figure 11). These patterns were clearly distinguishable histologically. HA APP staining was confined to the central necrotic region of the hemorrhage between the apparently burst vessel and the outer ring of extravasated infected and uninfected red blood cells. The degree of axonal damage in a given hemorrhage varied. In some hemorrhages, APP staining was strong and concentrated in the vicinity of the damaged vessel, while in other areas the APP staining appeared lighter with fewer axonal spheroids and dilated axons. NHA regions of APP positivity appeared as large, irregular, and diffuse areas with ill-defined boundaries. The NHA lesions often contained numerous dilated axons and axonal spheroids and appeared as waves of axonal damage across large sections of white matter. On higher magnification, many of these lesions appeared to be associated with a highly vacuolated neuropil. Although as a group the NHA lesions shared similar features from section to section, some lesions appeared to be more advanced than others, with darker staining, and more dilated axons.

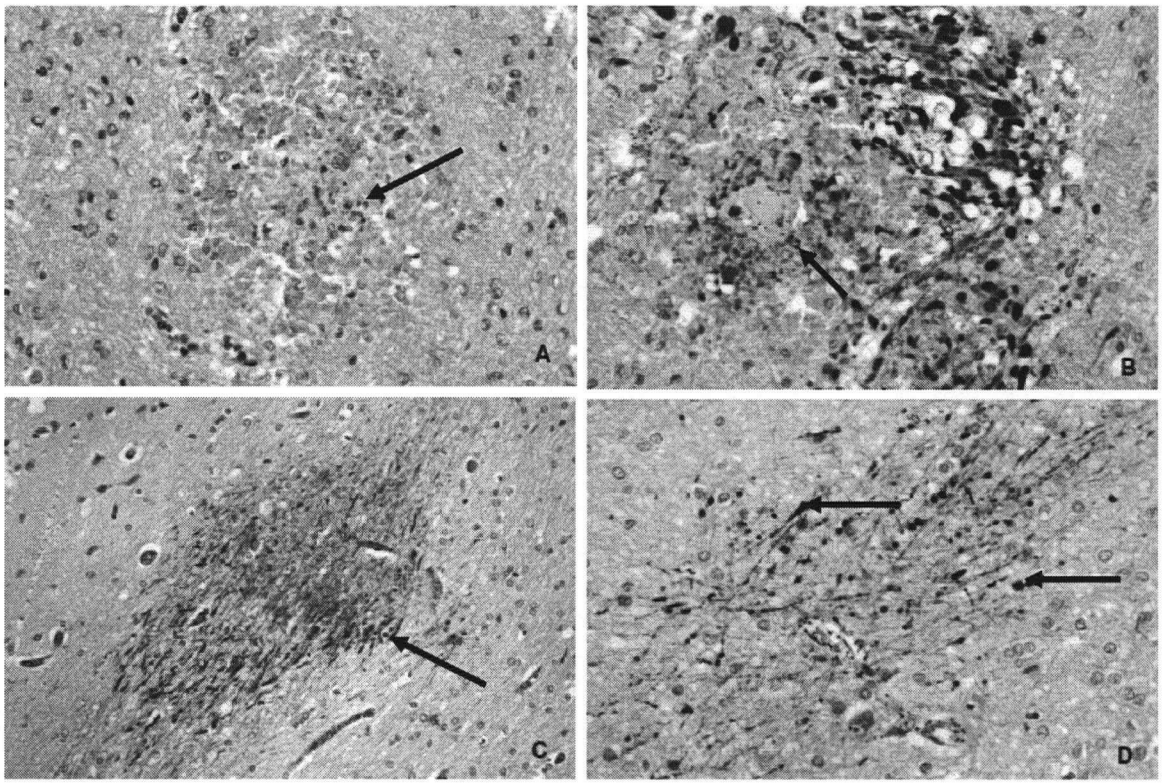

Figure 11 A-D: Types of axonal damage in CM. A-B: Axonal damage associated with hemorrhage (HA). A-40X and B-100X. Note the inner area of axonal damage close to the apparently burst vessel (arrow). C-D: Axonal damage not associated with hemorrhage (NHA). Lesion boundaries are not clearly demarcated and the lesions are associated with a significant number of axonal spheroids (arrows). C-40X and D-100X

Although both HA and NHA axonal pathology can be observed in individual CM patients, many patients had evidence of either HA or NHA pathology, suggesting that these types of axonal injury are independent of each other. As expected, SMA patients with axonal pathology only had the NHA type (hemorrhages are not a feature of this group). COCi patients had HA or NHA pathology or both. MP18, which under H&E staining appeared to not have hemorrhages showed evidence of both HA and NHA axonal pathology. MP12, which did not show hemorrhages under H&E showed hemorrhage associated APP staining, but not NHA APP staining. Only one case from the

COCni patients had APP staining (MP49), which was not associated with hemorrhage. We used morphometric analysis to determine the relative prevalence of NHA versus HA β-APP staining. The results indicate that most of the axonal damage in CM is not associated with hemorrhage (Figure 12).

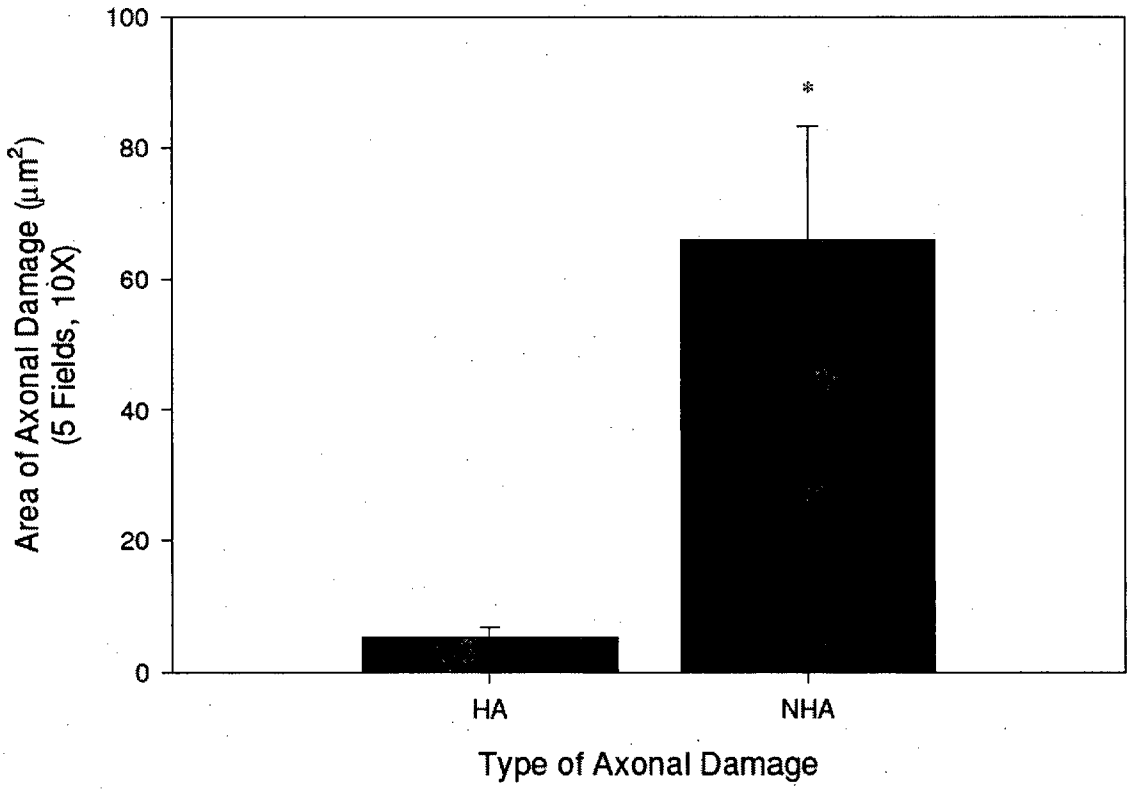

Figure 12. Relative frequency of HA and NHA axonal damage in CM patients. (*, $p<0.05$)

3.6.2. Relationship between Axonal Damage and Parasitological Data

A series of comparisons were made between counts of parasite sequestration and measures of NHA APP expression in CM patients. A strong correlation was found between the degree of NHA APP staining of CM patients and the number of pigment globules/vessel counted (Table 15 and Figure 13). Also, patients with high amounts of

NHA APP staining (>100 μm^2) had a statistically greater number of pigment globules in the parietal lobe compared to those with decreased amount of overall NHA APP (<100μm^2). Therefore, it appears that the only relevant parasitological association between sequestration and APP staining is the presence of pigment globules. Comparisons between Class I and Class II patients demonstrate that NHA axonal damage is common to both groups with the average NHA APP staining slightly greater in Class II patients, though not to a significant degree due potentially to a lack of cases (Figure 14).

Table 15 – Pearson correlations coefficients between NHA APP and parasite sequestration counts in CM.

Measure of Sequestration (Parietal Lobe)	NHA APP (R value, P-value)
Percentage of Sequestered Vessel	R=0.164, P=0,466
#Unpigmented Parasites/Vessel	-0.258, P=0.247
#Pigmented Parasites/Vessel	0.0416, P=0.854
#Pigment Globules/Vessel	**0.766, P<0.001**

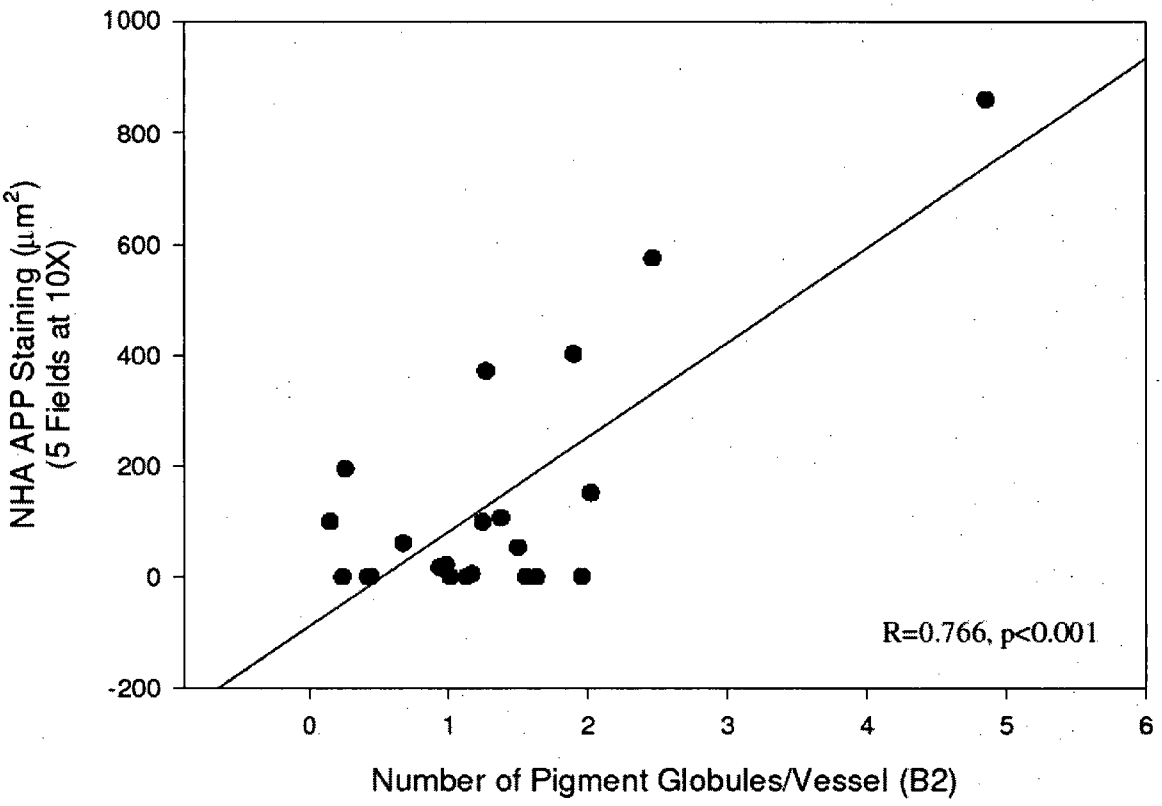

Figure 13. Correlation between degree of NHA axonal damage and the number of pigment globules/vessel. (R=0.766, p<0.001).

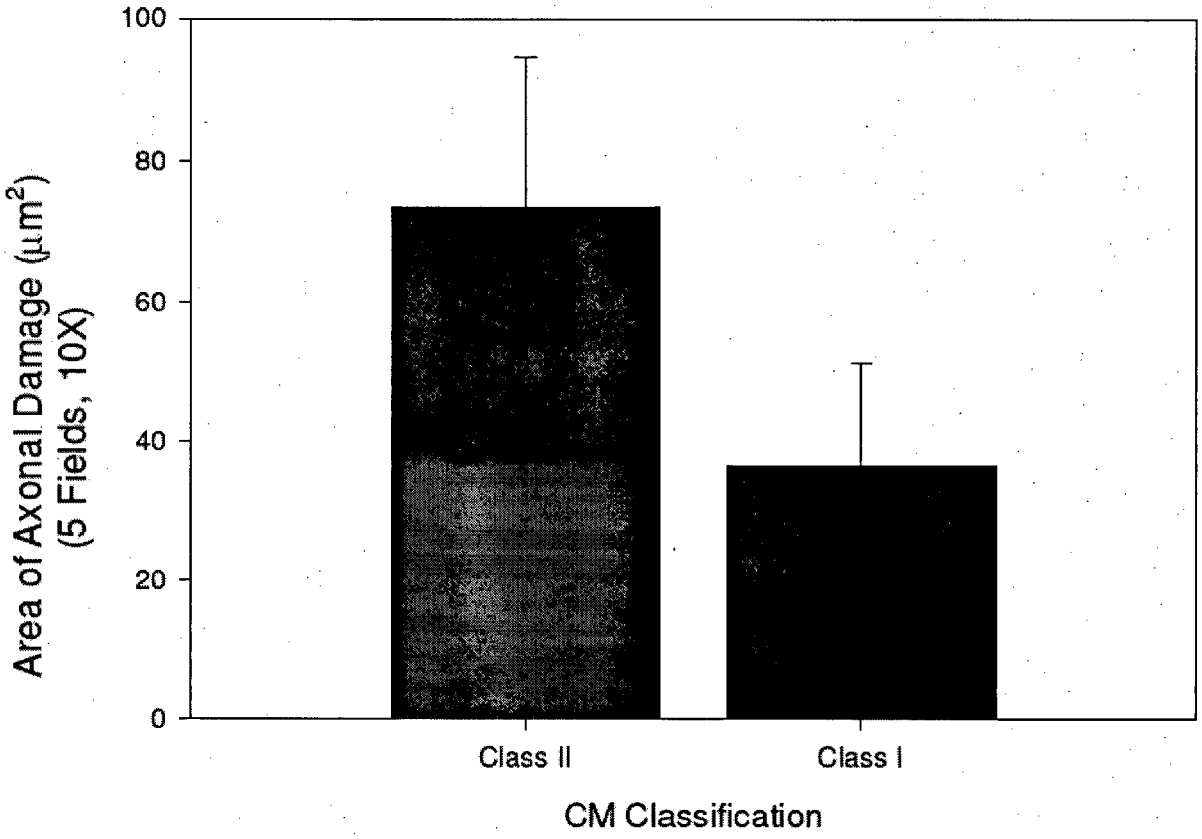

Figure 14. Axonal damage in class I and Class II patients.

3.6.3. Clinicopathological Correlation of Axonal Damage

To understand the role of axonal damage in the pathogenesis of CM we carried out detailed clinicopathological correlations between the extent of NHA APP staining in the brains of CM patients and several clinical and laboratory parameters. A number of correlations were observed including a negative correlation between the total NHA APP staining in the CNS of individual CM patients and the last blood glucose level before death ($R=-0.485$, $P<0.05$). This suggests that lower blood glucose levels may be predictive of more severe NHA axonal damage (Figure 15). There was also a positive correlation between the extent of axonal damage and duration of coma in that patients with areas of β-APP staining $>100\mu m^2$ had longer duration of coma and lower blood glucose levels ($P<0.05$, Table 16).

Table 16 – CM patients with severe axonal damage (NHA APP >100μm^2) have significantly longer coma duration and lower blood glucose levels than CM patients with less severe axonal damage (NHA APP <100μm^2).

Clinical Parameter	High NHA APP (>100μm^2, n=7)	Low NHA APP (<100μm^2, n=18)	P-value (t-test)
Coma Duration (hrs)	**31.86±5.02**	**20.25±2.47**	**P<0.05**
Last Blood Glucose (mmol/L)	**4.34±0.49**	**7.29±0.67**	**P<0.05**

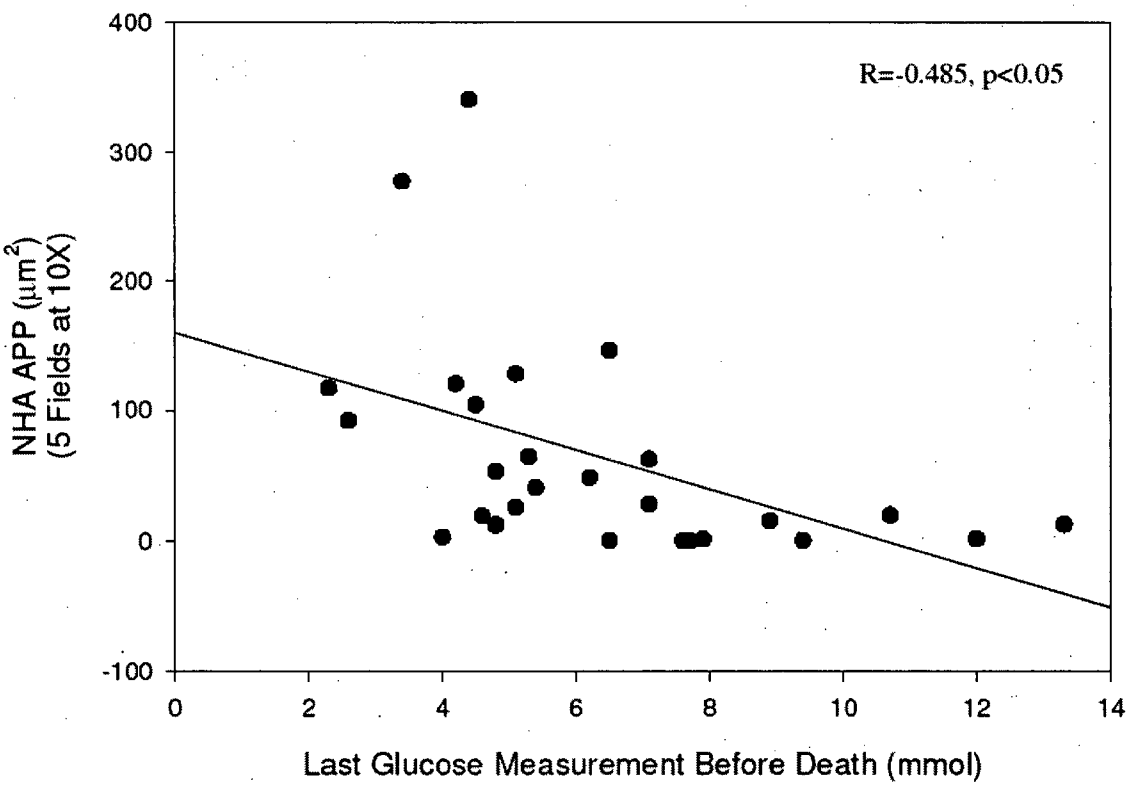

Figure 15. Correlation between NHA APP and last glucose measurement before death. (R=-0.485, p<0.05)

3.6.4. Relationship to Ophthalmic Pathology and Ocular Fundus Findings

β-APP immunohistochemical staining of retinal sections revealed that axonal damage is common in the retinas of CM patients, appearing in 15/23 cases (65.2%, $P<0.05$ compared to age-matched controls). SMA patients did not have evidence of axonal damage in their retina. Only one of the COC patients (from both infectious and non-infectious groups) had retinal APP staining (MP10, 1/16 6.25%). Age-matched controls did not have evidence of retinal axonal damage. Fisher's exact test revealed that based on the limiting number of patients per group, the only statistically significant difference that existed was between CM and COCi patients (Table 17).

Table 17 – Frequency of axonal damage in the retinas of CM patients and controls.

Patient Group	No. of Patients with Retinal Axonal Damage	No. of Patients without Retinal Axonal Damage	Fisher Exact Test (versus control)	Fisher Exact Test (versus CM)
CM	15	8	P=0.220	-
SMA	0	4	P=1.000	P=0.098
COCinfectious	1	12	P=1.000	**P<0.01**
COCother	0	4	P=1.000	P=0.098
Age-Matched Controls	0	2	-	P=0.220

To determine whether a positive correlation could be obtained between brain and retinal APP staining, the amount of APP staining in the retinas of individual CM patients was measured by morphometric analysis and compared to the total amount of axonal damage in the brains of CM patients (over all regions). A positive Pearson correlation was observed ($R=0.490$, $P<0.05$, Table 18). In a separate analysis, the degree of retinal APP staining was compared with the degree of APP staining in individual regions of the CNS. A positive correlation was found between the degree of retinal APP staining and the degree of brain APP in the CH ($R=0.476$, $P<0.05$). Similar associations could not be found in the C+T, BS or Cer. (Table 18). Therefore, there seems to be an association between the degree of retinal and brain APP staining in CM.

Table 18 – Correlation between brain and retinal axonal damage in CM.

Brain Region	Pearson Correlation Coefficient (Brain vs. Retina, R-value, P-value)
Overall	**R=0.490, P<0.05**
Cerebral Hemispheres (CH)	**0.476, P<0.05**
Caudate and Thalamus (C+T)	0.323, P=0.191
Brainstem (BS)	0.0346, P=0.892
Cerebellum (Cer.)	0.299, P=0.244

Further evidence for a link between brain and retinal APP staining came from the observation that the same two histological patterns of axonal damage observed in the brains of CM patients, namely, hemorrhage associated APP staining and non-hemorrhage associated APP staining (HA and NHA staining, see Section 3.6.1) were observed in the eyes of CM patients (Figure 16). Together, these findings suggest that at least at the histological level, there is a clear association between brain and retinal axonal damage in this disease.

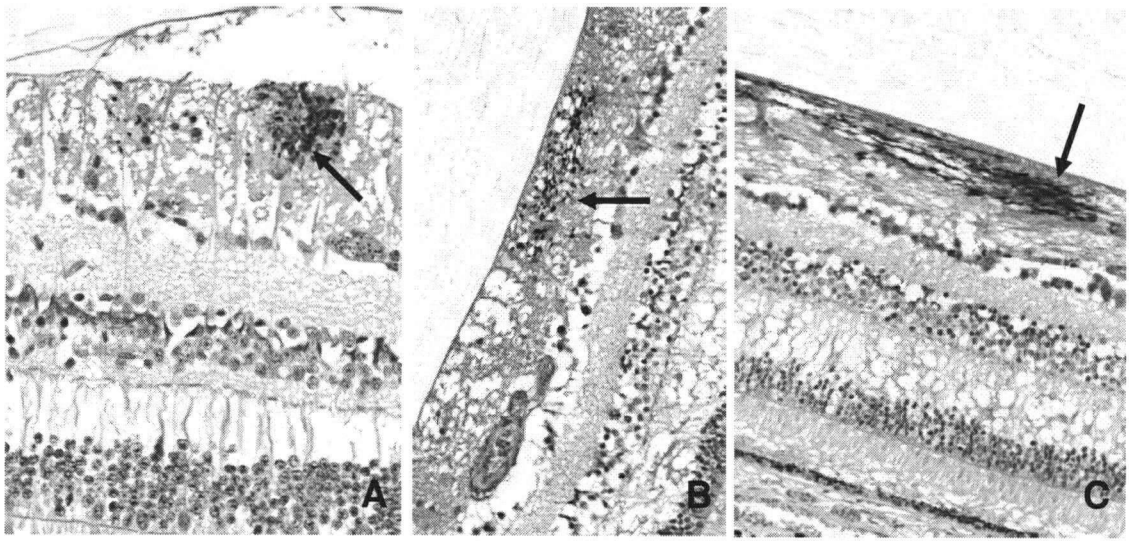

Figure 16 A-C: Patterns of axonal damage in the retinas of CM patients. A: Retinal hemorrhage with distinct axonal damage in the center of the lesion. 40X B-C: Axonal damage not associated with hemorrhage. Note the diffuse staining pattern and axonal spheroids similar to those observed in the brains of CM patients. B-40X and C-100X

Clinical ocular fundus findings observed during life (papilledema, retinal hemorrhage, retinal whitening, and retinal vessel abnormalities) were correlated with APP staining observed in both the eyes and brains of CM patients to determine whether eye findings during life are reflective of changes within the retinas and brains of CM patients. Although retinal whitening (macular and peripheral) on its own did not correlate positively with the total amount of axonal damage observed across the CNS of CM patients (Table 19), we found that patients with high NHA APP (>100μm^2) had significantly more macular whitening then those with less NHA APP staining (<100μm^2) (2.0$\pm$0.01 compared to 0.88$\pm$0.84, $P<0.05$). Retinal hemorrhage, a common ocular fundus feature in CM correlated positively with the total amount of APP staining observed in the brain of CM patients (Table 19). Interestingly, despite a positive correlation between brain and retinal APP staining, retinal APP staining alone was not found to correlate positively with the degree of either macular or peripheral whitening observed during life in CM patients or retinal hemorrhage. A relationship could not be found between measures of axonal damage and either papilledema, central foveal whitening, or vessel delineation. A comparison was also made between the amount of axonal damage in the retinas of CM patients and their parasitological classification (Class I and Class II). Slightly more axonal damage is observed in the retinas of Class II patients then Class I patients (7.67$\pm$2.93 μm^2 compared to 2.67$\pm$2.67 μm^2), but the difference was not statistically significant, due potentially to a lack of cases. Therefore, it seems that the types of axonal damage in the eyes of CM patients reflect those observed in their brains, and that some patients with severe CNS axonal damage also show retinal whitening and retinal hemorrhages on clinical exam.

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Table 19 – Comparison of brain and retinal ocular fundus findings to axonal pathology in CM.

Case (CM)	APP Eye	APP Brain	APP Eye (μm^2)	Total APP Brain (μm^2)	Retinal Hemorrhages (Scale 0-3)	Retinal Whitening (Scale 0-3)	Retinal Vessel Abnormalities
MP05	1	1	0.0092	106.24	1	3	1
MP06	-	1	n/a	37.32	n/a	n/a	n/a
MP09	1	1	0.0059	32.96	2	2	0
MP11	1	1	0.018	127.49	1	2	0
MP13	0	1	0	13.99	n/a	n/a	n/a
MP15	1	1	0.028	12.58	1	0	0
MP23	1	1	0.0037	25.53	1	2	1
MP26	0	1	0.016	150.21	1	2	1
MP27	1	1	0.00059	13.56	1	2	0
MP28	-	1	n/a	18.60	n/a	n/a	n/a
MP29	1	1	0.00040	58.32	2	1	1
MP32	1	1	0.00034	30.21	3	2	0
MP34	0	1	n/a	118.81	2	n/a	n/a
MP35	1	1	n/a	29.36	1	1	1
MP36	1	1	0	15.86	3	2	1
MP39	0	1	n/a	131.72	1	2	1
MP42	1	1	n/a	45.42	1	2	1
MP48	0	1	n/a	284.72	3	2	1
MP52	1	1	n/a	6.65	1	3	1
MP55	1	1	n/a	319.52	3	2	1
MP16	1	1	0	53.31	1	1	0
MP21	1	1	0.013	64.26	1	2	0
MP25	0	1	0	62.37	1	3	0
MP37	0	0	0	0	0	1	1
MP38	0	1	0	2.716363636	0	0	0
# Positive Patients	15/23	24/25	10/16	24/25	20/22	19/21	12/21
R-value, P-value	R=0.391, P=0.348		**R=0.490, P<0.05**		Brain	Eye	Brain	Eye	Brain	Eye
Nominal	-		-		R=0.909 P=0.091	R=0.541, P=0.091	R=0.714 P=1.000	R=0.428, P=0.500	R=0.047 P=1.000	R=-0.457 P=0.659
Pearson	-		-		**R=0.498, P<0.05**	R=-0.171 P=0.542	R=0.260 P=0.255	R=-0.142 P=0.613	-	-

3.7. Myelin Damage in Cerebral Malaria

Pallor of myelin staining indicating myelin damage was present to a degree in one or more brain regions in every case of CM in sections stained with Luxol Fast Blue /H&E. In contrast only one of the five SMA patients (MP19) showed myelin damage. Similarly, only 2/13 (15.4%) COCi patients showed evidence of myelin loss. Myelin loss was not a feature of either the COCni group or the normal age-matched controls (Table 20).

Table 20 – Frequency of myelin damage in CM and controls.

Patient Group	No. of Patients with Myelin Loss or Damage	Total Number of Patients	Fisher Exact Test (versus control)	Fisher Exact Test (versus CM)
CM	25	25	**P<0.001**	-
SMA	1	5	P=1.000	**P<0.001**
COCinfectious	2	13	P=1.000	**P<0.001**
COCnon-infectious	0	6	P=1.000	**P<0.001**
Age-Matched Controls	0	5	-	**P<0.001**

The CM group was the only group to differ significantly from age-matched controls based on the frequency of patients with myelin loss. In addition, a separate evaluation of the degree of myelin loss in each patient group, as determined by morphometric analysis, shows that although some myelin loss was present in SMA and COC patients, the amount of loss was significantly less than that observed in CM (Figure 17 ,$P<0.05$).

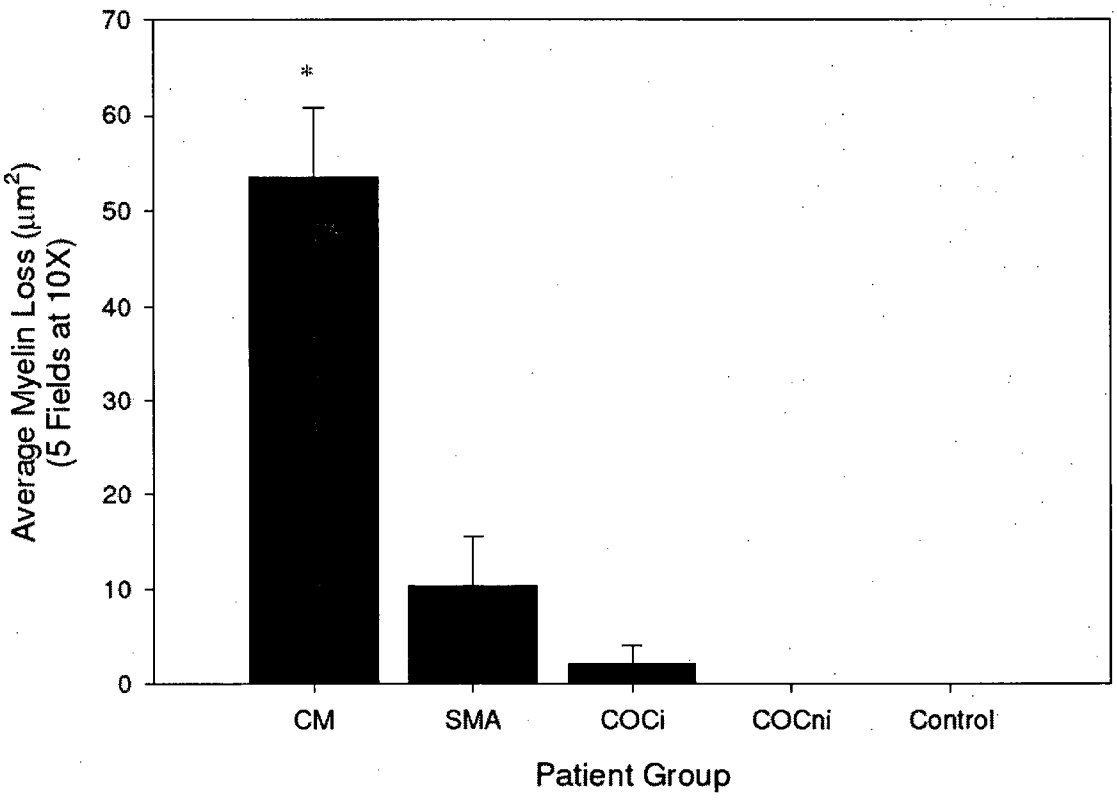

Figure 17. Comparison of the degree of myelin damage in CM patients and controls. (*, significant difference between CM and all groups).

The distribution of myelin loss was not uniform across the CNS of CM patients. A comparison of the total amount of myelin loss in the various regions of the brain of CM patients showed that myelin loss was most prominent in the cerebral hemispheres and cerebellum and least prominent in the brainstem (Figure 18). Statistical analysis showed that the degree of myelin loss in the BS was significantly lower than that observed in the CH ($P<0.001$), C+T ($P<0.05$) and the cerebellum ($P<0.05$). It was found that the degree of myelin loss in CM was significantly greater than that observed in SMA and COC patients and controls ($P<0.05$) in every region except the BS where no significant difference was found, and where the degree of myelin loss was minimal.

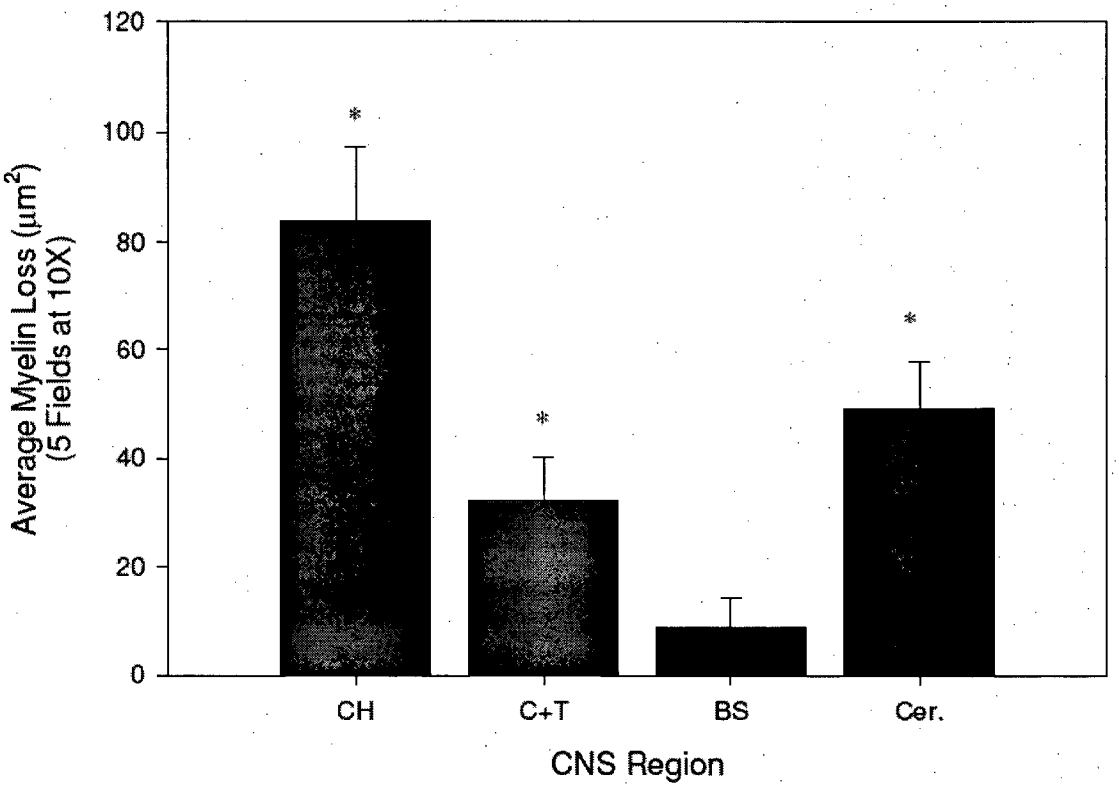

Figure 18. Distribution of myelin damage across the brains of CM patients. (*, significant difference between CM and all groups).

3.7.1. Patterns of Myelin Loss and Damage in CM

Two distinct histological patterns of myelin loss were observed in the CNS of CM patients, and corresponded to similar patterns observed under a separate analysis of axonal damage in this series (see Section 3.6.1.). As with axonal damage, myelin loss was (1) associated with perivascular ring hemorrhages (HA – hemorrhage associated) or (2) was independent of ring hemorrhage (NHA – non-hemorrhage associated) (Figure 19). Similar to the distribution of axonal damage, myelin loss associated with hemorrhage was localized to the central areas of hemorrhage between the central necrotic vessel and the outer ring of infected and uninfected erythrocytes. Non-hemorrhage associated myelin loss appeared as larger more diffuse, ill-defined areas of myelin pallor. These lesions were frequently associated with a vacuolated neuropil. NHA myelin damage predominated over HA lesions in all brains examined (Figure 20).

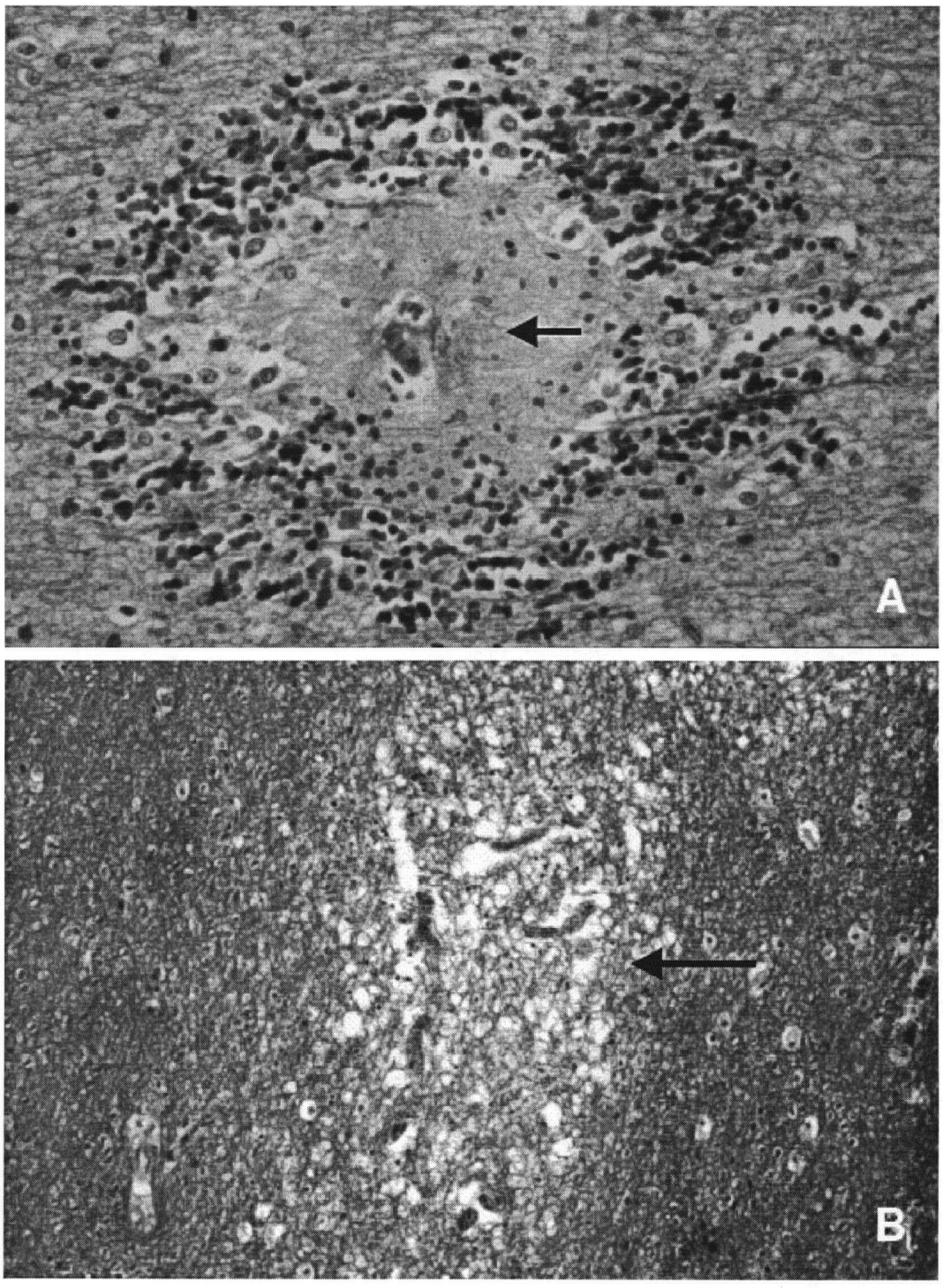

Figure 19 A-B: Types of myelin damage in CM. A: Myelin loss in the central necrotic region of hemorrhage (HA) 100X. B: Large diffuse patch of myelin loss not associated with hemorrhage (NHA) 100X.

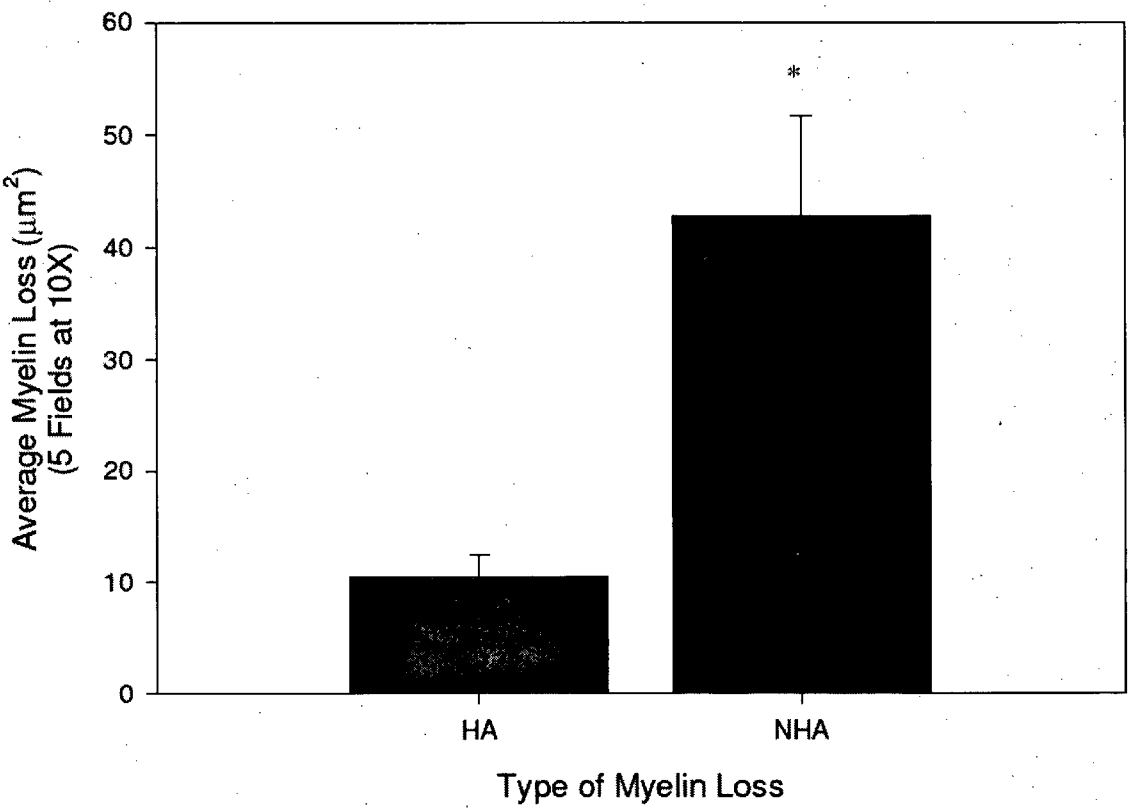

Figure 20. Comparison of the degree of HA and NHA myelin damage in CM patients. (*, $p<0.05$)

3.7.2. Relationship between Myelin Damage and Axonal Damage.

Having observed that similar histological patterns of axonal and myelin loss exist in patients with CM (see Section 3.6.1), we hypothesized that myelin loss and axonal damage might be related. To test this hypothesis, we carried out a series of correlations between the degree of axonal damage and the extent of myelin loss measured in the brains of individual CM patients. Strong positive linear correlations were observed between the two variables in all regions examined except the cerebellum (Table 21).

Table 21 – Pearson correlations coefficients between measures of total APP staining and total myelin loss in different brain regions in CM.

Brain Region	Total APP vs. Total LFB Myelin Loss
Overall	**R=0.440, P<0.05**
Cerebral Hemispheres (CH)	**0.560, P<0.01**
Caudate and Thalamus (C+T)	**0.737, P<0.001**
Brainstem (BS)	**0.861, P<0.001**
Cerebellum (Cer.)	-0.107, P=0.618

3.8. Blood-Brain-Barrier Permeability in Cerebral Malaria

Blood-brain-barrier dysfunction, as evidenced by increased vessel permeability to fibrinogen by immunohistochemistry, was observed in a majority of CM patients (24/25, 96%). Fibrinogen leakage was not unique to CM, however, and was a common feature in SMA and COC patients (infectious and non-infectious, Table 22).

Table 22 – Frequency of BBB dysfunction in CM patients and controls.

Clinical Group	Number of Patients with BBB Dysfunction	Number of Patients without BBB Dysfunction
CM	24	1
SMA	5	0
COCinfectious	12	1
COCother	6	0
Age-Matched Control	0	5

*significant differences were not found between clinical groups

Histologically, leaky vessels showed positive staining for fibrinogen around microvessels in areas of hemorrhage, around vessels containing microthrombi, as well as areas that otherwise appear to be intact (Figure 21). Increased permeability to fibrinogen was observed in both grey and white matter of the cerebral hemispheres and cerebellum as well as in the CT and BS. The percentage of vessels permeable to fibrinogen, as a measure of the degree of blood-brain-barrier dysfunction, was consistently greater in the white matter compared to the grey matter in all patient groups (P<0.05) with the exception of the COCinfectious patients where the difference between the white and grey matter was not significant (Figure 22). There was no statistically significant difference in

the average % of vessels permeable to fibrinogen in the WM between the CH, Cer., CT and BS in any of the CM patients. A comparison of the extent of BBB dysfunction in individual brain regions (% vessels permeable to fibrinogen) in CM using ANOVA revealed that the amount of BBB dysfunction was relatively uniform across the CNS in CM patients. A similar pattern of BBB dysfunction was observed in the SMA group. In addition, there was no statistically significant difference in the severity of BBB dysfunction between CM and SMA patients (Figure 23), suggesting that BBB dysfunction may be important in the pathogenesis of cerebral pathology in both CM and SMA.

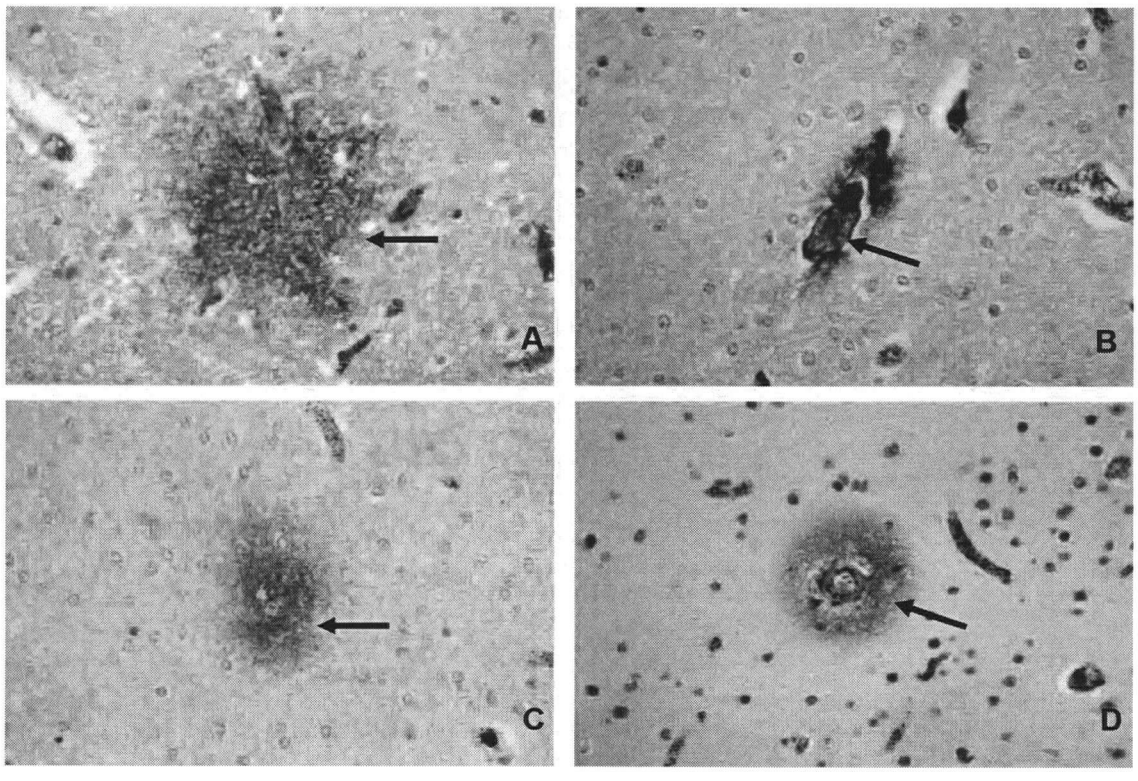

Figure 21 A-D: Types of fibrinogen leakage across the BBB in CM. A: Fibrinogen leakage present with perivascular ring hemorrhage. 40X. B: Fibrinogen leakage associated with thrombosis. 40X. C-D: Fibrinogen leakage present with sequestration and in the absence of hemorrhage, and near vessels that appear to be intact. 40X.

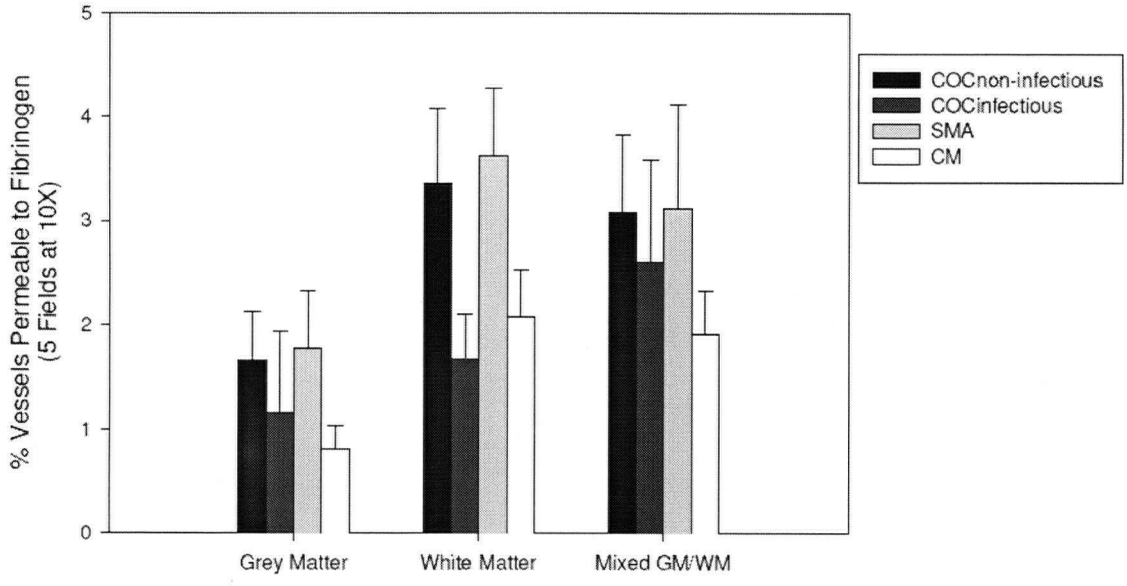

Figure 22. Fibrinogen leakage in CM, SMA and COC patients. The percent permeability to fibrinogen is consistently greater in the white matter over the grey matter in all patient groups except COCi ($p<0.05$).

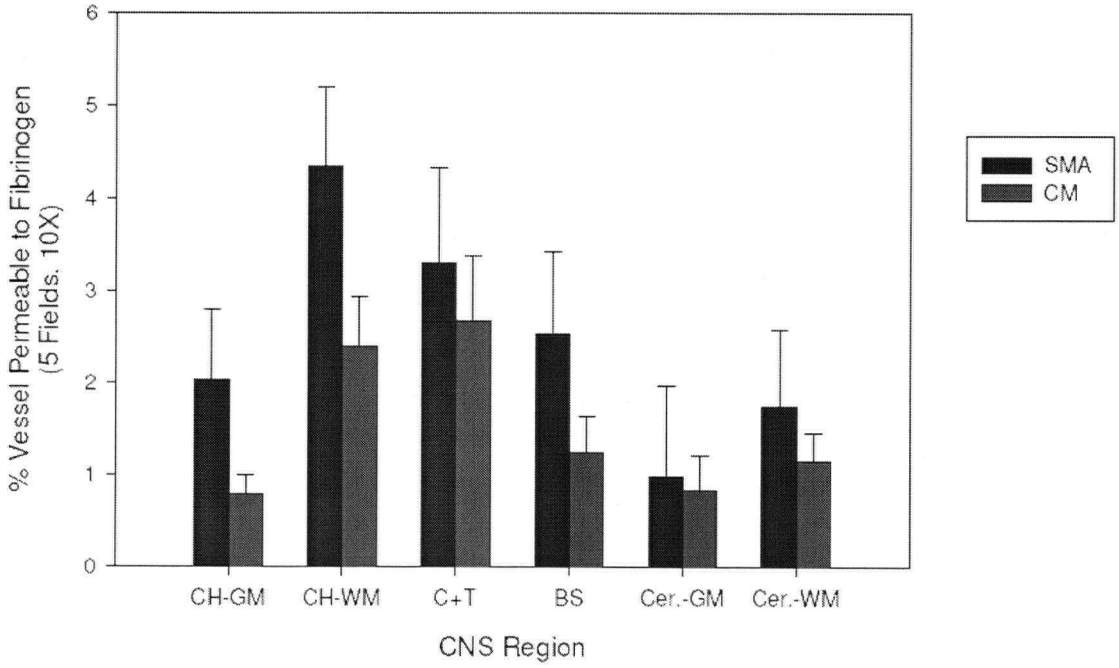

Figure 23. Distribution of fibrinogen leakage in the CNS of CM and SMA patients. There is no statistical significance between any two regions across the brains of CM or SMA patients.

3.8.1. Relationship between BBB Dysfunction and Parasitological Data

Comparisons were made between the various measures of parasite sequestration and the degree of fibrinogen leakage observed in CM patients. Of the various measures, a strong positive correlation was observed between the number of pigment globules and the total amount of fibrinogen leakage observed in the parietal lobe ($R=0.621$, $P<0.01$). Correlations were not found between fibrinogen leakage in either the white or grey matter (or mixed) and other measures of parasite sequestration. In a separate comparison, the degree of fibrinogen leakage across the CNS in Class I and Class II CM patients was compared, revealing no significant difference the degree of BBB dysfunction in either the white or grey matter between classes (Figure 24).

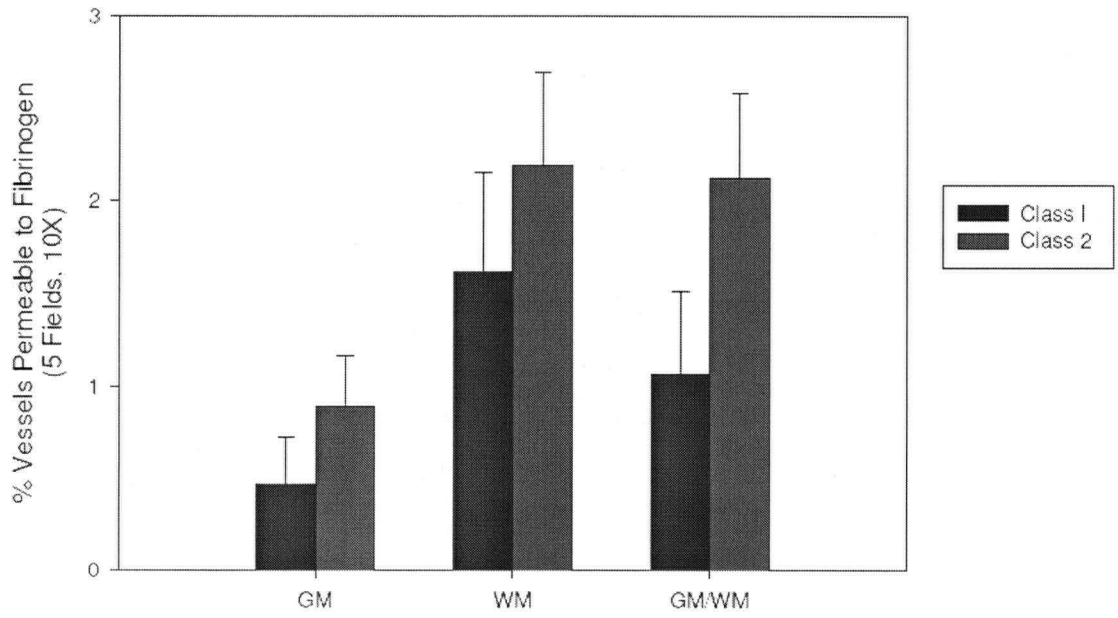

Figure 24. Fibrinogen leakage in grey and white matter and mixed grey-white matter of Class I and Class II CM patients. A statistically significant difference is not observed at any site between the two classes.

3.8.2. Clinicopathological Correlations of BBB Dysfunction

To understand the clinical relevance of BBB dysfunction, a detailed series of correlations were carried out between BBB dysfunction and a number of clinical and

laboratory parameters collected during life. Of the parameters observed, significant positive correlations were observed between BBB dysfunction and both the degree of the palpability of the spleen ($R=0.400$, $P<0.05$) and the blood platelet count on admission ($R=0.497$, $P<0.05$).

3.8.3. Relationship between Ophthalmic Pathology and Ocular Fundus Findings

Fibrinogen leakage in brain microvessels appeared to be a common histological finding in CM patients (see Section 3.8), so in a separate evaluation, the presence of extravascular leakage of fibrinogen across the retinas of CM patients was assessed. Leaky vessels were common in CM, occurring in 15/19 (78.9%) of the retinal specimens examined. Notably, every patient with the exception of MP25 that had retinal fibrinogen leakage also had fibrinogen leakage within their brains. The patterns of fibrinogen leakage observed in the retinas of CM patients also mirrored the patterns present in the brain. As observed in the brain, fibrinogen leakage was found to be associated with hemorrhage, independent of hemorrhage, or associated with the presence of fibrin thrombi (Figure 25). Despite the presence of fibrinogen leakage in the brains and retinas in individual CM patients, and a similar histological picture in the brains and eyes of CM patients, a positive correlation between brain and retinal fibrinogen leakage was not observed in this series ($R=0.474$, $P=1.000$). This may have been due to a lack of cases. Extravascular staining of fibrinogen was not found in the retinal specimens obtained from SMA or COC (infectious and non-infectious) patients despite BBB dysfunction. There was a statistical difference between the frequency of patients in CM with retinal fibrinogen staining compared to COCi patients but not SMA or COCni patients or controls (Table 23).

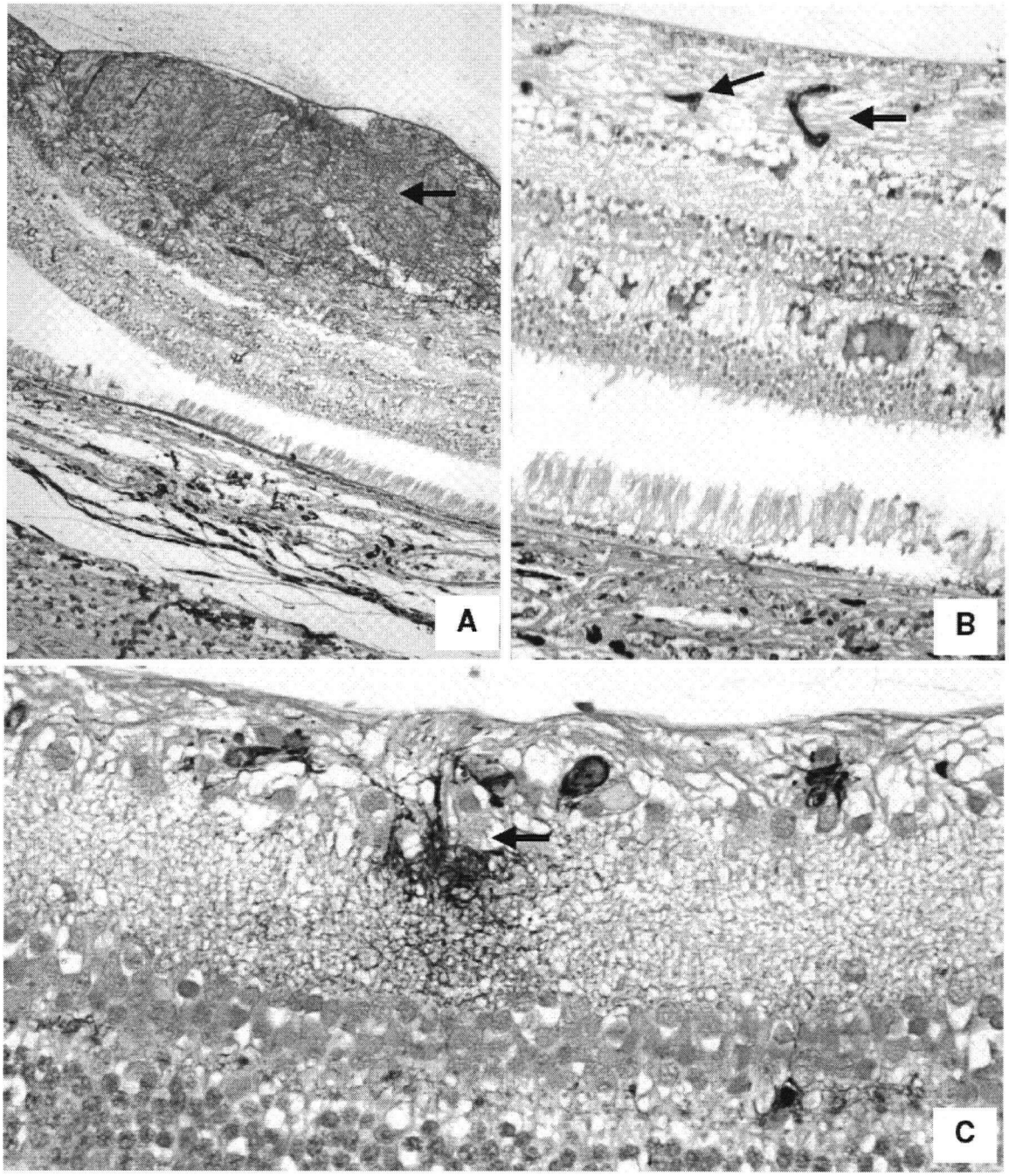

Figure 25 A-C: Fibrinogen leakage in the retinas of CM patients. A: Large retinal hemorrhage with intense fibrinogen leakage. 40X. B: Fibrin thrombi in retinal vessels. 100X. C: Fibrinogen leakage not associated with hemorrhage. 100X.

Table 23 – Frequency of fibrinogen leakage in the retinas of CM, SMA, COC patients and controls.

Clinical Group	Number of Patients with BRB Dysfunction	Number of Patients without BRB Dysfunction
CM	15	4
SMA	0	2
COCinfectious	0	9
COCother	0	2
Age-matched Control	0	2

*significant difference between COCi and CM ($P<0.001$)

In a separate evaluation, extravascular fibrinogen leakage in both the brains and retinas of CM patients was compared with the ocular fundus findings observed in these patients during life. A significant correlation using nominal scale values between brain or retinal fibrinogen leakage and retinal hemorrhage, papilledema, retinal whitening or retinal vessel abnormalities was not found (Table 24). Pearson correlations were also carried out between the % vessels permeable to fibrinogen over all the white matter, grey matter and mixed grey matter/white matter and both retinal hemorrhages and retinal whitening (because these values were recorded scale wise). No significant correlations were found.

Table 24 – Comparison of retinal fibrinogen leakage and ocular fundus findings in CM.

Case (CM)	Fibrinogen (Retina)	Fibrinogen (Brain)	Retinal Hemorrhages (Scale 0-3)	Retinal Whitening (Scale 0-3)	Retinal Vessel Abnormalities
Class II					
MP05	1	1	1	3	1
MP06	-	1	-	-	-
MP09	1	1	2	2	0
MP11	1	1	1	2	0
MP13	0	1	-	-	-
MP15	1	1	1	0	0
MP23	1	1	1	2	1
MP26	1	1	1	2	1
MP27	1	1	1	2	0
MP28	-	1	-	-	-
MP29	1	1	2	1	1
MP32	1	1	3	2	0
MP34	1	1	2	-	-
MP35	0	1	1	1	1
MP36	1	1	3	2	1
MP39	0	1	1	2	1
MP42	1	1	1	2	1
MP48	-	1	3	2	1
MP52	-	1	1	3	1
MP55	-	1	3	2	1
Class I					
MP16	1	1	1	1	0
MP21	-	1	1	2	0
MP25	1	2	1	3	0
MP37	1	1	0	1	1
MP38	0	1	0	0	0
No. Positive Patients	15/19	24/25	20/22	19/21	12/21
Correlation Coefficient (vs. Eye)	-	R=0.474, P=1.000	R=0.666, P=0.314	R=0.647, P=0.331	R=-0.059, P=1.000
Correlation Coefficient (vs. Brain)	R=0.474, P=1.000	-	R=0.727, P=1.000	R=0.714, P=1.000	R=0.455 P=0.181

*Retinal whitening = macular whitening and/or peripheral whitening
*Papilledema and central foveal whitening were not included as part of the correlation
*Correlation coefficients are calculated for nominal scale data despite ranking values
*0 or 1 means that it is either present or absent

3.9. Gliosis in Cerebral Malaria

Gliosis, indicated by an increase in the size and number of glial fibrillary acidic protein (GFAP) positive astrocytes in the brain, is indicative of CNS injury. Staining with an anti-GFAP antibody revealed that gliosis is a major feature in CM, present in 24/25 patients (96%). The pattern of staining in CM was similar from case to case, and was present primarily in the grey matter and white matter as well as subpial regions (Figure 26). Gliosis was not restricted to CM patients however, and was present in all of the SMA and COC patients (infectious and non-infectious). Age-matched control patients also showed staining (2/5, 40%), though the number of GFAP positive astrocytes was minimal in this group. Fisher's exact test revealed that only CM patients and COCi patients were significantly different from controls with respect to the number of patients with gliosis (Table 25).

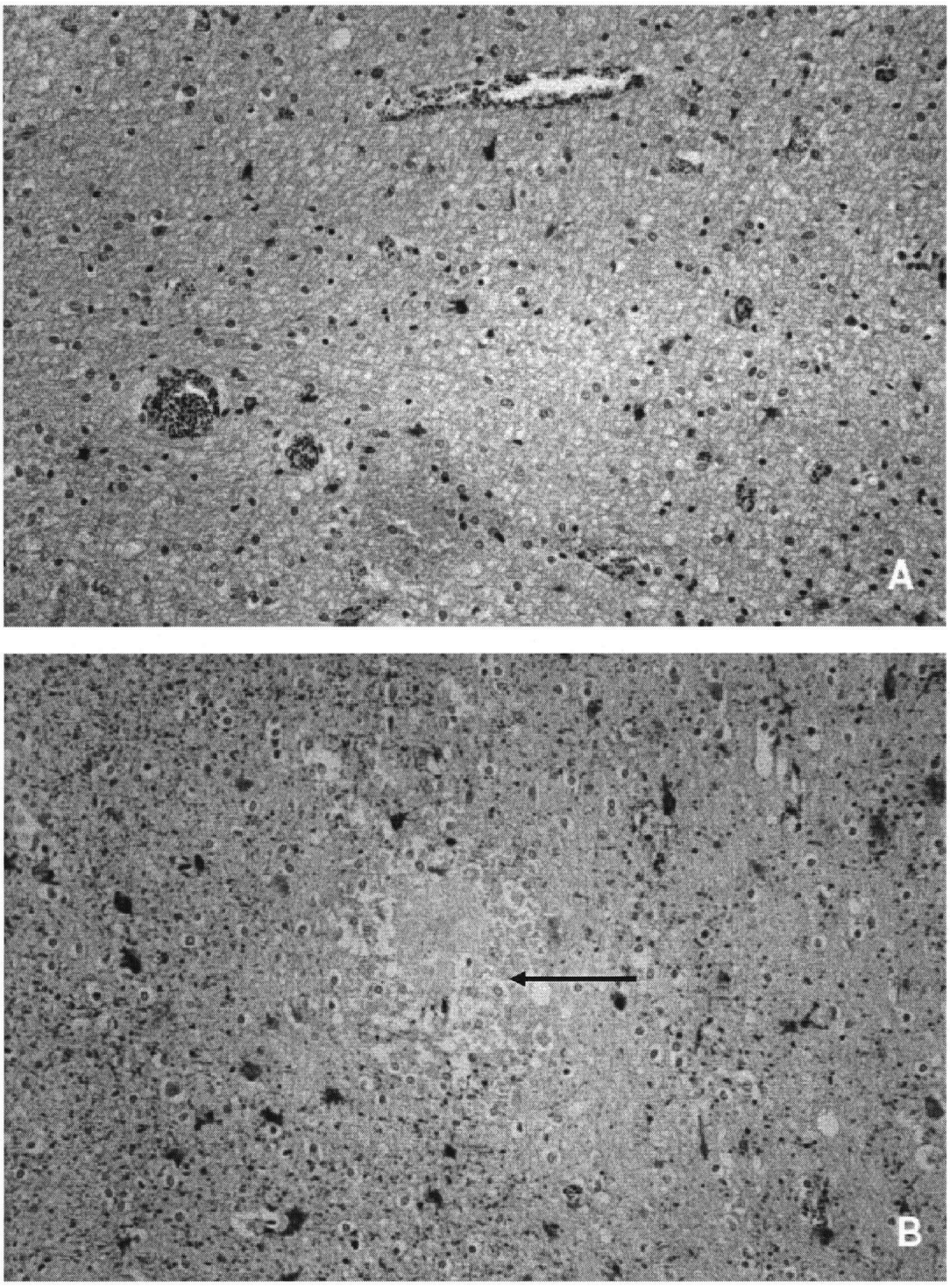

Figure 26 A-B: Gliosis in cerebral malaria patients. A: Gliosis present along with vacuolated neuropil. 40X. B: White matter gliosis surrounding a hemorrhage (arrow). 100X.

Table 25 – Frequency of gliosis in CM patients and controls.

Patient Group	No. of Patients with Gliosis	No. of Patients without Gliosis	Fisher Exact Test (versus control)	Fisher Exact Test (versus CM)
CM	24	1	**P<0.01**	-
SMA	5	0	P=0.167	P=1.000
COCinfectious	13	0	**P<0.05**	P=1.000
COCother	6	0	P=0.167	P=1.000
Age-Matched Controls	2	3	-	**P<0.001**

The positive SMA and COC cases (infectious and non-infectious) had a similar pattern of staining to that observed in CM, that is, staining in the grey and white matter and subpial regions. In CM, SMA, and COC patients the amount of gliosis was always observed to be statistically greater in the white matter than the grey matter (Figure 27). The distribution of gliosis across the CNS of CM patients and controls appeared to be constant, a histopathological feature that was confirmed using statistical analysis (Figure 28).

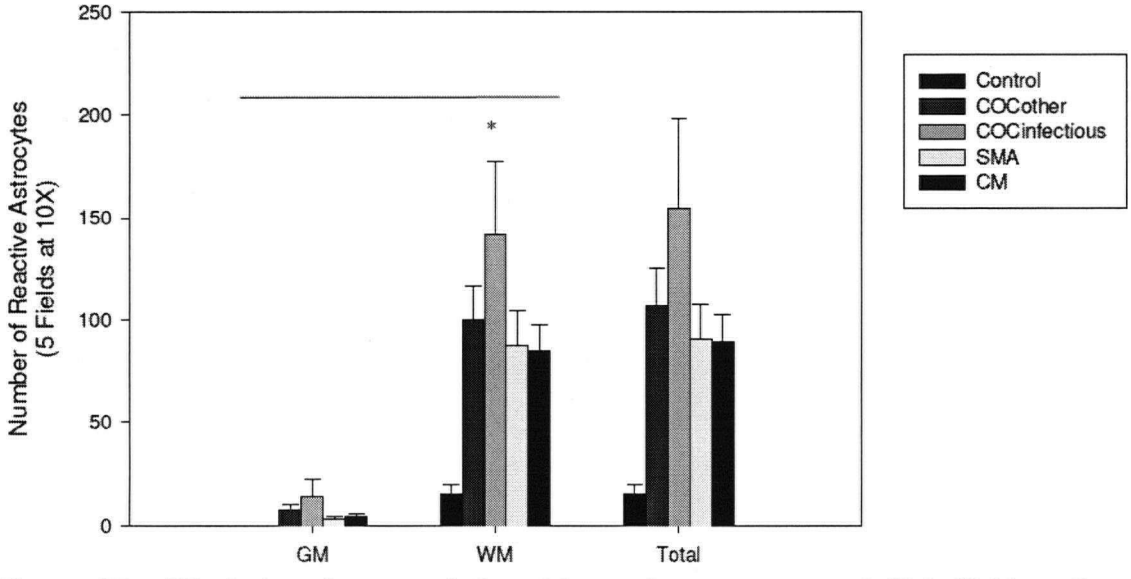

Figure 27. Gliosis is a feature of the white and grey matter of CM, SMA and COC patients. There was no statistically significant difference between the degree of gliosis between groups, but the amount of gliosis was consistently greater in the white matter as opposed to the grey matter (*, $p<0.05$).

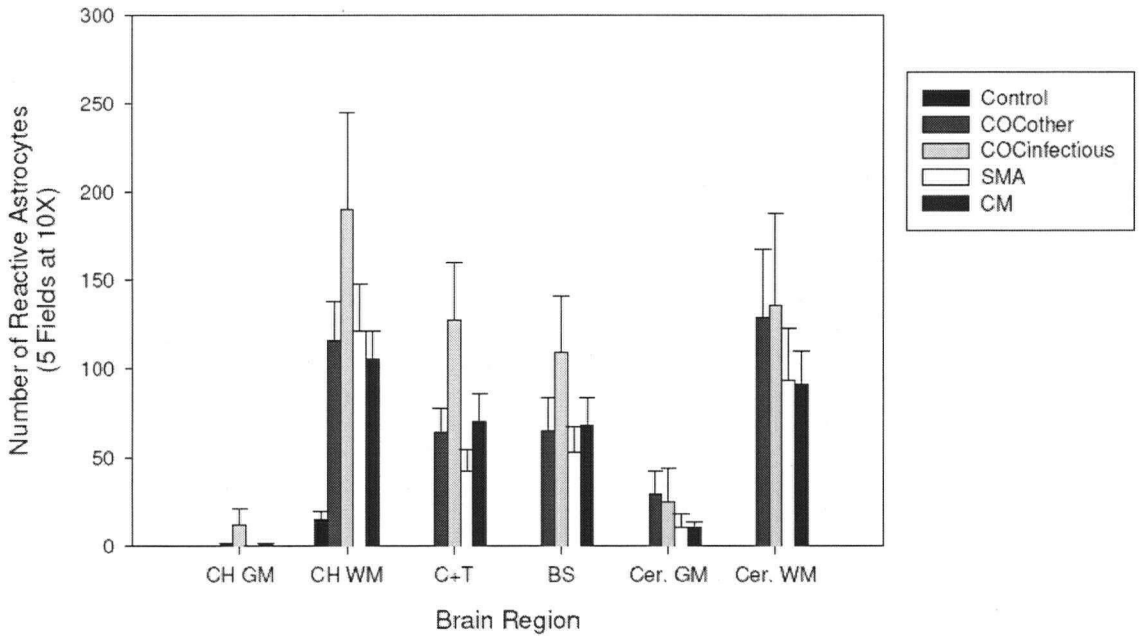

Figure 28. Distribution of gliosis across the CNS of CM, SMA, COC and control patients.

Histopathological examination and statistical analysis revealed that the degree of gliosis in CM patients and SMA patients appeared to be very similar from region to region. This suggests that CNS injury may be present in SMA patients even though they do not present clinically with neurologic dysfunction. An additional observation is that with respect to gliosis there was a correlation in the degree of gliosis between different brain regions in individual patients (Table 26). This confirms what was observed histologically. If there was significant gliosis in one region in a patient then gliosis was likely to be present in all sections examined

Table 26 - Strong correlations are observed between the total amount of gliosis observed in the various regions of the brain in CM.

Brain Region	CH	C+T	BS	Cer.
Cerebral Hemispheres (CH)	1	**R=0.630**	**0.537**	**0.613**
Caudate and Thalamus (C+T)		1	**0.590**	0.331, P=0.106
Brainstem (BS)			1	**0.560**
Cerebellum (Cer.)				1

*P<0.01 unless otherwise noted

3.9.1. Gliosis and Other Neuropathological and Parasitological Features

A series of comparisons were carried out between the amount of gliosis observed across the entire CNS and in individual brain regions in CM and other neuropathological features observed to be present in this disease. Correlations could not be found between gliosis and any of the observed neuropathological features. The same was true when gliosis was compared with the various measures of sequestration.

3.9.2. Clinicopathological Correlations of Gliosis

To understand the significance of gliosis in CM, patients were divided into three groups based on the number of GFAP positive astrocytes observed overall (grey and white matter, see Section): Scale 0 = Normal (0-50 Astrocytes/10 Fields at 10X), Scale 1 = Moderate Gliosis (50-100 Astrocytes/10 Fields at 10X) and Scale 2 = Dense Gliosis (>100 Astrocytes/10 Fields at 10X). With this, it appears that patients with dense gliosis (Scale 2) differed markedly from normal patients (scale 0) with respect to admission duration, parasitemia measure on admission, and number of doses of quinine. Significant differences could not be found between any clinical parameter when patients with normal gliosis or dense gliosis were compared with patients with moderate gliosis with respect to any clinical feature (Table 27). In addition, it was found that patients who arrived to the hospital with a history of coma also had higher amount of gliosis then those who did not (Table 28).

Table 27 - Patients with dense gliosis (scale 2) differed from patients without gliosis (scale 0) by a number of clinical parameters.

Clinical Parameter	Mild Gliosis (Scale 0)	Dense Gliosis (Scale 0)
Admission Duration (hrs)	**10.5±2.91**	**24.96±4.81**
Parasitemia (μl)	**125.32±47.50**	**39.25±10.76**
# Doses of Quinine	**1.33±0.41**	**2.71±0.42**

Table 28 - Cerebral malaria patients with a history of coma on admission have more severe gliosis.

Clinical Parameter	History of Coma on Admission	
	Yes	No
Gliosis (#Astrocytes/5 Fields at 10X)	**100.35±11.55**	**28.42±15.06**

3.9.3. Relationship to Ophthalmic Pathology and Ocular Fundus Findings

Gliosis appears to be a common finding in the brains of CM patient so a separate investigation was undertaken to determine the degree of gliosis in the retinas of CM patients. Retinal gliosis is marked by the presence of GFAP positive Muller cells extending posteriorly in the retina from the ora serrata towards the optic disk. 14/22 (64%) of CM patients were observed to have Muller cell staining. Retinal gliosis was present in the COC patients as well (7/14, 50%), but not SMA patients. The pattern of gliosis in the retinas of CM patients was striking and included gliosis both associated with hemorrhage and independent of hemorrhage (Figure 29). To determine whether gliosis might be related to ocular fundus findings observed during life a separate series of correlations was carried out (Table 29). Positive correlations were not found between any two features.

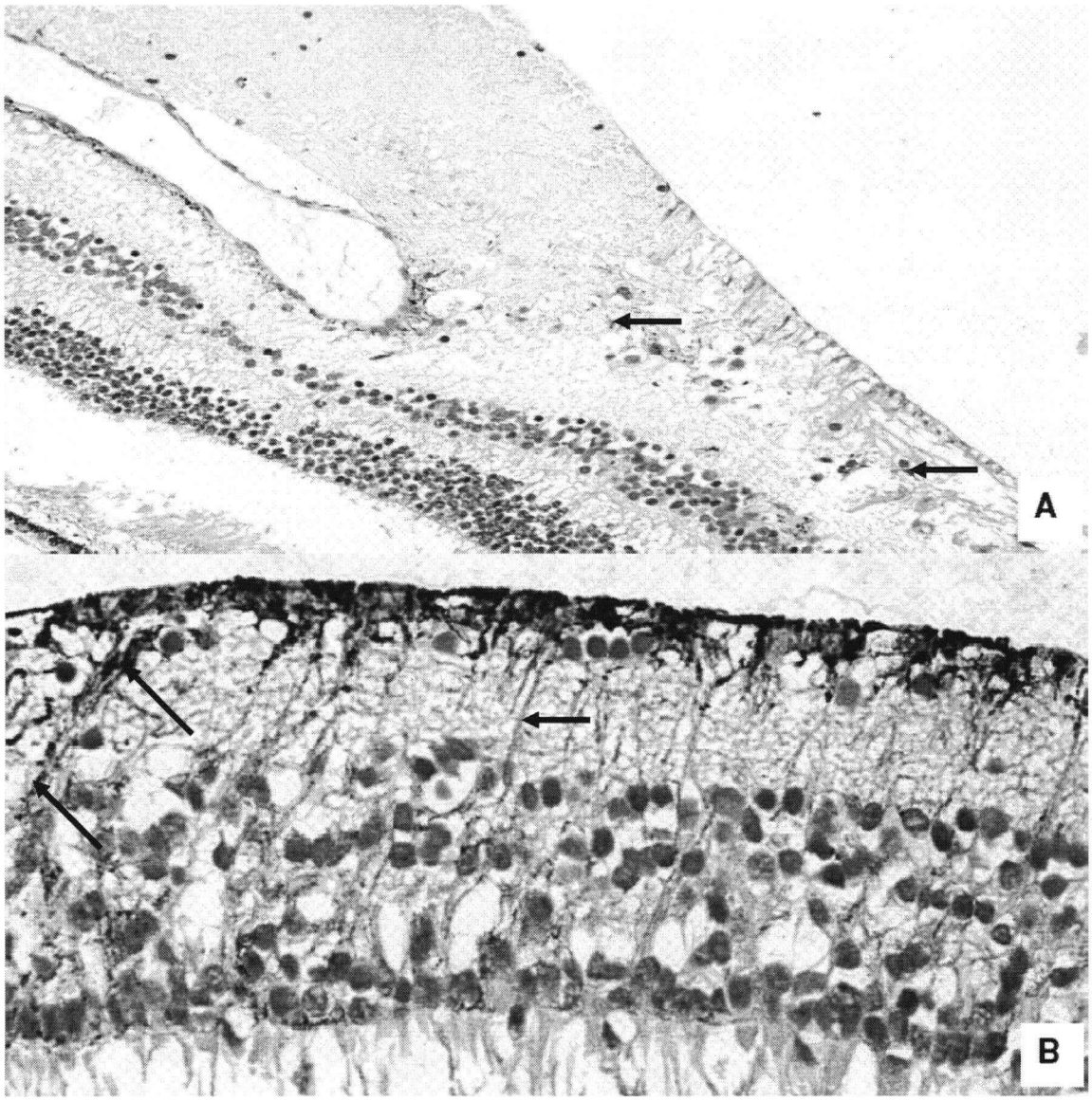

Figure 29 A-B: Gliosis in the retina in CM. A: GFAP positive Muller cells associated with a retinal hemorrhage Arrow points to individual Muller cells. 40X. B: Positive Muller cell staining in the peripheral retina in CM. 100X. Arrows point to Muller cell processes extending towards optic disk.

Table 29 – Comparison of brain and retinal GFAP staining in CM.

Case (CM)	GFAP* (Retina)	GFAP* (Brain)	Retinal Hemorrhages (Scale 0-3)	Retinal Whitening (Scale 0-3)	Retinal Vessel Abnormalities
MP05	0	1	1	3	1
MP06	1	1	n/a	n/a	n/a
MP09	0	1	2	2	0
MP11	1	1	1	2	0
MP13	1	1	n/a	n/a	n/a
MP15	0	1	1	0	0
MP23	1	1	1	2	1
MP26	0	1	1	2	1
MP27	1	1	1	2	0
MP28	0	1	n/a	n/a	n/a
MP29	n/a	1	2	1	1
MP32	0	1	3	2	0
MP34	1	1	2	n/a	n/a
MP35	n/a	1	1	1	1
MP36	0	1	3	2	1
MP39	n/a	1	1	2	1
MP42	1	1	1	2	1
MP48	1	1	3	2	1
MP52	0	0	1	3	1
MP55	1	1	3	2	1
MP16	1	1	1	1	0
MP21	1	1	1	2	0
MP25	1	1	1	3	0
MP37	1	1	0	1	1
MP38	1	1	0	0	0
No. Positive Patients	14/22	24/25	20/22	19/21	12/21

*GFAP was noted to be either present (score of 1) or absent (score of 0).

3.10. Correlation of Neuropathological Findings

To understand the relationship between the various neuropathological features under study in this investigation, a detailed series of correlations were carried out between data sets.

3.10.1. Perivascular Ring Hemorrhage and Other Neuropathological Features

To understand the role of hemorrhages in CM, attempts were made to determine whether the neuropathological features under study in this investigation were associated either histologically, or through statistical comparison, with hemorrhage. Histological exam using immunohistochemical staining for fibrinogen revealed intense fibrinogen leakage around apparent hemorrhages (Figure 21). Gliosis was focally associated with hemorrhage histologically. A statistically significant relationship was found between hemorrhages and the presence of fibrin thrombi in CM patients as revealed by the Fisher's Exact Test (Table 30).

Table 30 – Fibrin deposition and hemorrhage are significantly associated in CM ($P<0.05$).

	Presence of Hemorrhage	
Presence of Fibrin Deposition	+	-
+	n=16	3
-	1	3

Luxol fast blue staining and immunostaining for β-amyloid precursor protein demonstrated that perivascular hemorrhages were associated with myelin loss and axonal damage in the center of ring hemorrhages. The degree of axonal damage caused by hemorrhage varied from case to case, with some hemorrhages causing strong β-APP staining with large axonal spheroids and others with less drastic staining. The amount of myelin loss and axonal damage found to be associated with hemorrhage was measured and Pearson correlations were carried out between the total number of hemorrhages found in the brains of CM patients and the amount of hemorrhage associated axonal damage and myelin loss observed over the same areas. Pearson correlation coefficients of 0.653 ($P<0.001$) and 0.803 ($P<0.001$) for axonal damage and hemorrhage and myelin loss and hemorrhage were found respectively. Axonal damage and myelin loss appearing to be non-hemorrhage associated histologically (NHA) was shown to be unassociated with hemorrhage by statistical analysis (see Section 3.10.2.1).

3.10.2. Axonal Damage and Other Neuropathological Features

Pearson correlations and Chi-squared tests were carried out between measures of NHA APP and other neuropathological features: BBB dysfunction, gliosis and fibrin deposition. No statistically significant relationship could be found between any two variables in any brain region. However, a comparison between the degree of BBB dysfunction in CM patients with the most severe NHA axonal damage (>100 μm^2 NHA APP staining) and those with a low average NHA APP load (<100 μm^2) revealed that patients with high NHA axonal damage also had a greater degree of permeable vessels over all brain regions and in the white matter of the cerebral hemispheres and cerebellum (Table 31). A relationship could not be found between gliosis or fibrin deposition and the degree of NHA APP.

Table 31 – The percent permeability of vessels is greater overall and in the cerebral hemispheres and cerebellum of patients with high measures of NHA APP (>100μm^2).

Brain Region	High NHA APP (>100μm^2, n=7)	Low NHA APP (<100μm^2, n=18)	P-value (t-test)
	% Permeable Vessels	% Permeable Vessels	
Overall	**3.67±1.45**	**1.45±0.41**	**P<0.05**
Cerebral Hemispheres (CH)	**4.34±1.63**	**1.63±0.57**	**P<0.05**
Cerebellum (Cer.)	**2.14±0.74**	**0.77±0.28**	**P<0.05**

3.10.2.1. Relationship between HA and NHA Axonal Damage

Having determined that two types of axonal pathology exist, we sought to determine whether a causal relationship between the two could be determined. In particular, we were interested in determining whether NHA APP was independent of hemorrhage or possibly secondary to an adjacent focus of hemorrhage. Our findings indicate that the two forms of axonal damage may be independent since even though most CM patients appear to have had both HA and NHA pathology (15/24, 62.5%), a subset of CM patients appeared to have NHA APP staining only (8/24, 33.3%), suggesting that hemorrhage was not necessary to cause NHA axonal pathology in at least some patients (Table 32).

Table 32 – Types of axonal damage observed in individual malaria patients.

Patient Group	None	HA only	NHA only	HA and NHA
CM (n=25)	MP37	MP15	MP16, 21, 23, 25 27, 28, 38, 55	MP5, 6, 9, 11, 13, 26, 29, 32, 34, 35, 36, 39, 42, 48, 52
SMA (n=5)	MP7, 57	None	MP19, 30, 51	None
Age-Matched Controls (n=5)	All	None	None	None

We also carried out a series of correlations at each brain region between the amount of NHA axonal damage measured by morphometric analysis and both HA axonal damage and the number of hemorrhages observed. A significant correlation could not be found for the average APP load overall or for any individual region except for a significant correlation present between the number of hemorrhages in the brainstem and the amount of NHA APP (Table 33). This suggests that at least in most areas of the brain, the NHA APP staining observed in CM appears to be independent of hemorrhage.

Table 33 – Pearson correlations coefficients between measures of NHA APP and HA APP and number of hemorrhages over the different brain regions in CM.

Brain Region	NHA APP vs. HA APP	NHA APP vs. # Hemorrhages
Overall	R=-0.167, P=0.426	0.103, P=0.626
Cerebral Hemispheres (CH)	-0.206, P=0.323	0.154, P=0.462
Caudate and Thalamus (C+T)	-0.00909, P=0.966	-0.0675, P=0.748
Brainstem (BS)	0.076, P=0.721	**0.615, P<0.01**
Cerebellum (Cer.)	-0.192, P=0.358	0.164, P=0.435

3.10.2.2. Relationship between Axonal Damage and Myelin Loss

Histological and statistical evidence suggested that there was a strong relationship between the degree of axonal damage and the amount of myelin loss in CM patients. On histological evaluation it was apparent that the same patterns of axonal damage (HA and NHA) were observed when considering myelin loss by LFB staining. To confirm

whether the observed myelin loss was related to axonal damage we carried out a series of correlations between the amount of axonal damage and the amount of myelin loss measured in the brains of individual CM patients. Strong positive linear correlations are observed between the two variables over all regions except the cerebellum where a linear correlation was not apparent (Table 34).

Table 34 – Pearson correlations coefficients between measures of total APP staining and total myelin loss in different brain regions in CM.

Brain Region	Total APP vs. Total LFB Myelin Loss
Overall	**R=0.440, P<0.05**
Cerebral Hemispheres (CH)	**0.560, P<0.01**
Caudate and Thalamus (C+T)	**0.737, P<0.001**
Brainstem (BS)	**0.861, P<0.001**
Cerebellum (Cer.)	-0.107, P=0.618

3.10.3. Blood-Brain-Barrier Dysfunction and Other Neuropathological Features

To understand whether increased microvessel permeability in the brains of CM patients might affect other neuropathological features, we carried out a detailed series of histological comparisons and statistical correlations between the average % permeability to fibrinogen and hemorrhage, axonal damage, myelin loss, fibrin deposition and gliosis in individual sites and over all brain sites in the CNS. Immunohistochemical staining with antibodies to fibrinogen revealed intense fibrinogen leakage around apparent hemorrhages, evidence of the breakdown of the endothelial cells lining the vessels in hemorrhage. Despite the presence of fibrinogen leakage around hemorrhage, a significant correlation was not found between hemorrhage and BBB dysfunction at any brain site or across the CNS as a whole. Correlations were also absent between BBB dysfunction and axonal damage, myelin loss, fibrin deposition or gliosis. However, it was observed that patients with increased axonal damage (>100μm^2) had more BBB dysfunction then those patients with less axonal damage (<100μm^2) (Table 30).

3.10.4. Gliosis and Other Neuropathological Features

A series of correlations were carried out between the degree of gliosis observed across the entire CNS and in individual brain regions in CM and other neuropathological features observed to be present in this disease. Correlations could not be found between gliosis and any of the observed neuropathological features.

CHAPTER 4

DISCUSSION

4.1. Perivascular Ring Hemorrhages in Pediatric Cerebral Malaria and Controls

Perivascular ring hemorrhages have been noted in studies conducted in both adults and children who died of cerebral malaria (see Section 1.4.2.) but the findings have not been fully described and have not been included as part of a systematic neuropathological and clinicopathological study. In this work, we carried out a quantitative study of ring hemorrhages in sections from different parts of the brains of CM patients with particular emphasis on the number and distribution of hemorrhages while carrying out a series of neuropathological and clinicopathological correlations. We observed that perivascular ring hemorrhages were a common feature of the white matter of CM brains (18/25, 72%), observed primarily in the sub-cortical white matter and cerebellum, and are not present in SMA patients without neurologic dysfunction, suggesting that ring hemorrhages are unique and important to CM. Two COC patients showed evidence of ring hemorrhage in addition to parasite sequestration, emphasizing the importance of strict case definition and the potential for interfering pathologies in studies of CM, a problem that has been noted elsewhere (Taylor et al., 2004).

4.1.1. Significance of Ring Hemorrhages in Pediatric Cerebral Malaria

CM patients were the only malaria patients in this study that showed evidence of perivascular ring hemorrhages (Figure 4). Positive associations were observed between hemorrhages and both the duration of coma and the presence of seizures in CM patients (see Section 3.5.2.). These are the two most common neurological signs in CM, suggesting that ring hemorrhages are important to CM and may be directly related to neurologic dysfunction in this disease. Prior to this study, little data existed in the way of post-mortem examinations regarding the frequency of hemorrhages in CM and NCM patients, making it difficult to know the extent to which hemorrhages are restricted to CM. Rigdon (1944) described the histological features of the ring hemorrhages in CM in detail but did not allude to the frequency of hemorrhages in CM and NCM patients

(Rigdon, 1944). A more recent study that examined the neuropathology of a small group of adult CM and non-CM patients showed increased extravasation of erythrocytes into the brain parenchyma in addition to endothelial damage in both CM and NCM patients, with increased vascular pathology in the CM compared with SMA patients (MacPherson et al., 1985). The extent to which the extravasation of erythrocytes in the CM and NCM group in this study represented the characteristic perivascular ring hemorrhage unique to CM, and not the petechial hemorrhages which are non-specific (Turner, 1997) is not known. Most of what is known about the frequency of hemorrhages in CM and NCM is taken from studies on the ophthalmic pathology and ocular fundus features where retinal hemorrhages are observed in both CM patients and malaria patients without neurological abnormalities (Kochar et al., 2000; Schemann et al., 2002). However, the extent to which the hemorrhages observed in the retinas of CM patients are actual ring hemorrhages as opposed to the more commonly observed petechial hemorrhages was not clear from these studies. Our study shows that perivascular hemorrhages were unique and important to the CM, making this investigation an important starting point to understand the pathophysiology and importance of ring hemorrhages in CM.

Ring hemorrhages were directly associated with cell and tissue damage in CM. Every neuropathological feature examined in this study, with the exception of gliosis, and including axonal damage, myelin loss, fibrinogen leakage and the presence of fibrin thrombi, was directly associated with hemorrhages histologically and statistically. This is consistent with a similar role for hemorrhages observed in other studies of adult CM (Medana et al., 2002). In addition, this study shows that damage to axons and myelin and increased BBB permeability also occurs independently of hemorrhage. As an example, ring hemorrhages were associated with axonal damage (found in and around the areas of hemorrhages) though most of the axonal damage observed in this disease was not attributable to hemorrhage (see Section 3.6.1.). This suggests that even though hemorrhages cause significant damage in CM, hemorrhage is not the sole cause of pathological changes in this disease. It is also interesting that most but not all patients with clinically defined CM showed evidence of RH, suggesting that even though hemorrhages are common in CM, and may play a role in the development of neurologic

dysfunction, they are not the only pathologic abnormality leading to the development of this disease and the neurological findings observed in CM.

4.1.2. Perivascular Ring Hemorrhages and Multiple Pathologies

To understand the significance of hemorrhage in CM patients more specifically, we made a series of comparisons between CM patients with and without hemorrhages. No significant differences were observed in the degree of neuropathological insult in CM patients based on the presence or absence of hemorrhage. However, a striking finding was that the subset of CM patients with perivascular ring hemorrhages were the same patients who by regression analysis were observed to have an abundance of extra-erythrocytic pigment in their brain microvessels at autopsy (Figure 7) (Taylor et al., 2004). A significant positive correlation between the number of perivascular ring hemorrhages in the brains of CM patients and the number of extra-erythrocytic pigment globules observed in the brain at autopsy ($R=0.402$, $P<0.05$) was also observed. Therefore, a subset of CM patients showed vascular pathology in the form of hemorrhage that is related to the presence of pigment globules and was not present in other patients with clinically defined CM. Though the significance of pigment globules in CM pathology, and their specific relationship to hemorrhage is not yet known, pigment globules have been shown to have an immunomodulatory role that could contribute to the development of CM (see Section 4.1.2.1.). This suggests the possibility that this patient group may have more of an immune-mediated form of CM. Another interesting finding in this study, and one that has been observed elsewhere (White et al., 2001), was that a positive correlation between brain hemorrhages observed at autopsy and retinal hemorrhages observed on clinical examination (Table 12). This observation is especially powerful if differing pathologies related to the presence of hemorrhage exist in CM because it affords the clinician the opportunity to use ocular fundus findings to identify distinct pathophysiological mechanisms during life with implications on treatment and prognosis.

4.1.2.1. Perivascular Ring Hemorrhages and Extra-Erythrocytic Hemazoin

In this study, a positive association was observed between the number of extra-erythrocytic pigment globules measured histologically and hemorrhage ($R=0.402$, $P<0.05$). The exact nature of the relationship between malaria pigment, also known as hemazoin (Hz), and hemorrhage, is not known, but previous studies allude to Hz playing an immunomodulatory role in this disease. Hz is a polymer of hematin that is derived from the metabolism of hemoglobin in the food vacuole of *Plasmodium* (Taramelli et al., 2000). After schizogeny, the pigment is released from the erythrocyte and is taken up by circulating monocytes (Schwarzer et al., 2001) and other phagocytes (Olliaro et al., 2000). Studies using both native and synthetic pigment have shown that hemazoin can trigger a proinflammatory immune response. mRNA transcript expression for the proinflammatory chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2 and macrophage chemoattractant protein (MCP)-1 were upregulated in monocytes in response to Hz (Jaramillo et al., 2005), and co-culturing monocytes with isolated Hz induced the release of TNF-α and IL-1β *in vitro* (Biswas et al., 2001; Pichyangkul et al., 1994). Nitric oxide, a known mediator of coma, and understood to play a role in CM pathogenesis (Clark et al., 1997), can also be induced in cells treated with IFN-γ and Hz through the induction of iNOS (Jaramillo et al., 2003; Keller et al., 2004). Although the nature of the relationship between hemazoin, proinflammatory cytokine production and hemorrhage has not been established, several studies have suggested a relationship between hemorrhage and proinflammatory cytokines. In the *Plasmodium chabaudi* and *Plasmodium berghei* ANKA mouse models of CM, cerebral complications, and particularly the presence of hemorrhages, were closely related to TNF-α production (Rudin et al., 1997; Sanni et al., 2004), and immunohistochemical studies looking at the tissue specific expression of cytokines in human CM show that histopathological changes (hemorrhage included) were related to the presence of TNF-α, IFN-γ, IL-1β, and IL-10 (Maneerat et al., 1999).

4.1.3. Perivascular Ring Hemorrhages and Parasite Sequestration

Little is known about the pathophysiology of perivascular ring hemorrhages in CM though several authors argue that ring hemorrhages are likely to be a reperfusion injury secondary to microvessel blockage by PRBCs (Sein et al., 1993a; Sein et al.,

1993b; Turner, 1997). In adult patients, Sein *et al.* (1993) found the number of hemorrhages to be greatest in the cerebrum and cerebellum as compared to the brainstem, and related these changes to increased PRBC sequestration associated with increased vascularity in cerebral and cerebellar tissues (Sein et al., 1993b). Our study similarily shows that the majority of the hemorrhages occur in the sub-cortical white matter and the white matter of the cerebellum, with the number of hemorrhages decreasing towards the brainstem. We also observed a significant negative correlation between parasite counts taken from peripheral smears and the number of hemorrhages observed at autopsy ($R=-0.428$, $P<0.05$) that could reflect a redistribution of the bulk of PRBCs from peripheral blood to the brain microvasculature, resulting in microvessel blockage and the development of ring hemorrhages characteristic of this disease. It is known that infected erythrocytes move from the peripheral circulation and adhere to the microvasculature of major organs during the lifecycle of *P.falciparum* (see Section 1.3.1.). The reason for the increased numbers of hemorrhages in the white matter compared with the grey is not known. It has been suggested that if sequestration is important to the development of hemorrhages in CM through some type of reperfusion injury that the apparent predilection for white matter may be due to a relative lack of an anastomotic capillary network in the white matter compared to the grey in addition to the differences in capillary pressure at various sites in the CNS (White et al., 2001). Along the same lines, the increase in the number of hemorrhages in the subcortical white matter over other brain regions (brainstem) may also be related to vascular anatomy. A recent study into the vasculature throughout the CNS showed that cerebral arteries apparently travel lineally through the cortex and upon entering the sub-cortical white matter, begin to coil, loop and spiral. This feature of vascular anatomy could create a reservoir for *P.falciparum* in the sub-cortical white matter, leading to the development of neuropathological changes (Nonaka et al., 2003).

It is not known from this study, however, if sequestration is the cause of hemorrhages in CM. Even though decreased peripheral parasitemia is observed in patients with hemorrhage and may represent a redistribution of parasite to brain microvessels, peripheral parasitemia is not necessarily reflective of either parasite biomass or degree of organ sequestration (Lyke et al., 2003), making it difficult to relate

measures of peripheral blood parasite counts to local tissue pathology. Importantly, we did not observe positive correlations between hemorrhages and any measures of general parasite sequestration. This is consistent with other studies where sequestration was shown to be independent of hemorrhage (Boonpucknavig et al., 1990; Carvalho et al., 2000). The only parasitic element observed to be associated with hemorrhages in this study was extra-erythrocytic pigment (see Section 4.1.2.1.). Potentially more important was our finding of a significant correlation between the presence of fibrin thrombi in CM and hemorrhages. Some studies suggest that fibrin may play an important role in this disease (Boonpucknavig et al., 1990) and could be involved in the pathophysiology of hemorrhage. In this series, CM patients showed significantly decreased number of platelets in peripheral blood (Table 2), and several of the ring hemorrhages observed in the brains of CM patients contained a central thrombus, suggesting that hemorrhages may in some cases be the result of thrombus formation. However, many individual vessels also showed evidence of a thrombus without hemorrhage (Figure 21), making it difficult to determine the extent to which fibrin thrombi are important in the development of ring hemorrhages.

4.2. Axonal Damage in Pediatric Cerebral Malaria

Axonal injury is a major neuropathological feature and key predictor of outcome in a number of neurological disorders in both children and adults. It has been observed in severe head trauma, metabolic encephalopathies, autoimmune diseases such as multiple sclerosis, and infectious diseases such as HIV, and human lymphotropic virus type-1 (HTLV-1) (Medana and Esiri, 2003). The potential causes of axonal damage are numerous and include: mediators of coma (Conti et al., 2004), hypoglycemia (Dolinak et al., 2000), hypoxia/ischemia (Lambri et al., 2001), BBB dysfunction (Gray et al., 1998), and local CNS inflammation together with the production of pro-inflammatory cytokines (Bitsch et al., 2000; Sun et al., 2004). These are all clinical or pathological features previously observed in CM (see Sections 1.2. and 1.3.). Axonal injury has been previously described in a group of Southeast Asian adults who died of CM (Medana et al., 2002). The clinical presentation of adult and pediatric CM differs so widely, however, that an independent study into the relevance of axonal pathology in pediatric

CM was justified (see Section 1.2.3.). We hypothesized that pediatric cerebral malaria may be associated with significant axonal pathology, and tested this hypothesis by performing immunohistochemistry using a primary antibody to β-APP on brain tissue taken post-mortem from pediatric patients with strictly defined CM. In normal tissue, β-APP is transported along axons and is detectable only in the cell bodies of neurons. β-APP is only observable in axons when axonal injury allows it to accumulate to detectable levels. β-APP is detectable 2 hours after injury and remains visible for up to 2 weeks following an initial insult (Geddes et al., 1997; Gentleman et al., 1993). All of our patients died within two weeks of the onset of the illness, making β-APP immunohistochemistry useful for detecting axonal damage in this series.

4.2.1. Axonal Damage Compared in Pediatric Cerebral Malaria and Controls

Axonal damage was present in pediatric patients dying of either CM or SMA, with frequencies similar to those observed in adult patients (Medana et al., 2002). The presence of axonal damage in both CM and SMA patients suggests that axonal damage occurs in malaria patients independent of coma and neurological dysfunction. It is worth noting, however, that virtually the entire CM group showed some form of axonal damage while it was only present in a few SMA patients. The degree of axonal damage as quantitated by morphometric analysis of β-APP immunostaining was also significantly greater in the CM group compared to the SMA group. Furthermore, the CM group was the only one to differ from age-matched controls with respect to the amount and severity of damage. Of all the neuropathological features examined in this series, axonal damage was also the only one, besides perivascular ring hemorrhages, that distinguished CM from SMA patients. Together these results suggest that axonal damage plays a more central role in the pathophysiology of CM as compared to SMA.

We also compared the degree of axonal damage in CM patients to patients with comas caused by infectious agents other than *P.falciparum* (COCinfectious) including several patients who died of sepsis. It was important to compare CM patients to COCi patients because it has been suggested that the pathophysiology of CM is identical to sepsis where a host cell response to infection results in an over aggressive immune

response causing cell and tissue damage, multi-organ failure and eventually coma and death (see Section 1.3.2.2.) If CM and sepsis share a similar pathophysiology, we might expect similar patterns of pathology. In fact, we found that the degree of axonal damage was consistently and significantly greater in CM compared to patients with sepsis. This suggests that these illnesses differ at the level of axonal pathology and that they have distinct pathophysiology.

4.2.2. Distribution and Pattern of Axonal Damage in Pediatric Cerebral Malaria

The distribution of axonal damage in this series was non-uniform across the brains of CM patients, with most of the damage in the sub-cortical white matter and decreasing caudally towards the cerebellum. This finding is different from what was observed in SE Asian adults. Medana *et al.* showed that adult CM patients have the lowest APP load in the cortex and the greatest in the internal capsule and pons, with no mention of axonal damage in the sub-cortical white matter (Medana et al., 2002). In our study there was little evidence of axonal damage in the brainstem. Brainstem signs are common in pediatric CM (WHO, 2000) and axonal damage in the brainstem has been shown in other cases of coma (Smith et al., 2000). The limited axonal pathology in the brainstem suggests that brainstem signs may be linked to some other mechanism in this disease that has not been described. It is also worth noting that because axons travel long distances through the CNS, it is not possible to definitively assign a clinical outcome to axonal damage observed at a given site in the CNS. Therefore, the clinical relevance of the observed distribution of axonal damage in this study is unknown. The reason for the increased axonal damage in the sub-cortical white matter is also not clear. Several studies of parasite sequestration in CM have pointed out that there was an increased tendency towards sequestration in the cerebral hemispheres that may have been related to an increase in vasculature (Sein et al., 1993a; Sein et al., 1993b). In this work, however, no correlation was observed between axonal damage and general measures of sequestration. The only correlation between axonal damage and a parasitic element observed was a strong positive correlation between the number of extra-erythrocytic pigment globules observed at autopsy and the degree of non-hemorrhage associated

axonal damage (R=0.766, P<0.05, Figure 13). This suggests that a parasite-stage-specific insult may be the cause of the NHA axonal pathology.

Two distinct types of axonal pathology emerged in CM patients: axonal damage associated with hemorrhage (HA) and axonal damage unassociated with hemorrhage (NHA), with the latter group contributing to the bulk of the APP load in CM patients (Figure 12). Several lines of evidence suggest that these two pathologies are distinct. First, there was no correlation between the two pathologies (Table 32). Hemorrhages were not observed to be associated with NHA lesions histologically (Figure 11), and there was a subset of CM patients with NHA that did not have hemorrhages. HA axonal damage was secondary to hemorrhage, a feature that has been described in other disorders showing hemorrhage (Oehmichen et al., 2003). This suggests that treatments targeting hemorrhage in CM may influence the degree of HA axonal damage (see Section 4.1.). Little is known about the cause of axonal damage not associated with hemorrhage, a type of axonal damage also formerly observed in SE Asian adults (Medana et al., 2002). Morphologically, the NHA lesions appear to have a highly vacuolated neuropil which is consistent with axonal damage secondary to edema. We also found that patients with a significant degree of blood-brain-barrier dysfunction also had significant axonal damage (Table 30), which also suggest a role for edema in this disease. Medana *et al.* also found CSF protein to be related in part to APP load (Medana et al., 2002). CNS edema was not a major feature of CM patients in this series, however, and those patients with papilledema did not show increased axonal damage over CM patients without this feature. In addition, though edema has been observed in pediatric CM studies in general, several studies suggest that it is unlikely to be an important complicating factor in this disease (Sanni, 2001).

4.2.3. Clinicopathological Correlations of Axonal Damage in Pediatric CM

We carried out a detailed series of clinicopathological correlations, paying particular attention to the relationship of coma and convulsions, two features that are important to the clinical definition of CM, and the production of axonal damage. Importantly, no correlation was found between axonal damage and duration or presence

of convulsions in this series. This suggests that axonal damage was not secondary to physical trauma resulting from convulsion as observed previously in cases of head trauma (Wichert-Ana et al., 2004). Alternatively, it was observed that individual CM patients who experienced the longest coma duration had the most severe axonal pathology (Table 16), a feature that was independent of total illness duration and duration of hospital stay. Axonal damage in CM was also related to glucose levels in individual patients in this study with a negative correlation between the degree of axonal damage and the last glucose measurement before death (Figure 15). Patients with the most severe axonal damage had the lowest blood glucose levels (Table 16). This latter finding contradicts what was observed in adult CM patients where no relationship between blood glucose levels and axonal damage was found (Medana et al., 2002). Although this evidence suggests that hypoglycemia, coma, and axonal damage were related as has been observed in other diseases (Dolinak et al., 2000), it is worth noting that SMA patients in this series had lower glucose measurements than CM patients (Table 2) while also having less axonal damage. Consequently, if decreased glucose plays a role in the pathophysiology of CM, it does not likely do so independently of some other factor that has yet to be identified. It is also worth noting that the relationship between systemic glucose levels and local metabolic disturbances in the CNS of CM patients is not well understood.

4.2.4. Brain and Retinal Axonal Pathology in Pediatric Cerebral Malaria

We were also interested in determining the extent to which retinal axonal pathology might reflect brain axonal pathology in CM (see Section 1.5.). Axonal damage was a major feature in the retinas of CM patients, but it was observed much less frequently than it was in the brain. This may be due to the smaller area of the retina assessed for axonal damage compared to the brain. Both types of axonal damage characteristic to the brain were observed (HA and NHA) and an overall a positive correlation was found between brain and retinal axonal damage (see Section 3.6.4.). This suggests that the two pathologies are related, and may be due to the tissues sharing common cell types and developmental origin. Previous studies also showed that both tissues demonstrate parasite sequestration (Lewallen et al., 2000). The observation that retinal pathology reflects brain pathology in this study supports use of the retina as a

model tissue in studies of the pathophysiology of CM (Medana et al., 2001). No specific positive correlations were observed between β-APP staining and specific ocular fundus findings, though we found that those patients with appreciable brain axonal pathology had an increase in macular whitening. It has been suggested that macular whitening in the eyes of CM patients may be similar to cotton wool spots and could therefore represent localized accumulations of axonal debris collecting at areas of retinal axonal injury (Medana and Esiri, 2003). Thus, the presence of macular whitening may be a helpful clinical indicator of brain axonal injury in CM.

4.2.5. Axonal Damage and Myelin Loss in Pediatric CM

Myelin loss has been noted in studies of CM (Ma et al., 1997; Medana et al., 1996; Medana et al., 2002), and a previous neuropathological study of adult CM patients showed that myelin damage and axonal damage may be related in CM (Medana et al., 2002). In our study, myelin loss was present in every CM patient with axonal pathology, and the two types of axonal damage observed (HA and NHA) were also found in brain sections stained for LFB to identify myelin loss (Figure 19). In addition, a positive correlation was found between the total amount of myelin loss and axonal damage in individual brain regions CM (Table 21). Therefore, it appears that the myelin pallor observed using LFB in CM patients actually coexists with areas of axonal damage, and that a specific process directed at the myelin sheath is unlikely in this disease.

4.3. Blood-Brain-Barrier Dysfunction in Pediatric CM and SMA

The endothelial cells lining the microvessels of the brain are specially adapted to maintain a difference between the local environment of the CNS and events occurring in the periphery. Much evidence shows that disruption of the BBB is characteristic of several diseases of the CNS (Ballabh et al., 2004). BBB dysfunction has been considered in several studies of adult and pediatric CM in addition to the murine model of malaria. Despite this, the role of BBB dysfunction in the development of neurologic dysfunction in CM, and how BBB dysfunction relates to the range of clinical and neuropathological features in CM is not known (see Sections 1.3.2.3. and 1.4.7.). In this study, we assessed BBB dysfunction by anti-fibrinogen immunohistochemistry and BBB

dysfunction was found to be common in CM (24/25, 96%) and could therefore contribute to neurologic dysfunction and death in this disease. Interestingly, however, we also observed that all SMA patients in this series experienced similar degrees of BBB dysfunction, suggesting that BBB dysfunction may be important in the pathophysiology of SMA as well. It is not presently clear if the BBB opening observed in CM and SMA patients is related to a common pathophysiological mechanism. Several attempts have been made to explain the cause of the BBB dysfunction in CM. Recent evidence suggests that BBB dysfunction may be mediated through a signal transduction pathway where ligand proteins on the surface of parasite infected erythrocytes induce the opening of inter-endothelial tight junctions upon binding to ICAM-1 on the endothelium. Conceptually, this is similar in the same way that signal transduction occurs to allow the passage of white blood cells across the BBB (Adams et al., 2002). Studies have shown that ICAM-1 expression, necessary for a ligand-receptor interaction, was increased in CM patients but not SMA patients (Turner et al., 1994). Thus, if BBB dysfunction in this disease is ICAM-1 dependent, it is unlikely that this is also the case in SMA patients. Another potential source of BBB opening is the systemic and local production of cytokines observed in both CM and SMA patients (Clark et al., 1997), which have been shown to be capable of causing increased BBB permeability (Prat et al., 2001; Wong et al., 2004).

BBB dysfunction in CM appears to be associated with a number of neuropathological features. We found that BBB dysfunction could be found together with hemorrhages and microthrombi, or also in vessels that otherwise appeared intact. Thus, even though increased BBB permeability is common in CM, it is possible that it may not be the result of a single insult. The extent to which different insults may overlap in the development of BBB dysfunction is not known. Of the parasitological features observed, the only one to positively correlate with BBB dysfunction in CM patients was the number of pigment globules observed histologically ($R=0.621$, $P<0.01$), again suggesting a role for pigment-globules in the development of neuropathology. How extra-erythrocytic pigment globules might contribute to the development of BBB dysfunction is not known, however, some studies suggest that pigment globules may play

an immunomodulatory role in this disease (see Section 4.1.2.1.). As noted above, the malaria pigment hemazoin is able to induce the production of several pro-inflammatory cytokines *in vitro* that are able increase the permeability of the BBB (Prat et al., 2001; Wong et al., 2004). It is also interesting to note that in the entire patient group examined, the amount of BBB dysfunction appeared to be consistently greater in the white matter versus the grey matter.

4.4. Gliosis in Pediatric CM and SMA

Gliosis is characterized by an increase in the size and number of GFAP positive astrocytes and represents a reactive astrocytic response to non-specific CNS injury (Norenberg, 1994). A number of histological descriptions of gliosis in CM have been offered in previous studies (Medana et al., 2002), but the role of gliosis in the context of other neuropathological and clinical changes in this disease has not been considered. In this thesis work, an increased astrocyte response was observed in the majority of CM patients (24/25, 96%) and all the SMA patients under study. No specific correlations were observed between gliosis and either sequestration, axonal damage or any other neuropathological feature in this study. The only association between gliosis and the clinical data in CM was that patients with a history of coma on admission, and those patients who had longest hospitalization, also had the most severe gliosis.

Most of what is known about the role of astrocytes and gliosis in CM comes from studies in the fatal murine model of CM (FMCM). In this model, mice infected with *P.berghei* ANKA develop neurological symptoms at day 5 post-inoculation leading to coma and death at day 7 post-inoculation. A study by Ma *et al.* using horseradish peroxidase and GFAP immunohistochemistry of optic nerve sections as a model of brain white matter showed vascular congestion, blood-retinal-barrier breakdown, patchy axonal demyelination and astrogliosis beginning at day 3 post-inoculation and peaking at day 7 post-inoculation in FMCM mice. This finding suggested a mechanism where increased vascular permeability gives way to immune dysfunction and axonal damage (Chang-Ling et al., 1992; Ma et al., 1997). Other studies of the FMCM model by Medana *et al.* compare astrocyte responses in retinal wholemounts taken from: (1) FMCM mice (2) the resolving murine model of CM where mice develop neurologic dysfunction but recover,

and (3) a non-CM model where neurologic dysfunction is not seen (Medana et al., 1996). Similar results were found in the FMCM model, while mice with the resolving form experienced only mild vascular and astrocytic changes. Non-CM mice showed no change. Treatment of the FMCM, resolving mice, and non-CM mice with dexamethasone post-inoculation prevented the loss of vessel ensheathment by astrocytes characteristic of the FMCM model, suggesting a role for immune dysfunction in experimental cerebral malaria (ECM). In our study, reactive gliosis did not directly correlate with BBB dysfunction or any other neuropathological feature and was present in both CM and SMA patients. This suggests that astrocytes may play a very different role in human CM and SMA compared to the murine model.

CHAPTER 5

CONCLUSIONS & FUTURE DIRECTIONS

5.1. Perivascular Ring Hemorrhages and Pediatric Cerebral Malaria

The purpose of this study was to determine the nature and extent of the neuropathological changes in pediatric cerebral malaria. Perivascular ring hemorrhages have been noted in several other studies (see Section 1.4.2.), though the prevalence of this feature in pediatric CM, and the extent to which ring hemorrhages may contribute to CNS damage in this disease, are not known.

It is clear from this study that perivascular ring hemorrhages is positively associated with specific neuropathological changes and were a feature of CM patients alone. SMA patients did not have ring hemorrhages. Thus, the presence of hemorrhages at autopsy may therefore corroborate a diagnosis of CM. Perivascular ring hemorrhages were not present in all CM patients, however, making it difficult to rule out a diagnosis of CM in the absence of hemorrhage alone. We also observed occasional perivascular ring hemorrhages in COC patients, suggesting that the clinician must be aware of intervening pathologies in working with comatose patients thought to have CM, and that the potential for misdiagnosis exists in some instances (Taylor et al., 2004). To understand the potential for intervening pathology in comatose patients from malaria endemic zones more generally, it might therefore be worthwhile to examine a greater number of COC patients. A further study like this may help to establish systematic and accurate methods of determining cause of death in CM patients, making it easier to derive meaningful information from studies of CM pathogenesis.

A most interesting finding related to perivascular ring hemorrhages in this study is that hemorrhages were not a universal feature in CM. The presence of hemorrhages was related, to a large extent, to the degree of extra-erythrocytic pigment deposition observed histologically. It is also of interest to note that there was a positive correlation between the degree of hemorrhages in the retinas of CM patients and the degree of hemorrhages in

their brains, suggesting that patients with CNS hemorrhages can be distinguished from patients without hemorrhage by ophthalmologic examination during life. The latter finding would especially useful if hemorrhages are somehow reflective of a pathogenetic mechanism distinctive to a subset of CM patients. The only parameter unique to CM patients with hemorrhage in this study was the degree of extra-erythrocytic pigment, observed to play an immunomodulatory role in other CM studies (see Section 4.1.2.1.). To determine if an immunomodulatory process related to hemorrhage and pigment was involved in this series, further studies into the expression of pro-inflammatory cytokines (IFN-γ and TNF-α) and chemokines (MIP1-α, MIP1-β and MCP-1) using immunohistochemistry might be informative. Similar studies into the regulation of these mediators by measuring serum concentrations, and the expression pattern of these mediators in other tissues would also be helpful.

Although pigment has been shown to have an immunomodulatory response in CM, some authors have also suggested that the amount of extra-erythrocytic pigment may be the only reliable measure of total parasite biomass in malaria infected patients (Lyke et al., 2003). If this is the case, it might follow that hemorrhages are only present in those patients who have increased parasite biomass compared to other CM patients. Further studies into the role of pigment and total parasite biomass in CM patients would be helpful, albeit difficult, because no reliable way of determining total parasite biomass in CM exists. Another way to clarify further the role of hemorrhage would be to increase the total number of CM patients studied. Although the number of CM patients examined in this study (n=25) was sufficient to establish statistical differences between CM and SMA and COC groups, a statistically significant difference was not observed between Class I and Class II CM patients with respect to some parameters. This suggests perhaps that an increase in the number of patients might clarify the role of some of these parameters with respect to hemorrhage and pigment.

We also determined that perivascular ring hemorrhages were related to fibrin deposition in CM patients. A role for fibrin in CM pathogenesis has been offered previously, and some authors have suggested that hemorrhage may be related in part to

disseminated intravascular coagulation (DIC). A systematic investigation into DIC and the coagulation cascade might be helpful in clarifying how fibrin may be involved in the development of hemorrhage and how hemorrhage contributes to the pathogenesis of CM.

5.2. Axonal Damage and Pediatric Cerebral Malaria

Axonal damage was a major feature of CM patients, but was also present to a degree in SMA patients who did not exhibit neurologic dysfunction. Nevertheless, the frequency and extent of axonal damage was much greater in CM patients compared to SMA. Independent study of the cause of axonal damage in SMA is warranted, given that this study shows evidence of gliosis and blood-brain-barrier dysfunction in SMA patients (see Section 3.8. and 3.9.), a feature that is expected only in those patients who experience a cerebral insult. Some COC patients also showed evidence of axonal damage, and interestingly, the degree of axonal damage was much less in COC patients compared to CM patients. The latter finding is interesting because a major question in studies into CM pathogenesis is the extent to which CM patients are similar to patients experiencing general sepsis, and whether CM is mostly a sepsis-like illness. This study clearly shows that CM patients are different from sepsis patients at the neuropathological level. Further studies into the relationship between axonal damage and both systemic and local proinflammatory cytokine and chemokine production would bring valuable insight into this discussion by revealing whether a positive correlation exists between axonal damage and cytokine production, as might be the case in sepsis.

Two types of axonal damage were observed in CM patients in this series (HA and NHA). The two types of pathology were clearly independent and the NHA pathology was the more prevalent of the two. The cause of NHA axonal damage is not known, though some possibilities were ruled out; NHA axonal damage did not appear to be the result of edema and is not secondary to convulsions. NHA axonal damage did not seem to be related to sequestration in general, but may have been related to pigment production or to the presence of extra-erythrocytic pigment. The role of extra-erythrocytic pigment production is especially interesting given that it has been shown to mediate immunological dysfunction in CM, and may be a useful indicator of total parasite

biomass (see Section 6.1). A larger clinical cohort of CM patients would be helpful in clarifying which clinical parameters may be involved in axonal damage. Studies of axonal damage in SE Asian adults suggest that it may represent a final common syndrome in CM patients that, individually, may be experiencing independent pathogenetic events (Medana et al., 2002). Understanding how NHA axonal damage relates to cytokine production, excitotoxic damage, and metabolic change are of immediate interest (Medana et al., 2001).

A strong positive correlation was observed between the degree of brain and retinal axonal damage in CM patients. The nature of the lesions in the CNS and retina were the same, suggesting that retinal axonal pathology reflects brain axonal pathology in CM. The main difference between the axonal damage in the brain and retina was that retinal axonal pathology was not as common in the retina of CM patients compared to their brain. This is most likely due to the small sampling area of the retina compared to the brain. Obtaining whole globe samples from autopsy cases is difficult at QEHC. However, attempts could be made to produce retinal wholemounts from samples obtained for future studies. Retinal wholemounts allow the investigator to assess the total degree of pathology across the eye so that the degree of pathology is not underestimated. In addition, retinal wholemounts allow for the observation of the insult in the whole context of the retina so that the relationship between intervening lesions can be ascertained (Medana et al., 2001). The only relationship that existed between axonal damage and ocular fundus findings during life was between axonal damage and the degree of macular whitening observed in previous studies of retinal findings in pediatric CM (see Section 1.2.2.). The nature and cause of macular whitening in pediatric CM is currently not known. This study suggests that the insult may be related to axonal damage and that macular whitening may represent a pathologic finding similar to cotton wool spots, though further studies into this phenomenon must be undertaken.

5.3. Blood-Brain-Barrier Permeability in Pediatric Cerebral Malaria

Blood-brain-barrier dysfunction as measured by increased vessel permeability to fibrinogen was noted in both CM and SMA patients, suggesting that this may be

important to the pathogenesis of malaria infections more generally. The role of BBB dysfunction in CM has been studied previously, and some authors suggest that BBB dysfunction is likely to play a subtle role in CM pathophysiology. This is due mostly to the fact that widespread BBB dysfunction does not appear to be a common feature in the brains of CM patients (Gitau and Newton, 2005) and that widespread edema is relatively uncommon. Similar conclusions can be drawn from this work. BBB dysfunction, although present in most CM patients, was minimal. Further studies using additional immunohistochemistry markers to assess BBB permeability and endothelial cell junction proteins are warranted, so that the full extent of the BBB dysfunction in this group can be known.

5.4. Gliosis and Pediatric Cerebral Malaria

Gliosis is a non-specific response to CNS injury and was a major feature in the brains and retinas of CM patients, but was also present in SMA patients, suggesting that gliosis may not contribute specifically to CM without the involvement of some other factors not yet determined. Further studies including additional CM patients may serve to increase our understanding of the role of gliosis in CM and SMA patients.

CHAPTER 6

REFERENCES

Abeku, T. A., van Oortmarssen, G. J., Borsboom, G., de Vlas, S. J., and Habbema, J. D. (2003). Spatial and temporal variations of malaria epidemic risk in Ethiopia: factors involved and implications. Acta Trop *87*, 331-340.

Adams, S., Brown, H., and Turner, G. (2002). Breaking down the blood-brain barrier: signaling a path to cerebral malaria? Trends Parasitol *18*, 360-366.

Ahmed, A., Cerilli, L. A., and Sanchez, P. J. (1998). Congenital malaria in a preterm neonate: case report and review of the literature. Am J Perinatol *15*, 19-22.

Aikawa, M., Iseki, M., Barnwell, J. W., Taylor, D., Oo, M. M., and Howard, R. J. (1990). The pathology of human cerebral malaria. Am J Trop Med Hyg *43*, 30-37.

Ballabh, P., Braun, A., and Nedergaard, M. (2004). The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis *16*, 1-13.

Beeson, J. G., and Brown, G. V. (2002). Pathogenesis of Plasmodium falciparum malaria: the roles of parasite adhesion and antigenic variation. Cell Mol Life Sci *59*, 258-271.

Bellamy, R., Kwiatkowski, D., and Hill, A. V. (1998). Absence of an association between intercellular adhesion molecule 1, complement receptor 1 and interleukin 1 receptor antagonist gene polymorphisms and severe malaria in a West African population. Trans R Soc Trop Med Hyg *92*, 312-316.

Berendt, A. R., Ferguson, D. J., Gardner, J., Turner, G., Rowe, A., McCormick, C., Roberts, D., Craig, A., Pinches, R., Elford, B. C., and et al. (1994). Molecular mechanisms of sequestration in malaria. Parasitology *108 Suppl*, S19-28.

Bi, P., Tong, S., Donald, K., Parton, K. A., and Ni, J. (2003). Climatic variables and transmission of malaria: a 12-year data analysis in Shuchen County, China. Public Health Rep *118*, 65-71.

Biswas, S., Karmarkar, M. G., and Sharma, Y. D. (2001). Antibodies detected against Plasmodium falciparum haemozoin with inhibitory properties to cytokine production. FEMS Microbiol Lett *194*, 175-179.

Bitsch, A., Schuchardt, J., Bunkowski, S., Kuhlmann, T., and Bruck, W. (2000). Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain *123 (Pt 6)*, 1174-1183.

Boonpucknavig, V., Boonpucknavig, S., Udomsangpetch, R., and Nitiyanant, P. (1990). An immunofluorescence study of cerebral malaria. A correlation with histopathology. Arch Pathol Lab Med *114*, 1028-1034.

Brown, H., Hien, T. T., Day, N., Mai, N. T., Chuong, L. V., Chau, T. T., Loc, P. P., Phu, N. H., Bethell, D., Farrar, J., *et al.* (1999). Evidence of blood-brain barrier dysfunction in human cerebral malaria. Neuropathol Appl Neurobiol *25*, 331-340.

Brown, H., Rogerson, S., Taylor, T., Tembo, M., Mwenechanya, J., Molyneux, M., and Turner, G. (2001). Blood-brain barrier function in cerebral malaria in Malawian children. Am J Trop Med Hyg *64*, 207-213.

Brown, H. C., Chau, T. T., Mai, N. T., Day, N. P., Sinh, D. X., White, N. J., Hien, T. T., Farrar, J., and Turner, G. D. (2000). Blood-brain barrier function in cerebral malaria and CNS infections in Vietnam. Neurology *55*, 104-111.

Carvalho, L. J., Lenzi, H. L., Pelajo-Machado, M., Oliveira, D. N., Daniel-Ribeiro, C. T., and Ferreira-da-Cruz, M. F. (2000). Plasmodium berghei: cerebral malaria in CBA mice is not clearly related to plasma TNF levels or intensity of histopathological changes. Exp Parasitol *95*, 1-7.

Chang-Ling, T., Neill, A. L., and Hunt, N. H. (1992). Early microvascular changes in murine cerebral malaria detected in retinal wholemounts. Am J Pathol *140*, 1121-1130.

Chau, T. T., Mai, N. T., Phu, N. H., Luxemburger, C., Chuong, L. V., Loc, P. P., Trang, T. T., Vinh, H., Cuong, B. M., Waller, D. J., *et al.* (2002). Malaria in injection drug abusers in Vietnam. Clin Infect Dis *34*, 1317-1322.

Chotivanich, K., Udomsangpetch, R., McGready, R., Proux, S., Newton, P., Pukrittayakamee, S., Looareesuwan, S., and White, N. J. (2002). Central role of the spleen in malaria parasite clearance. J Infect Dis *185*, 1538-1541.

Choudhury, N., and Phadke, S. (2001). Transfusion transmitted diseases. Indian J Pediatr *68*, 951-958.

Clark, I., and Awburn, M. (2002). Migration inhibitory factor in the cerebral and systemic endothelium in sepsis and malaria. Crit Care Med *30*, S263-267.

Clark, I. A., al Yaman, F. M., and Jacobson, L. S. (1997). The biological basis of malarial disease. Int J Parasitol *27*, 1237-1249.

Clark, I. A., Awburn, M. M., Harper, C. G., Liomba, N. G., and Molyneux, M. E. (2003a). Induction of HO-1 in tissue macrophages and monocytes in fatal falciparum malaria and sepsis. Malar J *2*, 41.

Clark, I. A., Awburn, M. M., Whitten, R. O., Harper, C. G., Liomba, N. G., Molyneux, M. E., and Taylor, T. E. (2003b). Tissue distribution of migration inhibitory factor and inducible nitric oxide synthase in falciparum malaria and sepsis in African children. Malar J *2*, 6.

Clark, I. A., Rockett, K. A., and Cowden, W. B. (1991). Role of TNF in cerebral malaria. Lancet *337*, 302-303.

Conti, G., Rostami, A., Scarpini, E., Baron, P., Galimberti, D., Bresolin, N., Contri, M., Palumbo, C., and De Pol, A. (2004). Inducible nitric oxide synthase (iNOS) in immune-mediated demyelination and Wallerian degeneration of the rat peripheral nervous system. Exp Neurol *187*, 350-358.

Crawley, J., English, M., Waruiru, C., Mwangi, I., and Marsh, K. (1998). Abnormal respiratory patterns in childhood cerebral malaria. Trans R Soc Trop Med Hyg *92*, 305-308.

Crawley, J., Smith, S., Kirkham, F., Muthinji, P., Waruiru, C., and Marsh, K. (1996). Seizures and status epilepticus in childhood cerebral malaria. Qjm *89*, 591-597.

Davis, T. M., Suputtamongkol, Y., Spencer, J. L., Ford, S., Chienkul, N., Schulenburg, W. E., and White, N. J. (1992). Measures of capillary permeability in acute falciparum malaria: relation to severity of infection and treatment. Clin Infect Dis *15*, 256-266.

Day, N. P., Phu, N. H., Mai, N. T., Chau, T. T., Loc, P. P., Chuong, L. V., Sinh, D. X., Holloway, P., Hien, T. T., and White, N. J. (2000). The pathophysiologic and prognostic significance of acidosis in severe adult malaria. Crit Care Med *28*, 1833-1840.

de Souza, J. B., and Riley, E. M. (2002). Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis. Microbes Infect *4*, 291-300.

Deininger, M. H., Fimmen, B., Kremsner, P. G., Meyermann, R., and Schluesener, H. J. (2002). Accumulation of endostatin/collagenXVIII in brains of patients who died with cerebral malaria. J Neuroimmunol *131*, 216-221.

Deininger, M. H., Kremsner, P. G., Meyermann, R., and Schluesener, H. J. (2000a). Differential cellular accumulation of transforming growth factor-beta1, -beta2, and -beta3 in brains of patients who died with cerebral malaria. J Infect Dis *181*, 2111-2115.

Deininger, M. H., Kremsner, P. G., Meyermann, R., and Schluesener, H. J. (2000b). Focal accumulation of cyclooxygenase-1 (COX-1) and COX-2 expressing cells in cerebral malaria. J Neuroimmunol *106*, 198-205.

Deininger, M. H., Winkler, S., Kremsner, P. G., Meyermann, R., and Schluesener, H. J. (2003). Angiogenic proteins in brains of patients who died with cerebral malaria. J Neuroimmunol *142*, 101-111.

Dobbie, M. S., Hurst, R. D., Klein, N. J., and Surtees, R. A. (1999). Upregulation of intercellular adhesion molecule-1 expression on human endothelial cells by tumour necrosis factor-alpha in an in vitro model of the blood-brain barrier. Brain Res *830*, 330-336.

Dolinak, D., Smith, C., and Graham, D. I. (2000). Hypoglycaemia is a cause of axonal injury. Neuropathol Appl Neurobiol *26*, 448-453.

Ekvall, H. (2003). Malaria and anemia. Curr Opin Hematol *10*, 108-114.

English, M., Waruiru, C., Amukoye, E., Murphy, S., Crawley, J., Mwangi, I., Peshu, N., and Marsh, K. (1996). Deep breathing in children with severe malaria: indicator of metabolic acidosis and poor outcome. Am J Trop Med Hyg *55*, 521-524.

Fauser, S., Deininger, M. H., Kremsner, P. G., Magdolen, V., Luther, T., Meyermann, R., and Schluesener, H. J. (2000). Lesion associated expression of urokinase-type plasminogen activator receptor (uPAR, CD87) in human cerebral malaria. J Neuroimmunol *111*, 234-240.

Flick, K., and Chen, Q. (2004). var genes, PfEMP1 and the human host. Mol Biochem Parasitol *134*, 3-9.

Fontenille, D., and Lochouarn, L. (1999). The complexity of the malaria vectorial system in Africa. Parassitologia *41*, 267-271.

Freeman, W. R., McCutchan, J. A., Arevalo, J. F., Wolfson, T., Marcotte, T. D., Heaton, R. K., and Grant, I. (2004). The relationship between AIDS retinal cotton wool spots and neuropsychological impairment in HIV-positive individuals in the pre-highly active antiretroviral therapy era. Ocul Immunol Inflamm *12*, 25-33.

Furlan, R., Villa, P., Senaldi, G., and Martino, G. (2004). TNFalpha in experimental diseases of the CNS. Methods Mol Med *98*, 171-190.

Geddes, J. F., Vowles, G. H., Beer, T. W., and Ellison, D. W. (1997). The diagnosis of diffuse axonal injury: implications for forensic practice. Neuropathol Appl Neurobiol *23*, 339-347.

Gentleman, S. M., Nash, M. J., Sweeting, C. J., Graham, D. I., and Roberts, G. W. (1993). Beta-amyloid precursor protein (beta APP) as a marker for axonal injury after head injury. Neurosci Lett *160*, 139-144.

Genton, B., al-Yaman, F., Alpers, M. P., and Mokela, D. (1997). Indicators of fatal outcome in paediatric cerebral malaria: a study of 134 comatose Papua New Guinean children. Int J Epidemiol *26*, 670-676.

Ghigo, D., Todde, R., Ginsburg, H., Costamagna, C., Gautret, P., Bussolino, F., Ulliers, D., Giribaldi, G., Deharo, E., Gabrielli, G., and et al. (1995). Erythrocyte stages of Plasmodium falciparum exhibit a high nitric oxide synthase (NOS) activity and release an NOS-inducing soluble factor. J Exp Med *182*, 677-688.

Gitau, E. N., and Newton, C. R. (2005). Review Article: blood-brain barrier in falciparum malaria. Trop Med Int Health *10*, 285-292.

Grau, G. E., Piguet, P. F., Vassalli, P., and Lambert, P. H. (1989). Tumor-necrosis factor and other cytokines in cerebral malaria: experimental and clinical data. Immunol Rev *112*, 49-70.

Gray, F., Belec, L., Chretien, F., Dubreuil-Lemaire, M. L., Ricolfi, F., Wingertsmann, L., Poron, F., and Gherardi, R. (1998). Acute, relapsing brain oedema with diffuse blood-brain barrier alteration and axonal damage in the acquired immunodeficiency syndrome. Neuropathol Appl Neurobiol *24*, 209-216.

Gruenberg, J., Allred, D. R., and Sherman, I. W. (1983). Scanning electron microscope-analysis of the protrusions (knobs) present on the surface of Plasmodium falciparum-infected erythrocytes. J Cell Biol *97*, 795-802.

Hamer, D. H., and Wyler, D. J. (1993). Cerebral malaria. Semin Neurol *13*, 180-188.

Hay, S. I., Noor, A. M., Simba, M., Busolo, M., Guyatt, H. L., Ochola, S. A., and Snow, R. W. (2002). Clinical epidemiology of malaria in the highlands of western Kenya. Emerg Infect Dis *8*, 543-548.

Hay, S. I., Rogers, D. J., Shanks, G. D., Myers, M. F., and Snow, R. W. (2001). Malaria early warning in Kenya. Trends Parasitol *17*, 95-99.

Hearn, J., Rayment, N., Landon, D. N., Katz, D. R., and de Souza, J. B. (2000). Immunopathology of cerebral malaria: morphological evidence of parasite sequestration in murine brain microvasculature. Infect Immun *68*, 5364-5376.

Heremans, H., Dillen, C., Dijkmans, R., Grau, G., and Billiau, A. (1989). The role of cytokines in various animal models of inflammation. Lymphokine Res *8*, 329-333.

Hermsen, C. C., Crommert, J. V., Fredix, H., Sauerwein, R. W., and Eling, W. M. (1997). Circulating tumour necrosis factor alpha is not involved in the development of cerebral malaria in Plasmodium berghei-infected C57Bl mice. Parasite Immunol *19*, 571-577.

Hermsen, C. C., Mommers, E., van de Wiel, T., Sauerwein, R. W., and Eling, W. M. (1998). Convulsions due to increased permeability of the blood-brain barrier in experimental cerebral malaria can be prevented by splenectomy or anti-T cell treatment. J Infect Dis *178*, 1225-1227.

Ijumba, J. N., and Lindsay, S. W. (2001). Impact of irrigation on malaria in Africa: paddies paradox. Med Vet Entomol *15*, 1-11.

Ijumba, J. N., Shenton, F. C., Clarke, S. E., Mosha, F. W., and Lindsay, S. W. (2002). Irrigated crop production is associated with less malaria than traditional agricultural practices in Tanzania. Trans R Soc Trop Med Hyg *96*, 476-480.

Jabs, D. A. (1995). Ocular manifestations of HIV infection. Trans Am Ophthalmol Soc *93*, 623-683.

Janota, I., and Doshi, B. (1979). Cerebral malaria in the United Kingdom. J Clin Pathol *32*, 769-772.

Jaramillo, M., Godbout, M., and Olivier, M. (2005). Hemozoin induces macrophage chemokine expression through oxidative stress-dependent and -independent mechanisms. J Immunol *174*, 475-484.

Jaramillo, M., Gowda, D. C., Radzioch, D., and Olivier, M. (2003). Hemozoin increases IFN-gamma-inducible macrophage nitric oxide generation through extracellular signal-regulated kinase- and NF-kappa B-dependent pathways. J Immunol *171*, 4243-4253.

Keller, C. C., Kremsner, P. G., Hittner, J. B., Misukonis, M. A., Weinberg, J. B., and Perkins, D. J. (2004). Elevated nitric oxide production in children with malarial anemia: hemozoin-induced nitric oxide synthase type 2 transcripts and nitric oxide in blood mononuclear cells. Infect Immun *72*, 4868-4873.

Kochar, D. K., Shubhakaran, Kumawat, B. L., and Vyas, S. P. (2000). Prognostic significance of eye changes in cerebral malaria. J Assoc Physicians India *48*, 473-477.

Koram, K. A., Owusu-Agyei, S., Utz, G., Binka, F. N., Baird, J. K., Hoffman, S. L., and Nkrumah, F. K. (2000). Severe anemia in young children after high and low malaria transmission seasons in the Kassena-Nankana district of northern Ghana. Am J Trop Med Hyg *62*, 670-674.

Kristensson, K., Mhlanga, J. D., and Bentivoglio, M. (2002). Parasites and the brain: neuroinvasion, immunopathogenesis and neuronal dysfunctions. Curr Top Microbiol Immunol *265*, 227-257.

Kwiatkowski, D. (1990). Tumour necrosis factor, fever and fatality in falciparum malaria. Immunol Lett *25*, 213-216.

Kwiatkowski, D., Hill, A. V., Sambou, I., Twumasi, P., Castracane, J., Manogue, K. R., Cerami, A., Brewster, D. R., and Greenwood, B. M. (1990). TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated Plasmodium falciparum malaria. Lancet *336*, 1201-1204.

Lambri, M., Djurovic, V., Kibble, M., Cairns, N., and Al-Sarraj, S. (2001). Specificity and sensitivity of betaAPP in head injury. Clin Neuropathol *20*, 263-271.

Lewallen, S., Bakker, H., Taylor, T. E., Wills, B. A., Courtright, P., and Molyneux, M. E. (1996). Retinal findings predictive of outcome in cerebral malaria. Trans R Soc Trop Med Hyg *90*, 144-146.

Lewallen, S., Harding, S., Ajewole, J., W., S., Molyneux, M., Marsh, K., S., U., White, N., and Taylor, T. (1999a). A review of the spectrum of clinical ocular fundus findings in P.falciparum malaria in African children with a proposed classification and grading system. Trans R Soc Trop Med Hyg *93*, 619-622.

Lewallen, S., Harding, S. P., Ajewole, J., Schulenburg, W. E., Molyneux, M. E., Marsh, K., Usen, S., White, N. J., and Taylor, T. E. (1999b). A review of the spectrum of clinical

ocular fundus findings in P. falciparum malaria in African children with a proposed classification and grading system. Trans R Soc Trop Med Hyg *93*, 619-622.

Lewallen, S., Taylor, T. E., Molyneux, M. E., Wills, B. A., and Courtright, P. (1993). Ocular fundus findings in Malawian children with cerebral malaria. Ophthalmology *100*, 857-861.

Lewallen, S., White, V. A., Whitten, R. O., Gardiner, J., Hoar, B., Lindley, J., Lochhead, J., McCormick, A., Wade, K., Tembo, M., *et al.* (2000). Clinical-histopathological correlation of the abnormal retinal vessels in cerebral malaria. Arch Ophthalmol *118*, 924-928.

Lim, S. A., Heng, W. J., Lim, T. H., Leo, Y. S., and Wong, S. Y. (1997). Ophthalmic manifestations in human immunodeficiency virus infection in Singapore. Ann Acad Med Singapore *26*, 575-580.

Looareesuwan, S., Warrell, D. A., White, N. J., Chanthavanich, P., Warrell, M. J., Chantaratherakitti, S., Changswek, S., Chongmankongcheep, L., and Kanchanaranya, C. (1983). Retinal hemorrhage, a common sign of prognostic significance in cerebral malaria. Am J Trop Med Hyg *32*, 911-915.

Looareesuwan, S., Wilairatana, P., Krishna, S., Kendall, B., Vannaphan, S., Viravan, C., and White, N. J. (1995). Magnetic resonance imaging of the brain in patients with cerebral malaria. Clin Infect Dis *21*, 300-309.

Lou, J., Lucas, R., and Grau, G. E. (2001). Pathogenesis of cerebral malaria: recent experimental data and possible applications for humans. Clin Microbiol Rev *14*, 810-820, table of contents.

Lyke, K. E., Diallo, D. A., Dicko, A., Kone, A., Coulibaly, D., Guindo, A., Cissoko, Y., Sangare, L., Coulibaly, S., Dakouo, B., *et al.* (2003). Association of intraleukocytic Plasmodium falciparum malaria pigment with disease severity, clinical manifestations, and prognosis in severe malaria. Am J Trop Med Hyg *69*, 253-259.

Ma, N., Madigan, M. C., Chan-Ling, T., and Hunt, N. H. (1997). Compromised blood-nerve barrier, astrogliosis, and myelin disruption in optic nerves during fatal murine cerebral malaria. Glia *19*, 135-151.

MacPherson, G. G., Warrell, M. J., White, N. J., Looareesuwan, S., and Warrell, D. A. (1985). Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. Am J Pathol *119*, 385-401.

Maitland, K., Levin, M., English, M., Mithwani, S., Peshu, N., Marsh, K., and Newton, C. R. (2003). Severe P. falciparum malaria in Kenyan children: evidence for hypovolaemia. Qjm *96*, 427-434.

Maitland, K., and Marsh, K. (2004). Pathophysiology of severe malaria in children. Acta Trop *90*, 131-140.

Maneerat, Y., Pongponratn, E., Viriyavejakul, P., Punpoowong, B., Looareesuwan, S., and Udomsangpetch, R. (1999). Cytokines associated with pathology in the brain tissue of fatal malaria. Southeast Asian J Trop Med Public Health *30*, 643-649.

Maneerat, Y., Viriyavejakul, P., Punpoowong, B., Jones, M., Wilairatana, P., Pongponratn, E., Turner, G. D., and Udomsangpetch, R. (2000). Inducible nitric oxide synthase expression is increased in the brain in fatal cerebral malaria. Histopathology *37*, 269-277.

Manga, L., Toto, J. C., and Carnevale, P. (1995). Malaria vectors and transmission in an area deforested for a new international airport in southern Cameroon. Ann Soc Belg Med Trop *75*, 43-49.

Marsh, K., English, M., Crawley, J., and Peshu, N. (1996). The pathogenesis of severe malaria in African children. Ann Trop Med Parasitol *90*, 395-402.

Marsh, K., Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., Newton, C., Winstanley, P., Warn, P., Peshu, N., and et al. (1995). Indicators of life-threatening malaria in African children. N Engl J Med *332*, 1399-1404.

Mbogo, C. M., Mwangangi, J. M., Nzovu, J., Gu, W., Yan, G., Gunter, J. T., Swalm, C., Keating, J., Regens, J. L., Shililu, J. I., *et al.* (2003). Spatial and temporal heterogeneity of Anopheles mosquitoes and Plasmodium falciparum transmission along the Kenyan coast. Am J Trop Med Hyg *68*, 734-742.

McGuire, W., D'Alessandro, U., Stephens, S., Olaleye, B. O., Langerock, P., Greenwood, B. M., and Kwiatkowski, D. (1998). Levels of tumour necrosis factor and soluble TNF receptors during malaria fever episodes in the community. Trans R Soc Trop Med Hyg *92*, 50-53.

Medana, I. M., Chan-Ling, T., and Hunt, N. H. (1996). Redistribution and degeneration of retinal astrocytes in experimental murine cerebral malaria: relationship to disruption of the blood-retinal barrier. Glia *16*, 51-64.

Medana, I. M., Chan-Ling, T., and Hunt, N. H. (2000). Reactive changes of retinal microglia during fatal murine cerebral malaria: effects of dexamethasone and experimental permeabilization of the blood-brain barrier. Am J Pathol *156*, 1055-1065.

Medana, I. M., Chaudhri, G., Chan-Ling, T., and Hunt, N. H. (2001). Central nervous system in cerebral malaria: 'Innocent bystander' or active participant in the induction of immunopathology? Immunol Cell Biol *79*, 101-120.

Medana, I. M., Day, N. P., Hien, T. T., Mai, N. T., Bethell, D., Phu, N. H., Farrar, J., Esiri, M. M., White, N. J., and Turner, G. D. (2002). Axonal injury in cerebral malaria. Am J Pathol *160*, 655-666.

Medana, I. M., and Esiri, M. M. (2003). Axonal damage: a key predictor of outcome in human CNS diseases. Brain *126*, 515-530.

Migasena, P., and Maegraith, B. G. (1968a). Factor affecting on the movement of protein across the blood: brain: C.S.F. barriers in Plasmodium knowlesi infected Macaca mulatta. Med J Malaya *22*, 251.

Migasena, P., and Maegraith, B. G. (1968b). The movement of the dye (disulphine blue) from blood into brain tissue examined by dye method in normal and Plasmodium berghei infected mice. Med J Malaya *22*, 252.

Miller, L. H., Baruch, D. I., Marsh, K., and Doumbo, O. K. (2002). The pathogenic basis of malaria. Nature *415*, 673-679.

Miller, L. H., Good, M. F., and Milon, G. (1994). Malaria pathogenesis. Science *264*, 1878-1883.

Molyneux, M. E., Taylor, T. E., Wirima, J. J., and Borgstein, A. (1989). Clinical features and prognostic indicators in paediatric cerebral malaria: a study of 131 comatose Malawian children. Q J Med *71*, 441-459.

Mons, B. (1990). Preferential invasion of malarial merozoites into young red blood cells. Blood Cells *16*, 299-312.

Mordmuller, B. G., Metzger, W. G., Juillard, P., Brinkman, B. M., Verweij, C. L., Grau, G. E., and Kremsner, P. G. (1997). Tumor necrosis factor in Plasmodium falciparum malaria: high plasma level is associated with fever, but high production capacity is associated with rapid fever clearance. Eur Cytokine Netw *8*, 29-35.

Murphy, S. C., and Breman, J. G. (2001). Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. Am J Trop Med Hyg *64*, 57-67.

Nagel, R. L. (2002). Malarial anemia. Hemoglobin *26*, 329-343.

Neuwelt, E. A. (2004). Mechanisms of disease: the blood-brain barrier. Neurosurgery *54*, 131-140; discussion 141-132.

Newton, C. R., Peshu, N., Kendall, B., Kirkham, F. J., Sowunmi, A., Waruiru, C., Mwangi, I., Murphy, S. A., and Marsh, K. (1994). Brain swelling and ischaemia in Kenyans with cerebral malaria. Arch Dis Child *70*, 281-287.

Newton, C. R., Taylor, T. E., and Whitten, R. O. (1998). Pathophysiology of fatal falciparum malaria in African children. Am J Trop Med Hyg *58*, 673-683.

Newton, C. R., and Warrell, D. A. (1998). Neurological manifestations of falciparum malaria. Ann Neurol *43*, 695-702.

Nonaka, H., Akima, M., Hatori, T., Nagayama, T., Zhang, Z., and Ihara, F. (2003). The microvasculature of the cerebral white matter: arteries of the subcortical white matter. J Neuropathol Exp Neurol *62*, 154-161.

Norenberg, M. D. (1994). Astrocyte responses to CNS injury. J Neuropathol Exp Neurol *53*, 213-220.

Odeh, M. (2001). The role of tumour necrosis factor-alpha in the pathogenesis of complicated falciparum malaria. Cytokine *14*, 11-18.

Oehmichen, M., Walter, T., Meissner, C., and Friedrich, H. J. (2003). Time course of cortical hemorrhages after closed traumatic brain injury: statistical analysis of posttraumatic histomorphological alterations. J Neurotrauma *20*, 87-103.

Olliaro, P., Lombardi, L., Frigerio, S., Basilico, N., Taramelli, D., and Monti, D. (2000). Phagocytosis of hemozoin (native and synthetic malaria pigment), and Plasmodium falciparum intraerythrocyte-stage parasites by human and mouse phagocytes. Ultrastruct Pathol *24*, 9-13.

Patankar, T. F., Karnad, D. R., Shetty, P. G., Desai, A. P., and Prasad, S. R. (2002). Adult cerebral malaria: prognostic importance of imaging findings and correlation with postmortem findings. Radiology *224*, 811-816.

Patnaik, J. K., Das, B. S., Mishra, S. K., Mohanty, S., Satpathy, S. K., and Mohanty, D. (1994). Vascular clogging, mononuclear cell margination, and enhanced vascular permeability in the pathogenesis of human cerebral malaria. Am J Trop Med Hyg *51*, 642-647.

Phillips, R. E., Looareesuwan, S., Warrell, D. A., Lee, S. H., Karbwang, J., Warrell, M. J., White, N. J., Swasdichai, C., and Weatherall, D. J. (1986). The importance of anaemia in cerebral and uncomplicated falciparum malaria: role of complications, dyserythropoiesis and iron sequestration. Q J Med *58*, 305-323.

Phillips, R. E., and Solomon, T. (1990). Cerebral malaria in children. Lancet *336*, 1355-1360.

Pichyangkul, S., Saengkrai, P., and Webster, H. K. (1994). Plasmodium falciparum pigment induces monocytes to release high levels of tumor necrosis factor-alpha and interleukin-1 beta. Am J Trop Med Hyg *51*, 430-435.

Pongponratn, E., Riganti, M., Punpoowong, B., and Aikawa, M. (1991). Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. Am J Trop Med Hyg *44*, 168-175.

Pongponratn, E., Turner, G. D., Day, N. P., Phu, N. H., Simpson, J. A., Stepniewska, K., Mai, N. T., Viriyavejakul, P., Looareesuwan, S., Hien, T. T., *et al.* (2003). An ultrastructural study of the brain in fatal Plasmodium falciparum malaria. Am J Trop Med Hyg *69*, 345-359.

Prat, A., Biernacki, K., Wosik, K., and Antel, J. P. (2001). Glial cell influence on the human blood-brain barrier. Glia *36*, 145-155.

Rigdon, R. (1944). The Pathological Lesions in the Brain in Malaria. Southern Med J *37*, 687-694.

Rudin, W., Eugster, H. P., Bordmann, G., Bonato, J., Muller, M., Yamage, M., and Ryffel, B. (1997). Resistance to cerebral malaria in tumor necrosis factor-alpha/beta-deficient mice is associated with a reduction of intercellular adhesion molecule-1 up-regulation and T helper type 1 response. Am J Pathol *150*, 257-266.

Sanni, L. A. (2001). The role of cerebral oedema in the pathogenesis of cerebral malaria. Redox Rep *6*, 137-142.

Sanni, L. A., Jarra, W., Li, C., and Langhorne, J. (2004). Cerebral edema and cerebral hemorrhages in interleukin-10-deficient mice infected with Plasmodium chabaudi. Infect Immun *72*, 3054-3058.

Sanni, L. A., Rae, C., Maitland, A., Stocker, R., and Hunt, N. H. (2001). Is ischemia involved in the pathogenesis of murine cerebral malaria? Am J Pathol *159*, 1105-1112.

Schellenberg, D., Menendez, C., Kahigwa, E., Font, F., Galindo, C., Acosta, C., Schellenberg, J. A., Aponte, J. J., Kimario, J., Urassa, H., *et al.* (1999). African children with malaria in an area of intense Plasmodium falciparum transmission: features on admission to the hospital and risk factors for death. Am J Trop Med Hyg *61*, 431-438.

Schelp, F. P., Migasena, P., Vudhivai, N., Boonperm, P., Supawan, V., Pongpaew, P., Harinasuta, C., Intraprasert, R., and Tharakul, C. (1977). Alterations of human serum proteins and other biochemical parameters after five to ten days of untreated acute falciparum malaria. Tropenmed Parasitol *28*, 319-322.

Schemann, J. F., Doumbo, O., Malvy, D., Traore, L., Kone, A., Sidibe, T., and Keita, M. (2002). Ocular lesions associated with malaria in children in Mali. Am J Trop Med Hyg *67*, 61-63.

Schluesener, H. J., Kremsner, P. G., and Meyermann, R. (2001). Heme oxygenase-1 in lesions of human cerebral malaria. Acta Neuropathol (Berl) *101*, 65-68.

Schwarzer, E., Bellomo, G., Giribaldi, G., Ulliers, D., and Arese, P. (2001). Phagocytosis of malarial pigment haemozoin by human monocytes: a confocal microscopy study. Parasitology *123*, 125-131.

Sein, K. K., Brown, A. E., Maeno, Y., Smith, C. D., Corcoran, K. D., Hansukjariya, P., Webster, H. K., and Aikawa, M. (1993a). Sequestration pattern of parasitized erythrocytes in cerebrum, mid-brain, and cerebellum of Plasmodium coatneyi-infected rhesus monkeys (Macaca mulatta). Am J Trop Med Hyg *49*, 513-519.

Sein, K. K., Maeno, Y., Thuc, H. V., Anh, T. K., and Aikawa, M. (1993b). Differential sequestration of parasitized erythrocytes in the cerebrum and cerebellum in human cerebral malaria. Am J Trop Med Hyg *48*, 504-511.

SenGupta, S. K., and Naraqi, S. (1992). The brain in cerebral malaria: a pathological study of 24 fatal cases in Papua New Guinea. P N G Med J *35*, 270-274.

Sharief, M. K., Ciardi, M., and Thompson, E. J. (1992). Blood-brain barrier damage in patients with bacterial meningitis: association with tumor necrosis factor-alpha but not interleukin-1 beta. J Infect Dis *166*, 350-358.

Sharief, M. K., and Thompson, E. J. (1992). In vivo relationship of tumor necrosis factor-alpha to blood-brain barrier damage in patients with active multiple sclerosis. J Neuroimmunol *38*, 27-33.

Sherman, I. W., Crandall, I., and Smith, H. (1992). Membrane proteins involved in the adherence of Plasmodium falciparum-infected erythrocytes to the endothelium. Biol Cell *74*, 161-178.

Silamut, K., Phu, N. H., Whitty, C., Turner, G. D., Louwrier, K., Mai, N. T., Simpson, J. A., Hien, T. T., and White, N. J. (1999). A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain. Am J Pathol *155*, 395-410.

Smith, D. H., Nonaka, M., Miller, R., Leoni, M., Chen, X. H., Alsop, D., and Meaney, D. F. (2000). Immediate coma following inertial brain injury dependent on axonal damage in the brainstem. J Neurosurg *93*, 315-322.

Snow, R. W., and Marsh, K. (2002). The consequences of reducing transmission of Plasmodium falciparum in Africa. Adv Parasitol *52*, 235-264.

Snow, R. W., Omumbo, J. A., Lowe, B., Molyneux, C. S., Obiero, J. O., Palmer, A., Weber, M. W., Pinder, M., Nahlen, B., Obonyo, C., *et al.* (1997). Relation between severe malaria morbidity in children and level of Plasmodium falciparum transmission in Africa. Lancet *349*, 1650-1654.

Snow, R. W., Trape, J. F., and Marsh, K. (2001). The past, present and future of childhood malaria mortality in Africa. Trends Parasitol *17*, 593-597.

Somboon, P., Aramrattana, A., Lines, J., and Webber, R. (1998). Entomological and epidemiological investigations of malaria transmission in relation to population movements in forest areas of north-west Thailand. Southeast Asian J Trop Med Public Health *29*, 3-9.

Sun, D., Newman, T. A., Perry, V. H., and Weller, R. O. (2004). Cytokine-induced enhancement of autoimmune inflammation in the brain and spinal cord: implications for multiple sclerosis. Neuropathol Appl Neurobiol *30*, 374-384.

Tachado, S. D., Gerold, P., McConville, M. J., Baldwin, T., Quilici, D., Schwarz, R. T., and Schofield, L. (1996). Glycosylphosphatidylinositol toxin of Plasmodium induces nitric oxide synthase expression in macrophages and vascular endothelial cells by a protein tyrosine kinase-dependent and protein kinase C-dependent signaling pathway. J Immunol *156*, 1897-1907.

Taramelli, D., Recalcati, S., Basilico, N., Olliaro, P., and Cairo, G. (2000). Macrophage preconditioning with synthetic malaria pigment reduces cytokine production via heme iron-dependent oxidative stress. Lab Invest *80*, 1781-1788.

Taylor, T. E., Borgstein, A., and Molyneux, M. E. (1993). Acid-base status in paediatric Plasmodium falciparum malaria. Q J Med *86*, 99-109.

Taylor, T. E., Fu, W. J., Carr, R. A., Whitten, R. O., Mueller, J. S., Fosiko, N. G., Lewallen, S., Liomba, N. G., and Molyneux, M. E. (2004). Differentiating the pathologies of cerebral malaria by postmortem parasite counts. Nat Med *10*, 143-145.

Taylor, T. E., Molyneux, M. E., Wirima, J. J., Borgstein, A., Goldring, J. D., and Hommel, M. (1992). Intravenous immunoglobulin in the treatment of paediatric cerebral malaria. Clin Exp Immunol *90*, 357-362.

Thumwood, C. M., Hunt, N. H., Clark, I. A., and Cowden, W. B. (1988). Breakdown of the blood-brain barrier in murine cerebral malaria. Parasitology *96 (Pt 3)*, 579-589.

Toro, G., and Roman, G. (1978). Cerebral malaria. A disseminated vasculomyelinopathy. Arch Neurol *35*, 271-275.

Turner, G. (1997). Cerebral malaria. Brain Pathol *7*, 569-582.

Turner, G. D., Morrison, H., Jones, M., Davis, T. M., Looareesuwan, S., Buley, I. D., Gatter, K. C., Newbold, C. I., Pukritayakamee, S., Nagachinta, B., and et al. (1994). An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Am J Pathol *145*, 1057-1069.

van Hensbroek, M. B., Palmer, A., Onyiorah, E., Schneider, G., Jaffar, S., Dolan, G., Memming, H., Frenkel, J., Enwere, G., Bennett, S., *et al.* (1996). The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. J Infect Dis *174*, 1091-1097.

Vanderwal, T., and Paulton, R. (2000). Malaria in the Limbe River valley of northern Haiti: a hospital-based retrospective study, 1975-1997. Rev Panam Salud Publica *7*, 162-167.

Varandas, L., Julien, M., Van Lerberghe, W., Goncalves, L., and Ferrinho, P. (2000). Independent indicators of outcome in severe paediatric malaria: maternal education, acidotic breathing and convulsions on admission. Ann Trop Paediatr *20*, 265-271.

Vass, K., and Lassmann, H. (1990). Intrathecal application of interferon gamma. Progressive appearance of MHC antigens within the rat nervous system. Am J Pathol *137*, 789-800.

Walker, O., Salako, L. A., Sowunmi, A., Thomas, J. O., Sodeine, O., and Bondi, F. S. (1992). Prognostic risk factors and post mortem findings in cerebral malaria in children. Trans R Soc Trop Med Hyg *86*, 491-493.

Waller, D., Krishna, S., Crawley, J., Miller, K., Nosten, F., Chapman, D., ter Kuile, F. O., Craddock, C., Berry, C., Holloway, P. A., and et al. (1995). Clinical features and outcome of severe malaria in Gambian children. Clin Infect Dis *21*, 577-587.

Warrell, D. A. (1987). Pathophysiology of severe falciparum malaria in man. Parasitology *94 Suppl*, S53-76.

Warrell, D. A., White, N. J., Veall, N., Looareesuwan, S., Chanthavanich, P., Phillips, R. E., Karbwang, J., Pongpaew, P., and Krishna, S. (1988). Cerebral anaerobic glycolysis and reduced cerebral oxygen transport in human cerebral malaria. Lancet *2*, 534-538.

Weatherall, D. J., Miller, L. H., Baruch, D. I., Marsh, K., Doumbo, O. K., Casals-Pascual, C., and Roberts, D. J. (2002). Malaria and the red cell. Hematology (Am Soc Hematol Educ Program), 35-57.

White, N. J., Warrell, D. A., Looareesuwan, S., Chanthavanich, P., Phillips, R. E., and Pongpaew, P. (1985). Pathophysiological and prognostic significance of cerebrospinal-fluid lactate in cerebral malaria. Lancet *1*, 776-778.

White, V. A., Lewallen, S., Beare, N., Kayira, K., Carr, R. A., and Taylor, T. E. (2001). Correlation of retinal haemorrhages with brain haemorrhages in children dying of cerebral malaria in Malawi. Trans R Soc Trop Med Hyg *95*, 618-621.

Whitty, C. J., Rowland, M., Sanderson, F., and Mutabingwa, T. K. (2002). Malaria. Bmj *325*, 1221-1224.

WHO (1998). World Health Organization Fact Sheet *Number 94*.

WHO (2000). Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster. Trans R Soc Trop Med Hyg *94 Suppl 1*, S1-90.

Wichert-Ana, L., Velasco, T. R., Terra-Bustamante, V. C., Alexandre, V., Jr., Guarnieri, R., Walz, R., Kato, M., Araujo, W. M., Carlotti, C. G., Jr., Araujo, D., *et al.* (2004). Ictal chronology and interictal spikes predict perfusion patterns in temporal lobe epilepsy: a multivariate study. Seizure *13*, 346-357.

Williams, K. E., and Johnson, L. N. (2004). Neuroretinitis in patients with multiple sclerosis. Ophthalmology *111*, 335-340; discussion 340-331.

Wong, D., Dorovini-Zis, K., and Vincent, S. R. (2004). Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood-brain barrier. Exp Neurol *190*, 446-455.