ASSOCIATION OF INTRALEUKOCYTIC MALARIA PIGMENT WITH DISEASE SEVERITY IN CHILDREN WITH *PLASMODIUM FALCIPARUM* MALARIA

by

Nzooma Munkwangu SHIMAPONDA

A dissertation submitted to the University of Zambia in partial fulfillment of the requirements for the degeree of Master of Science MSc in Medical Parasitology.

The University of Zambia

LUSAKA

June, 2008

Declaration

I, **NZOOMA MUNKWANGU SHIMAPONDA** this 20th day of June 2008, do declare that this dissertation represents my own work. This work has not been done in Zambia before and neither has it been submitted for any qualification at the University of Zambia or any other University.

Candidate Name: **NZOOMA MUNKWANGU SHIMAPONDA**

Signature: N. M. Shimaponda

Date: 25.06.08

Certificate of Approval

DISSERTATION TITLE: ASSOCIATION OF INTRALEUKOCYTIC MALARIA PIGMENT WITH DISEASE SEVERITY IN CHILDREN WITH *PLASMODIUM FALCIPARUM* MALARIA

This dissertation of **NZOOMA MUNKWANGU SHIMAPONDA** has been approved as partial fulfillment of the requirements for the award of the Master of Science degree in Medical Parasitology at the University of Zambia.

CHAIRPERSON – (Board of examiners)	Signature	Month/ Date/Year
Dr. C. J. Shinondo		26/06/08
INTERNAL EXAMINER	Signature	Month/ Date/Year
Dr. James Chipeta		26/06/08
INTERNAL EXAMINER	Signature	Month/ Date/Year
EXTERNAL EXAMINER	Signature	Month/ Date/Year

Abstract

Key Words: Haemozoin, Leukocytes, Severe Malaria.

In malaria diagnosis, peripheral parasitaemia is used as an indicator of malaria severity. However, this does not quantify central sequestration, which is important in the pathogenesis of severe malaria. Intraleukocytic malaria pigment or haemozoin, recognizable within the cytoplasm of phagocytic cells by light microscopy, may represent a peripheral marker for parasite biomass. This study evaluated the association of intraleukocytic haemozoin with the severity of *Plasmodium falciparum* malaria in Zambian children. The study sought to determine peripheral *Plasmodium falciparum* malaria parasite densities in blood slides of children with parasitaemia, determine malaria severity, assess presence and quantities of pigment-laden leukocytes and evaluate the association of intraleukocytic malaria pigment with malaria severity. A sample size of 204 children divided into 37 severe malaria, 80 uncomplicated malaria and 87 controls were analyzed prospectively for presence of intraleukocytic haemozoin. Previous studies on the association of intraleukocytic malaria pigment with disease severity and other markers of malaria disease severity were reviewed. The study was conducted at the University Teaching Hospital and Chilenje Health Center in Lusaka, Mpongwe District Hospital on the Copperbelt and Mpulungu Health Center in the Northern Province of Zambia from December 2006 to May 2007. This was a hospital based case-control study. Cases were classified as severe or uncomplicated malaria based on the modified criteria put forth by the World Health Organization. Patients whose blood slides were negative for malaria parasites and were without severe disease were enrolled into the control category. Severity of malaria was determined based on haemoglobin concentrations and coma scores. Three blood slides of thick and thin films were prepared at enrollment of each patient prior to therapy. The thick blood smears were dehaemoglobinized and stained with 10% Giemsa at pH 7.2 for 15 minutes. Peripheral parasite density was determined from the thick films and calculations were based on the number of asexual forms/mm^3 per 200 leukocytes and later converted to asexual forms per µl. Thin smears were fixed in absolute methanol for one minute and then stained at pH 6.8 using the MayGrunwald-Giemsa stain method; for 15 minutes in Maygrunwald stain, 15 minutes in Giemsa stain and two minutes in Sorensen's phosphate buffer. Differential counts were determined manually by two expert microscopists. Malaria pigment was detected on thin films by counting 500 leucocytes and determining the proportions of pigment-laden monocytes, lymphocytes and neutrophils. The study observes that the rate of severe malaria is significantly higher in children under five years than in children above five years of age due to the less developed immunity at that stage in life. Secondly, that the presence of neutrophilic, lymphocytic and monocytic malaria pigment is strongly associated with severe and not uncomplicated malaria. Thirdly, that there is a significant association between pigment-laden neutrophils, lymphocytes and monocytes with severe anaemia and pigment-laden neutrophils with coma and with coma/severe anaemia. Fourthly, our study concludes that high parasitaemia (> 250,000 asexual forms per µl) is not associated with pigment-laden leukocytes and neither is it associated with severe malaria. Our data shows justification to reject the null hypothesis that the presence of intraleukocytic pigment is

Dedications

I dedicate this work to my family. I am so thankful to my dear **husband WAMULUME** for being so understanding, tolerating and supportive through out my 2-year period of study but more so during the extended Dissertation/ Research process. Our lovely children **KULUMBEKA** and **KUYAMBEKWA** - you may not have understood but your being there eased my work. I was not there for you much during this time but it's now done!

To my **Mum** – God add more days- I owe you lots. My late **Dad** - how I wish he lived to see me get this far. The rest of my family members (Ba Ireen- SS Liemba!) all sisters and brothers, Uncles and Aunties and friends, I say thanks a lot for your support.

Acknowledgements

This work was quite involving and to see it to its completion required the guidance, support and co-operation of many.

Therefore, I wish to acknowledge Dr C. J. Shinondo my Supervisor and Dr J. Chipeta Co- Supervisor for their tireless efforts, guidance and indeed chastening to the accomplishment of this work.

I also acknowledge Mr. R. Makasa, Ms. R. Mbaya, Mr. N. Mwanza, Sten, Mimosa, Mr. W. Mbuzi, Mr. W. Siame, Mr. Mubiana, for assistance with sample collection and clinical care, Prof. S. S. Siziya, Prof. K. Mbata, Mr. L. Simbangala, Mr. Y. Banda, Dr. S. Mharakurwa, for guidance and assistance with statistical analysis, and Dr. R. Chilengi, Mr. W. Mataa, Dr. P. Kelly, Dr. S. A. Feresu, for comments on the manuscript.

I further wish to acknowledge Mr. W. Mataa, for the proof reading and thorough editing of the final script.

I acknowledge also my sponsors, The University of Zambia - Staff development Fellow Office, without which this work would not have materialized.

I acknowledge the Ministry of Health, University Teaching Hospital (UTH) and Lusaka District Health Management Team authorities for the permission to carry out this work. In the same vein, I acknowledge the Staff at UTH, Chilenje Health Center, Mpongwe District Hospital, Mpulungu Health Center and UNZA Ridgeway Campus for their help, co-operation and support.

Above all, I wish to acknowledge the Ever present, All powerful, Great provider and sustainer, my able God who guided me through all the 'thick and thins' of this work.

Table of Contents

List of Figures

List of Tables

List of Illustrations

List of Symbols

$<$	-	Less than
$\leq$	-	Less than or equal to
µl	-	Microlitre
$>$	-	Greater than
°c	-	Degrees centigrade
%	-	Percent
χ^2	-	Chi-square
R^2	-	R Square value (Regression)
Z	-	Z value (Mann-Whitney)

Acronyms and Abbreviations

ACTs	-	Artemisinin based Combination therapies
C/Sa	-	Coma with severe anaemia
C.S.O	-	Central Statistical Office
g/dL	-	Grams per deciliter
HB	-	Haemoglobin concentration in grams per deciliter
HRP-II	-	Histidine-rich protein II
H.P.	-	High parasitaemia
IMCI	-	Integrated Management of Childhood Illnesses
LDMS	-	Laser Desorption Mass Spectrometry
N.O.	-	Nitric Oxide
OR	-	Odds Ratio
pLDH	-	*Plasmodium lactate dehyrogenase*
PLN	-	Pigment-laden neutrophils
PLL	-	Pigment-laden lymphocytes
PLM	-	Pigment-laden monocytes
RBC	-	Red Blood Cell
RDTs	-	Rapid Diagnostic Tests
S.A.	-	Severe anaemia
S.M.	-	Severe malaria
SPSS	-	Statistical Package for Social Sciences
U.M.	-	Uncomplicated malaria
UNZA	-	University Of Zambia
UTH	-	University Teaching Hospital
WBC	-	White blood Cell

Chapter one Introduction

1.1 General Introduction

Malaria is one of the most common and important parasitic diseases worldwide. It affects the lives of almost all people living in the area of Africa defined by the southern Sahara desert in the north, and latitude of about 28° in the south (WHO/UNICEF 2003). About 40 percent of the world's population lives in malaria-endemic areas (Sturchler 1989). Over 90 percent of all cases of life-threatening malaria occur in African children. Majority of deaths occur in children under five years of age. Severe malaria is the commonest cause of death, particularly in rural areas that are not serviced by formal health systems.

Severe malaria, conveniently defined by two major syndromes of cerebral malaria and severe malarial anaemia (Snow *et al.* 2003) often results in fatal outcomes. Those who are at most risk of severe malaria include children in areas of high endemicity (especially those aged six months to six years) and pregnant women (WHO/UNICEF 2003). Severe malaria is responsible for about 200 000 infant deaths worldwide per year (Steketee *et al.* 2001; Murphy and Breman 2001).

In Zambia, malaria is the most significant health problem. It is endemic through out the country with transmission ranging from holo endemic in the major river valleys to hypo endemic in large urban areas. Highland plateau areas and industrial towns are prone to epidemic malaria. Parasite prevalence rates in children below 14 years of age range from 28.9 percent in rural areas to 4.9 percent in urban areas (MACEPA 2006). Admission to children's wards for malaria in Lusaka was quite rare in the 2005 / 2006 malaria season compared to previous years and of the 14 children admitted with malaria, only four were the severe cases (Chanda *et al.* 2007). Pre-study sampling for malaria in 2007 showed that prevalence rates had dropped to two percent for severe malaria and six percent for uncomplicated malaria (unpublished data 2007). Over 95 percent of malaria in Zambia is due to *P. falciparum,* with *P. malariae* and *P. ovale*

accounting for the remaining five percent (Johnson 2000). Transmission of *P. falciparum* malaria in Zambia is mild all the year through and gets to its peak during the period October to April.

Data from health facilities in Zambia shows that malaria accounts for 32 percent and 36 percent of all hospital, and health center admissions, respectively and 40 percent of all outpatient attendances. Hospital deaths range from between 14 percent in hospitals and 20 percent in health centers (Johnson 2000). The greatest burden of disease falls on children who, in 1995, accounted for 44 percent of all reported cases and 47 percent of all hospital admissions. In 2001 hospital admissions due to malaria had risen to 62 percent. Malaria cases were 204 per 1000 for out-patients and 15 per 1000 for in-patients (WHO/UNICEF 2003). The incidence rates vary from province to province; they are normally lower in the urban provinces around Lusaka and the Copperbelt, with the highest incidence rates (468.3 per 1,000 population) in 1999 being reported from Northwestern Province (Johnson 2000).

There are a number of factors contributing to high child case fatality in Zambia, which is estimated at 4.3 percent (Petit and van Ginneken 1995). Some of these factors are the sudden progression from the simple form of the disease to severe malaria and malaria-induced anaemia, exacerbated by a lack of timely or inappropriate treatment (Kaona and Tuba 2005). Such mortalities from treated severe malaria in children have been shown to range from five to 15 percent worldwide (Waller and Krishna 1995; Marsh and Foster 1995). The current management of severe malaria has changed surprisingly little, in spite of the rapid scientific advances in malariology and measures to eradicate malaria have been ineffective (Newton and Krishna 1998).

Plasmodium falciparum, being the major cause of malaria, may infect humans at any time from conception to adulthood. Most people at risk of the disease live in areas of relatively stable malaria transmission. These are areas where infection is common and occurs with sufficient frequency that some level of immunity develops (WHO/UNICEF 2003). Children in Tropical regions particularly living in sub-Saharan Africa are greatly

affected by the disease, as they are exposed to it frequently after birth. These children either experience clinical episodes of infection or die from complications (Newton and Krishna 1998) in their early years of life when they have not yet acquired natural immunity. This occurrence of severe disease goes on for many years until the slow and capricious development of immunity (Newton and Krishna 1998). This balance between occurrence of severe disease and acquisition of immunity has continued for thousands of years.

Malaria-associated immunosuppression has been widely investigated. It has been suggested that malaria pigment, also known as haemozoin, may participate in the mechanisms underlying this immunosuppression (Coban *et al.* 2002).

Severe and complicated malaria in Zambia is responsible for the high mortality rate in adults and children alike. In their study, Biemba and colleagues showed mortalities of about 90 percent of all under-five children admitted to a Hospital with severe malarial anaemia, despite aggressive management with blood transfusion and anti-malarials (Biemba *et al.* 2000).

In areas like Lusaka where malaria transmission is mild, the severity of malaria has been attributed to lack of immunity. A study conducted in Lusaka concluded that many people in Lusaka do not develop immunity and are therefore at risk of severe attacks if they do become infected (Watts *et al.* 1990).

Rapid diagnosis and recognition of severity, leading to effective treatment is essential to the control of escalating infectious diseases such as malaria. A number of markers for severity and methods have been advanced in this regard.

Malaria pigment (haemozoin) detection by Laser Desorption Mass Spectrometry (LDMS) was recently shown to be a sensitive technique for detecting *Plasmodium falciparum* parasites cultured in human blood (Scholl *et al.* 2004). The in vitro detection of *Plasmodium* is another diagnostic technique (Demirev *et al.* 2002). Nitric Oxide

(NO) had also been considered a marker of malaria severity until when it was discovered that NO appears to have a protective rather than pathological role in African children with malaria (Anstey *et al.* 1996).

Most markers and methods advanced are rather expensive and time consuming. The use of intraleukocytic malaria pigment to indicate *Plasmodium falciparum* malaria severity may be quite useful in stratifying patients for timely and appropriate treatment. Studies in other countries have shown that the quantity of malaria pigment liberated into the circulation at schizogony reflects the pathogenic sequestered parasite burden in *Plasmodium falciparum* malaria (Nguyen *et al.* 1995). This may therefore be a measure of disease severity. This method employs counting the pigment-laden leukocytes in peripheral blood. This is actually considered to be a rapid, simple and practical prognostic test. Additionally, given that the typical half-life of a neutrophil is six to eight hours and that of a monocyte is several days, the quantity and distribution of engulfed pigment within these phagocytic cells may reflect the chronology of a patient's infection (Lyke *et al.* 2003).

1.2 Statement of the Problem

Disease severity in *Plasmodium falciparum* malaria infections varies from patient to patient but not all cases, in the past, have been stratified accordingly to give priority and appropriate treatment in Zambia. This is because parasitaemia alone has been considered the marker of disease severity and as an indicator of prognosis in *Plasmodium falciparum* malaria. Thus malaria patients with hyper parasitaemia are given more attention in treatment and yet such patients may not even present with remarkable signs of illness. On the other hand, patients with low or negative parasitaemia are considered to have a less severe condition. Some patients may have low parasitaemia, which may be missed by the laboratory staff. Such low, negative or indeed missed parasitaemia contribute greatly to the morbidity and mortality in patie

(Amodu *et al.* 1998). Such patients may end up with severe malaria or even death merely because of lack of appropriate stratification and timely treatment.

Patients with low or negative parasitaemia usually have the intraleukocytic malarial pigment (Amodu *et al.* 1998). Some studies have shown that intraleukocytic malaria pigment and not parasitaemia alone, is responsible for disease severity (Lell *et al.* 2005; Amodu *et al.* 1998; Nguyen *et al.* 1995). Our study sought to relate intraleukocytic malaria pigment with disease severity in Zambian children. The study aimed at providing evidence to form the basis for modification of guidelines on the stratification of cases according to the severity for appropriate treatment.

1.3 Justification of the Study

Mortality due to *Plasmodium falciparum* malaria in children in Zambia has been on the increase partly due to delayed identification of severe cases. Mortality due to malaria can be reduced by stratification of patients for appropriate treatment and medical attention. All this can only be achieved with correct identification of disease severity. Currently, the use of hyper parasitaemia alone as a criterion for diagnosing severe malaria is being practiced in some parts of Zambia. World Health Organization (WHO) recommends the use of hyper parasitaemia ($\geq$ 5, 000 asexual forms per µl) as part of the criteria for diagnosing severe malaria (WHO 2006). However, the clinical course in children who present with this manifestation may actually appear less acute than those with lower parasitaemia. Malaria pigment has been associated with severity of *Plasmodium falciparum* malaria and mortality. The presence of this pigment in leukocytes has been taken as a useful diagnostic indicator in anaemic children and patients with negative *Plasmodium falciparum* malaria blood smears (Lell *et al.* 2005; Amodu *et al.* 1998; Nguyen *et al.* 1995). This approach may have an advantage over microscopy and other methods, which are time consuming, require trained staff, electricity, reagents and correct procedures to detect, identify and or count parasites. Recognizing and counting leukocytes with malaria pigment is easier since leukocytes

are larger than malaria parasites in size. It is possible that there could have been delayed identification of some cases of severe malaria in Zambia in the past due to lack of prompt techniques to recognize disease severity.

This study sought to evaluate the association of the malaria pigment with severity of *Plasmodium falciparum* malaria and the role it could play in severe malaria diagnosis in children in Zambia. The presence and quantities of malaria pigment-laden leukocytes were evaluated and the association with disease severity assessed in Zambian children.

1.4 Review of Literature

The *Plasmodium* parasite life cycle is complex and proceeds through several asexual and sexual stages (Demirev *et al.* 2002). The process of RBC infection and destruction and haemoglobin digestion by *Plasmodia* produces haemozoin, also known as malaria pigment (Lyke *et al.* 2003). Malaria parasites catabolize haemoglobin as an important source of amino acids in the metabolic pathway (Hempelmann and Egan 2002). The parasites then detoxify haem molecules in the food vacuole into haemozoin (Coban *et al.* 2002), which persists inside the parasite.

This metabolic end product of haemoglobin is sequestered in the *P. falciparum* digestive vacuole within infected red blood cells and released into host circulation at schizogony. Once released into host circulation, haemozoin subsequently gets concentrated in the reticulo-endothelial system of the host, where it may persist unchanged in macrophages for several months (Coban *et al.* 2002). Scavenger neutrophils and monocytes engulf the haemozoin in circulation. It is easily visible by light microscopy, appearing as a black-brown pigment (Lyke *et al.* 2003). In vivo experiments have shown that during malaria infection, haemozoin loading greatly impairs the function of phagocytes. Haemozoin loaded monocytes are impaired in the generation of the oxidative burst, the ability to repeat phagocytosis, and the protein kinase C activity (Coban *et al.* 2002). Other studies have also revealed that phagocytosis of opsonised haemozoin impairs the

expression of major histo-compatibility complex class II antigen, CD54, and CD11c in human monocytes (Coban *et al.* 2002).

The existence of haemozoin has been recognized for centuries, and the complexities of its formation and biological relevance quite understood. However, its clinical relevance is incompletely understood (Lyke *et al.* 2003). As part of parasite erythrocytic invasion, haemoglobin is proteolysed to release toxic haem. Due to the absence of haem oxygenase, *Plasmodia* are unable to cleave haem or haematin into open-chain tetra-pyrrole to allow cellular excretion. To detoxify soluble haem, a novel breakdown product known as haemozoin is created intracellularly. Haemozoin is chemically and structurally identical to synthetic β- haematin (Hempelmann and Egan 2002). Until recently, this material was believed to be a polymer of haematin. However, the structure determination has revealed that it is a dimer with hydrogen bonding between the dimer units in the crystal (Hempelmann and Egan 2002). Haemozoin is composed of Fe^{III}-porphyrin units. These units are linked by propionate oxygen-iron bonds into polymers accompanied by additional host and parasite nucleic acids and lipids (Lyke *et al.* 2003).

Some studies refer to malaria pigment formation as a biomineralization-type process (Hempelmann and Egan 2002). Strictly speaking, biomineralization refers to the formation of insoluble inorganic salts. This term, biocrystallization, is probably the more appropriate term to describe malaria pigment formation.

Currently, the mechanism of malaria pigment formation *in vivo* is unknown, although proteins (in the form of histidine rich proteins) and lipids have been implicated. Both proteins and lipid membranes are typically involved in biomineralization processes. The detailed role of such constituents in malaria pigment formation and the precise effect of chloroquine and other anti-malarials remain to be determined (Hempelmann and Egan 2002).

The pathophysiology of severe *Plasmodium falciparum* malaria is complex and results in a broad spectrum of disease manifestations. Sequestration of parasites is thought to be

central to these disease processes. Anaemia, cytokine production, and metabolic distortion may also contribute to the spectrum of disease (Lyke *et al.* 2003).

Over the years, studies to illuminate our understanding of malaria disease processes have been conducted although none have succeeded so far in reducing mortality from severe infection (Newton and Krishna 1998).

The association of intraleukocytic malaria pigment with severity of malaria may facilitate in the reduction of mortality from severe infection. Recently, studies associating severe and fatal malaria with the increased presence of malaria haemozoin in peripheral phagocytes (Lell *et al.* 2005; Amodou *et al.* 1998; Nguyen *et al.* 1995) have been conducted in a number of places. These places include Gabon, Ghana, Nigeria, Mali and sites for the multi center, prospective, observational study in The Gambia, Ghana, Kenya, Malawi and Gabon. Similar studies had not been conducted in Zambia before. This association employing the pigment-laden leukocyte count has been considered to be a simple marker of disease severity in malaria in addition to the parasite count. A lot of work in this regard has been done in adults unlike in children (Lyke *et al.* 2003).

Parasite density generally correlates with disease severity, but peripheral parasitaemia does not necessarily reflect the burden of sequestered parasites. In a study done in Mali, children whose sole defining criterion for severe malaria was hyper parasitaemia appeared to fall somewhere between uncomplicated malaria and severe malaria in the spectrum of illness. A lower peripheral parasite density was noted in children who died compared with children that survived. Children with hyper parasitaemia alone are presumably healthier children who are more likely to survive compared to those with low peripheral parasitaemia (Lyke *et al.* 2003).

In hospital-based studies, malaria therapy taken prior to admission may further hamper efforts to properly diagnose and characterize acute illness. Methods of estimating malaria disease severity and predicting prognosis may be useful in stratifying patients in the early stages of admission and assessment (Lyke *et al.* 2003).

One of the Strategies to Roll Back malaria is early Diagnosis and prompt and effective treatment. Rapid, sensitive, and reliable methods for malaria detection are a factor that determines the ultimate success in controlling, restricting, and eradicating this disease.

Diagnosis of malaria mainly involves Clinical, Microscopic, Rapid Diagnostic Tests and molecular methods like Polymerase chain reaction (PCR). The main diagnostic method in developing countries is empirical as it is the only feasible diagnostic method in many rural areas. An overtly high sensitivity and non specific case definition means that there is a lot of drug wastage which can not be sustained now that efficacious treatments are much more costly. Therefore, there is need to streamline treatment practices to improve rational usage of drugs without necessarily increasing the risk of malaria disease becoming severe. However, attempts to differentiate malaria from other febrile illnesses using clinical features have not been quite successful because they are not sensitive enough or too specific to guide treatment. This complexity is further complicated by the common overlap between malaria and other febrile illnesses (Bojang 2005). These complexities led to the introduction of the Integrated Management of Childhood Illnesses (IMCI) guideline that Anti-malarial treatment could be given to febrile children if parasitological diagnosis is not possible. Microscopic diagnosis, which is the "gold standard" for detecting and identifying malaria parasites is relatively inexpensive, provides a permanent record, allows for characterization of parasites and it is a technique that can be shared with other disease control programmes. The disadvantages of microscopic diagnosis are that it is labor intensive, time consuming and it requires good techniques, reagents, microscopes and well-trained technicians. There are often delays in providing results to clinicians. It is important also to note that detection of malaria parasites does not necessarily mean that they are responsible for the patient's illness (Bojang 2005). Intraleukocytic malaria pigment produced by parasites during intraerythrocytic development, and is associated with severe disease and mortality may be a useful diagnostic indicator in anaemic children and patients with negative blood smears (Bojang 2005). Malaria Rapid Diagnostic Tests (RDTs) detect antigens derived from malaria parasites using immunochromatographic methods. The antigens are Histidine-rich protein II (HRP-II),

specific to *P. falciparum, Plasmodium lactate dehyrogenase* (pLDH), used in products that include *P. falciparum*-specific, pan-specific, and *P. vivax* specific pLDH antibodies and *Aldolase* (pan-specific) (Bojang 2005). The advantages of RDTs are that they are both sensitive and specific and easy to perform and interpret. These RDTs do not require electricity or other special equipment and can detect *P. falciparum* even when parasites are sequestered in the deep vascular compartment and thus not detectable by microscopy. The disadvantages of RDTs are that HRP-II based tests can give positive results up to two weeks following parasite clearance. RDTs are also disadvantageous in that they are expensive, they are not quantitative and the kits that detect *P. falciparum* and non- *P. falciparum* species cannot differentiate among *P. vivax*, *P. ovale*, and *P. malariae.* The LDH based RDTs are able to detect active infection with *P. falciparum*, however, they tend to be very sensitive to storage conditions and require to be stable for effective performance. In general, and perhaps unimportantly, RDTs can also detect antigens produced by gametocytes. Potential use of RDTs in malaria endemic countries is in cases of suspected severe malaria in peripheral locations when microscopy is not available, and the cost of the first line treatment is high. Indeed in Zambia changes of treatment policy to Artemisinin based Combination therapies (ACTs), which cost much more, empirically treatment of all fevers, as malaria can no longer be sustainable. The RDTs can also be used during complex emergencies, outbreak investigation and malaria prevalence surveys. RDTs are also suitable for Private-sector health providers in urban areas of low malaria transmission (Bojang 2005).

Polymerase chain reaction (PCR) is another diagnostic method. It is highly sensitive and specific. PCR allows for species identification, detection of markers of resistance, and diagnosis of patients with suspected malaria despite repeated negative thick-blood-films. However, it is expensive (Bojang, 2005) and not suitable for routine use.

A recently discovered, rapid, new malaria-screening assay is the use of the Laser Desorption Mass Spectrometry (LDMS) (Scholl *et al.* 2004). The LDMS detection of haemozoin was found to be a potentially more rapid screen, than light microscopy, for detecting malaria infection in the mouse model (Scholl *et al.* 2004).

Demirev and others (2002) developed a method for the in vitro detection of *Plasmodium*. This method comprises a protocol for cleanup of whole blood samples, followed by direct ultraviolet Laser Desorption (LD) time-of-flight mass spectrometry. Intense ion signals are observed from intact ferriprotoporphyrin IX (haem), sequestered by malaria parasites during their growth in human red blood cells. The LD mass spectrum of the haem is structure-specific, and the signal intensities are correlated with the sample parasitaemia (number of parasites per unit volume of blood). Parasitaemia levels on the order of ten parasites per microlitre of blood can be unambiguously detected by this method. Consideration of laser beam parameters (spot size, rastering across the sample surface) and actual sample consumption suggests that the detection limits can be further improved by at least an order of magnitude. The influence of experimental factors, such as desorbed ion polarity, laser exposure and fluence, sample size, and parasite growth stage, on the threshold for parasite detection is also addressed (Demirev *et al.* 2002).

Nitric oxide had been considered as a marker for malaria severity but now studies have shown that it appears to have a protective rather than pathological role in African children with malaria. Nitric oxide in Tanzanian children with malaria showed an inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2-expression (Anstey *et al.* 1996).

Recent studies have also demonstrated a method of detection of *P. falciparum* in human urine and saliva using PCR in Zambia. Regularly matching polymorphic PSP2 genotypes were found between the corresponding urine, saliva and peripheral blood amplicons of each individual, with different inter-individual polymorphic genotypes. The author concludes that *P. falciparum* is detectable by PCR on human urine and saliva sample, although the extraction techniques need further refinement (Mharakurwa *et al.* 2006).

Most of these detection techniques are either time-consuming, have low sensitivity or specificity, or are expensive for mass screening and routine examination (Scholl *et al.* 2004).

The varied complexities of the current diagnostic methods make the method of pigment determination appropriate and useful. This method of course utilizes the time-consuming microscopy. However, it is not by the counting of parasites that are small and not very easy to recognize; it involves the counting of leukocytes, which are larger in size and more discernable under the microscope.

In the past decade, apart from investigators demonstrating the utility of assessing pigment formation for disease characterization, the malaria pigment has also been shown to be a useful diagnostic indicator in anaemic children and patients with negative blood smears (Bojang 2005).

1.5 Hypothesis

1.5.1 Null Hypothesis

This study tested the null hypothesis that the presence of intraleukocytic pigment is not associated with disease severity in children with *Plasmodium falciparum* malaria in Zambia.

1.6 Objectives

1.6.1 General objective

The general objective of the study was to evaluate the association of intraleukocytic malarial pigment (haemozoin) with disease severity in children with *Plasmodium falciparum* malaria in Zambia.

1.6.2 Specific Objectives

The specific objectives set for this study were to:

i. Identify children with *Plasmodium falciparum* malaria infections and recruit them into the study
ii. Determine peripheral malaria parasite densities in blood slides of the recruited children
iii. Determine the severity of malaria in the recruited children
iv. Determine the presence and quantities of pigment-laden leukocytes in the recruited children
v. Determine the association of intraleukocytic pigment with disease severity

Chapter two Materials and Methods

2.1 Study Site

The study was conducted over a period of six months from December 2006 to May 2007 with recruitment sites at the University Teaching Hospital and Chilenje Health Center in Lusaka, Mpongwe District Hospital on the Copperbelt and Mpulungu Health Center in the Northern Province of Zambia.

Both rural and urban areas in Zambia have seasonal transmission of *Plasmodium falciparum* malaria during the period from October to April yearly with peak transmission in April. However, transmission is higher in rural than urban areas. Parasite rates in one season were 2.6 percent in November and 10.3 percent in April in urban areas and 10 percent in November and 27 percent in April in rural areas respectively (Watts *et al.* 1990). Mpulungu and Mpongwe, as in most rural areas of Zambia, have the peak transmission of *Plasmodium falciparum* malaria in April yearly. *Plasmodium falciparum* malaria in Lusaka is hypo-endemic with peak transmission in April (Watts *et al.* 1990).

The University Teaching Hospital is a referral center and receives cases from all clinics in the city of Lusaka. Lusaka is the capital city of Zambia and is located at latitude, 15°24'S and longitude, 28°17'E.

Mpulungu is a town in northern Zambia at the southern tip of Lake Tanganyika and is located at latitude, 8°46'S and longitude, 31°08'E. The town is Zambia's only port and the gateway to the North of Africa by the Lake Tanganyika and provides an important outlet for exports and imports by ship from Burundi and Tanzania. Mpulungu has a population of 70, 000 and is a fishing port. The town lies in a natural bay along the lakeshore, protected by a large island just a few hundred meters out from the lake. The population consists mainly of fishermen. There is also a tropical fishing industry that exports aquarium fishes and fish stakes all over the world.

Mpulungu has a Health Center that services the surrounding villages namely Musende, Muzabwela, Posa, Tonga, Kaizya, Mupata, Kapata and Chilila, among others. There are also a few inadequately serviced Rural Health Centers.

Mpongwe is a district on the Copperbelt province of Zambia. It is situated about 320km north of Lusaka. This district is basically a rural setting and it shares boundaries with Masaiti, Lufwanyama, Kapiri Mposhi, Chibombo and Kasempa districts. The population of Mpongwe was estimated to be about 67,972 in 2000 (CSO 2002). The district has two hospitals, nine health centers and two health posts. Mpongwe Mission hospital is the major referral facility in the District and has a bed capacity of 90 beds. The catchment area has a population of about 12, 006 and most of the people are involved in subsistence farming of maize, sorghum and groundnuts. Commercial farmers in the district grow maize, soya beans, coffee and wheat mainly by irrigation schemes.

2.2 Study Design

A prospective study design was used to assess intraleukocytic malaria pigment as it relates to malaria severity. This was a hospital based case-control study conducted in Lusaka, Mpulungu and Mpongwe Districts in Zambia.

All children between three months and 14 years of age, who attended or were admitted to the University Teaching Hospital, Mpongwe District Hospital, Chilenje and Mpulungu Health centers with diagnosis sûggestive of malaria, from January to April 2007, were evaluated for inclusion in the study. Criteria for diagnosis and enrolment included the modified criteria by the World Health Organization for severe malaria (WHO 2006). Children who satisfied the inclusion criteria were enrolled. Parents or guardians were interviewed about the presenting symptoms and signs and the Study Clinicians documented the clinical data including weight and vital signs after examining the patients. Children infected with *Plasmodium falciparum* malaria, were the cases.

The cases were divided into severe and uncomplicated malaria. The controls were children between three months and 14 years of age, without severe disease, attending the same Hospitals and Health facilities as the cases. Laboratory tests for malaria diagnosis, haematocrit and intraleukocytic pigment were performed within four hours of presentation.

2.3 Study Population

A total of 204 study participants were drawn from the population of Zambian children (three months to 14 years old) who attended the University Teaching Hospital, Mpongwe District Hospital, Chilenje and Mpulungu Health centers at the time of the study. The participants were recruited into severe malaria, uncomplicated malaria or control categories.

2.3.1 Cases

Cases were classified as severe or uncomplicated malaria based on the modified criteria put forth by the World Health Organization [Appendix A (WHO 2006)]. In this study, severe malaria was defined as Blantyre coma score <5 or severe anaemia ≤5g/dl when they co-exist with parasitaemia and an auxiliary temperature of ≥37.5°C and other criteria as listed in the WHO criteria for severe malaria. Uncomplicated malaria was defined as *P. falciparum* parasitaemia and an axillary temperature of ≤37.5°C, or parasitaemia and symptoms suggestive of malaria.

2.3.2 Controls

Patients with negative malaria slides and without severe disease were recruited as controls.

2.3.3.1 Inclusion criteria

Children between three months and 14 years of age with *Plasmodium falciparum* asexual forms of malaria parasites were recruited into the study as cases.

2.3.3.2 Exclusion criteria

Children under three months or above 14 years of age with or without malaria but in coma or with severe disease due to causes other than malaria were not enrolled in to the study.

2.4 Sample Size

2.4.1 Severe Malaria

Assuming that two percent of clinical malaria cases would result in severe malaria (unpublished 2007), if this was correct at 95 percent confidence level, and if wrong considering the Standard deviation of +/- five percent, the sample size of the study was calculated by the sample size formula;

Sample size (n) $=Z^2 [P (1-P)]/D^2$

Where

Z= the critical value,

P= represents proportion of children with severe malaria (two percent),

D= standard deviation (Ralph *et al.* 2002)

Sample size (n) $=\frac{1.96X1.96X2 (100-2)}{25}$

= 30 severe cases

2.4.2 Uncomplicated Malaria

Assuming that six percent of clinical malaria cases would result in uncomplicated malaria (unpublished 2007), if this was correct at 95 percent confidence level, and if wrong considering the Standard deviation of +/- five percent, the sample size of the study was calculated by the sample size formula;

Sample size (n) $=Z^2 [P (1-P)]/D^2$

Where

Z= the critical value,

P= represents proportion of children with severe malaria (six percent),

D= standard deviation (Ralph *et al.* 2002)

Sample size (n) $=\frac{1.96X1.96X6\ (100-6)}{25}$

= 87 uncomplicated cases and 87 controls

2.5 Clinical Assessment

Clinical and or admission data were collected after blood slide results or within four hours of admission to hospital. The Clinical data collected included demographic details of the patients and presenting symptoms and signs. This data was obtained from the medical records of the participants, interviews with parents or guardians and by clinical examinations performed by the Zambia Medical Council registered Clinicians in the Hospitals and Health Centers. The data was recorded on the Clinical Data Form (Appendix C).

2.6 Determination of Parasitaemia

Finger prick blood specimens were obtained from each study participant at enrollment prior to therapy. For each finger prick blood specimen collected, three blood slides of

both thick and thin films were prepared. The thick blood smears were dehaemoglobinized for transparency of the red blood cells, by placing them into buffer for 30 to 60 seconds before staining. These smears were stained with ten percent Giemsa at pH 7.2 – 7.4 for 15 minutes (Shute 1988). Peripheral parasite density was determined from thick films by two expert microscopists. Parasite density calculations were based on the number of asexual forms/mm^3 per 200 leukocytes (Shute 1988) and converted into parasites per microlitre of blood based on the participants total white cell count. Geometric mean parasite densities were determined using the SPSS 11.5 version. Parasite densities were represented as high parasitaemia when there were $\geq$ 250000 asexual forms per microlitre of blood and low parasitaemia when there were < 250000 asexual forms per microlitre of blood (Lell *et al.* 2005). Two hundred microscope fields of the thick films were examined at 1,000× magnification before assigning a negative result.

2.7 Determination of malaria severity

The children in this study were recruited into severe malaria, uncomplicated malaria or control categories. In this study, haemoglobin concentrations and coma scores were the main determinants of malaria severity.

2.7.1 Determination of haemoglobin concentrations

Haemoglobin concentrations in the patients were determined by the haematocrit/haemoglobin method (Williams 1989; Robergs 2007). Finger prick blood was collected in heparinized micro capillary tube, sealing one end of the tube, and centrifuging the tube to pack the red blood cells. After packing, which takes about three to five minutes in a micro centrifuge, the length of the packed red blood cell column and the total filled length were measured against a ruler, and the haematocrit, expressed as a percentage, was determined. Haematocrit of

<15 percent or haemoglobin concentration of ≤ 5 g/dl is interpreted as severe anaemia (WHO 2006; Lell *et al.* 2005; Lyke *et al.* 2003).

2.7.2 Determination of coma or impaired level of consciousness

Coma scores in the patients were determined using the Blantyre Coma Scale for children (Molyneux *et al.* 1989). The Blantyre coma scale employs scores for motor response, verbal response and eye movements. A minimum score of 0 is interpreted as poor, a maximum score of 5 is interpreted as good and any score ≤ 4 is interpreted as an abnormal score.

2.8 Determination of presence and quantities of pigment-laden leukocytes.

Thin smears were fixed in absolute methanol for one minute before staining. These smears were then stained at pH 6.8 using the MayGrunwald-Giemsa method (Howen 2000). The smears were stained for 15 minutes in MayGrunwald stain, for 15 minutes in Giemsa stain and for two minutes in Sorensen's phosphate buffer. Two expert microscopists determined the white blood cell differential counts manually. Intraleukocytic malaria pigment was detected on thin films by counting 500 leukocytes and determining the proportions of haemozoin-containing neutrophils, lymphocytes and monocytes. The two expert microscopists were blinded to clinical presentation and outcome. To determine inter observer variability, an average of the slide readings by the two expert microscopists was taken. Total pigment-laden leukocytes per microlitre (WHO 2006) were calculated as follows:

Total pigment-laden leukocytes* per microlitre = (percent pigment-laden leukocytes* [in a count of 500WBCs]) x (absolute WBC count per µl) x (percent leukocytes* in differential count of peripheral blood).

leukocytes* = **neutrophils, lymphocytes or monocytes**

Laboratory data comprising haemoglobin concentrations of the patients, parasite species and peripheral parasite densities in the blood smears, differential white blood cell counts and presence and quantity of pigment-laden leukocytes were recorded on Laboratory Form (Appendix D) and the results were made available to the Clinicians for treatment or management of the patients using the routine Laboratory forms.

2.9 Data Entry, analysis and presentation

All study data were captured on structured clinical and laboratory forms (Appendices C and D) bearing participant demographic details and identification numbers. Microsoft Excel and SPSS spreadsheets were used to enter these data for analysis. The raw data was checked for completeness and internal consistency and analyzed by quantitative methods. Data is presented in the Results as Tables and Figures.

2.9.1 Statistical analysis

Data was analysed using version 11.5 of SPSS and Microsoft Excel Office 2003. Pooled analyses between the clinical groups were made using the Mann-Whitney U / Wilcoxon test for continuous variables and the Chi-square test for categorical variables using SPSS version 11.5 (SPSS Inc., Chicago, IL). The Mann-Whitney U – test of the SPSS version 11.5 (SPSS Inc., Chicago, IL) was used to determine the significance of the differences observed in quantities of the pigment-laden leukocytes in various severity categories and hence test the hypothesis. The Chi-square test of the SPSS version 11.5 (SPSS Inc., Chicago, IL) was used to determine association of the intraleukocytic malaria pigment with disease severity and Odds Ratios of the SPSS version 11.5 (SPSS Inc., Chicago, IL) were used to determine the level of association. Linear regression analysis of Microsoft Excel Office 2003 was used to determine correlation of

malaria severity with parasitaemia and intraleukocytic malaria pigment. A level of statistical significance (two sided) was set at $P<0.05$.

2.10 Ethical considerations

The study did not pose any significant added risk than is routinely faced by the patients and neither did it alter the course of treatment patients would normally receive. The UNZA School of Medicine Research Ethics Committee reviewed and approved the study proposal. The Ethics committee waived the Informed Consent process in this study with the understanding that the study would utilize routine specimens.

Confidentiality of the participant personal information was and will be assured in that nothing that identifies an individual participant shall be published, and all such information remains in the local facility files.

Permission to conduct the study was sought from and granted by the relevant authorities in the Ministry of Health, University Teaching Hospital (UTH) and Lusaka District Health Management Team.

2.11 Funding

The Staff Development Office of the University of Zambia funded the study.

Chapter three Results

3.1 Study Population

A total of 204 children were enrolled in to the study. These children were enrolled from UTH and Chilenje Health Center in Lusaka, Mpongwe District Hospital on the Copperbelt and Mpulungu Health Center in the Northern Province of Zambia. The study participants were recruited into the severe malaria, uncomplicated malaria or control categories. Table 1 shows that a total of 204 children were enrolled into the study with 80 children enrolled from Mpulungu and 49 children from Mpongwe. In Lusaka, the study enrolled 17 children from UTH and 58 children from Chilenje Health Center. The children in the study comprised of 101 males and 103 females, with a mean age of three years (range, 3 months – 14 years).

Table 1. Demographic details of the Study Participants in the four study locations enrolled using inclusion and exclusion criteria of the study.

		LOCATION				Total
		Mpulungu	Mpongwe	UTH	Chilenje	
Mean age (range)		(3m –11yr)	(6m-9yr)	(6m-14yr)	(3m-14yr)	
Sex	Male	41	23	9	28	101
	Female	39	26	8	30	103
Total patients		80	49	17	58	204
Severity	SM	27	3	6	1	37
	UM	27	46	1	6	80
	Control	26	0	10	51	87
Total patients		80	49	17	58	204

3.2 Assessment of Peripheral Parasitaemia

In this study, all the malaria infected children (100 percent) harbored *P. falciparum*, while *P. malariae* was present in two children (1.7 percent). A total of 115 out of 117 malaria infected children (98.3 percent) were exclusively infected with *P. falciparum*. The two children with *P. malariae* had a co-infection with *P. falciparum*. Figure 1 shows that peripheral parasite densities ranged from as low as 80 and as high as 447040 asexual forms per microlitre of blood.

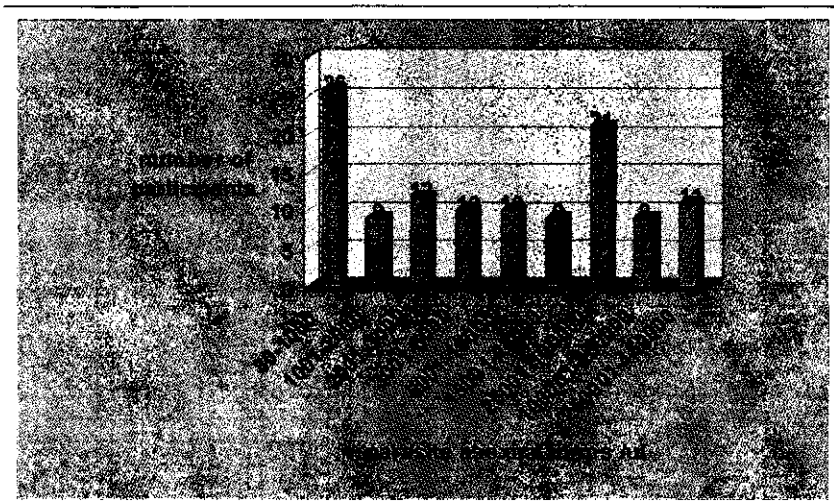

Figure 1 Distribution of peripheral malaria parasite densities in malaria infected children. Peripheral parasitaemia was determined from Giemsa stained thick blood smears.

Figure 1 shows that among the 117 malaria-infected children, 11 (9.4 percent) had high parasitaemia (asexual forms $\geq$ 250000 per µl) and 106 (90.6 percent) had low parasitaemia (asexual forms < 250000 per µl).

Figure 2 shows that among the malaria-infected children, low parasitaemia was observed in all age groups but high parasitaemia was only observed in children aged between one to five years. There was no record of high parasitaemia in children above five years and in children under one year of age.

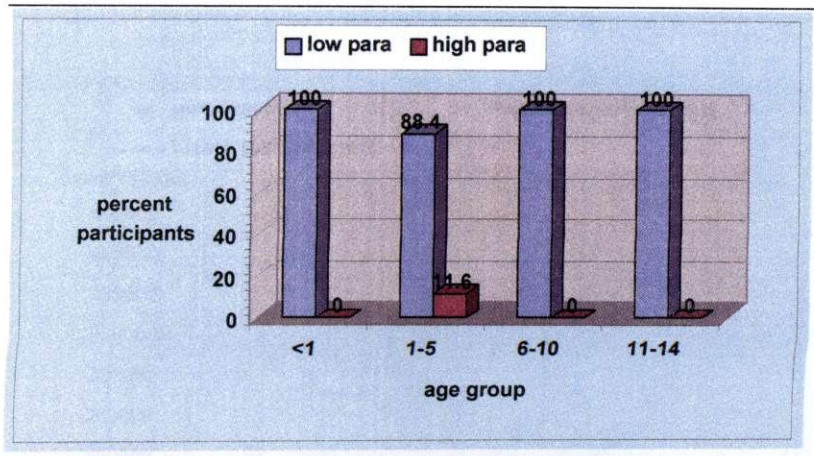

Figure 2 Distribution of low and high parasitaemia by age group. Peripheral parasitaemia was determined from Giemsa stained thick blood smears. Low parasitaemia was represented by < 250000 asexual forms per µl and high parasitaemia by ≥ 250000 asexual forms per µl.

Of the 11 children with high parasitaemia, three children (27.3 percent) had severe malaria and eight children (72.7 percent) had uncomplicated malaria. Figure 3 and Table 2 show that the parasitaemia levels did not differ significantly between children with severe malaria and children with uncomplicated malaria (χ^2; p=1.000, R^2; p=0.95).

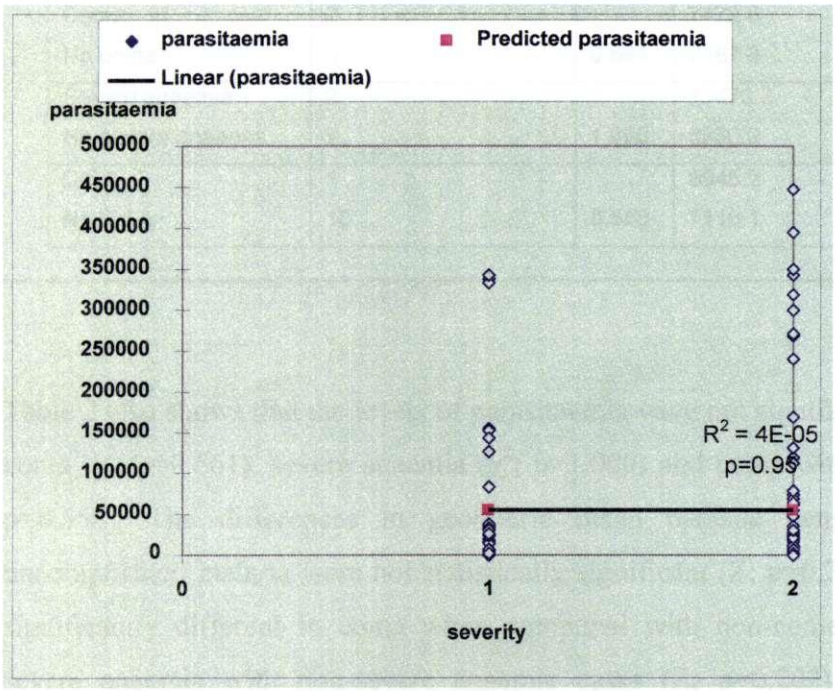

Figure 3 Correlation between parasitaemia and severity of malaria. Peripheral parasitaemia was determined from Giemsa stained thick blood smears. On the x-axis of the variable severity, category 1 represents severe malaria and category 2 represents uncomplicated malaria.

Table 2. Association of Parasite density levels with Malaria Severity.

	Participants with high parasitaemia	**P value**	**Geometric mean parasite densities**	**P value**
Severe malaria	3		9940.7	
Uncomplicated	8	**1.000**	6237.8	**0.282**
Coma	2		7472.6	
No coma	9	**0.661**	7187.6	**0.908**
Severe anaemia	2		11470.3	
No severe anaemia	9	**1.000**	6250.9	**0.202**
C/SA	1		8945.2	
No C/SA	10	**0.558**	7116.1	**0.956**

Table 2 also shows that the levels of parasitaemia were not significantly associated with coma (χ^2; p=0.661), severe anaemia (χ^2; p=1.000) and coma with severe anaemia (χ^2; p=0.558). The differences in geometric mean parasite densities in severe and uncomplicated malaria were not statistically significant (Z; p=0.282) neither were they significantly different in coma when compared with non-coma cases (Z; p=0.908), severe anaemia with non-severe anaemic cases (Z; p=0.202) and in coma/severe anaemia with non coma/severe anaemic cases (Z; p=0.956).

The odds of having severe malaria were highest in children with the least parasite densities (OR= 3.02; p<0.05; 95%CI) and least in children with the highest parasite densities (OR= 1.72; p<0.05; 95% CI). Lŏw parasitaemia was more likely to occur in severe malaria than was high parasitaemia.

3.3 Severity of Malaria based on haemoglobin concentrations and coma scores

3.3.1 Haemoglobin concentrations in the Study participants

Haemoglobin concentrations ranged from 1g/dl to 16g/dl with a mode of Hb 10g/dl as shown in Figure 4. The mean haemoglobin concentrations in children with severe malaria were significantly lower (5.2g/dl - Z; $p<0.001$) than the mean haemoglobin concentrations in children with uncomplicated malaria (9.1 g/dl - Z; $p<0.001$) and children without malaria (11.1g/dl – Z; $p<0.001$).

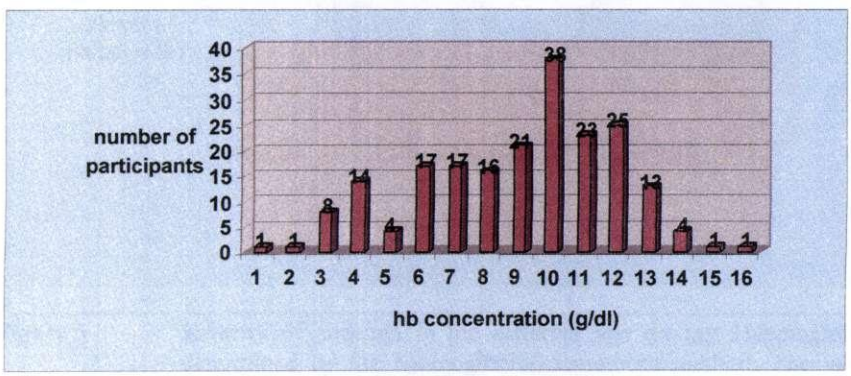

Figure 4 Distribution of haemoglobin concentrations in the Study Participants. Haemoglobin concentrations were determined using the haemoglobin/haematocrit method.

Figure 5 shows that children under 11 years of age had severe anaemia as opposed to the older children in the study. The rate of severe anaemia was highest (20 percent) in children under one year of age compared with the older children. The mean haemoglobin concentrations in children under five years of age (9g/dl) were significantly lower than the mean haemoglobin concentrations in children above five years of age (10.5g/dl) [Z; p=0.001].

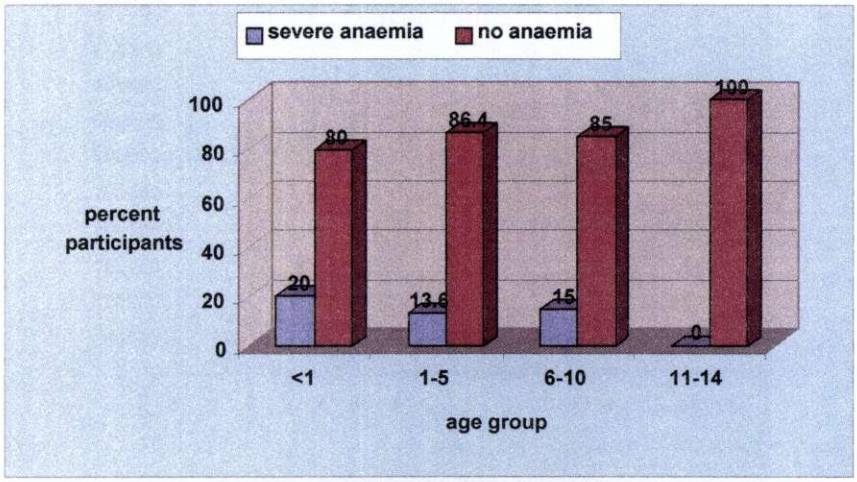

Figure 5 Severity of anaemia in the different age groups. Haemoglobin concentrations were determined by the haemoglobin/haematocrit method. The variable 'severe anaemia' represented children with haemoglobin concentrations ≤5 and the variable 'no anaemia' represented children with haemoglobin concentrations >5.

Twenty-eight of the 117 malaria –infected participants were severely anaemic. Figure 6 shows that haemoglobin concentrations were high in children with low or no parasitaemia at all but parasitaemia was not associated (χ^2; p=1.000) nor correlated (R^2; p=0.34) with haemoglobin concentrations.

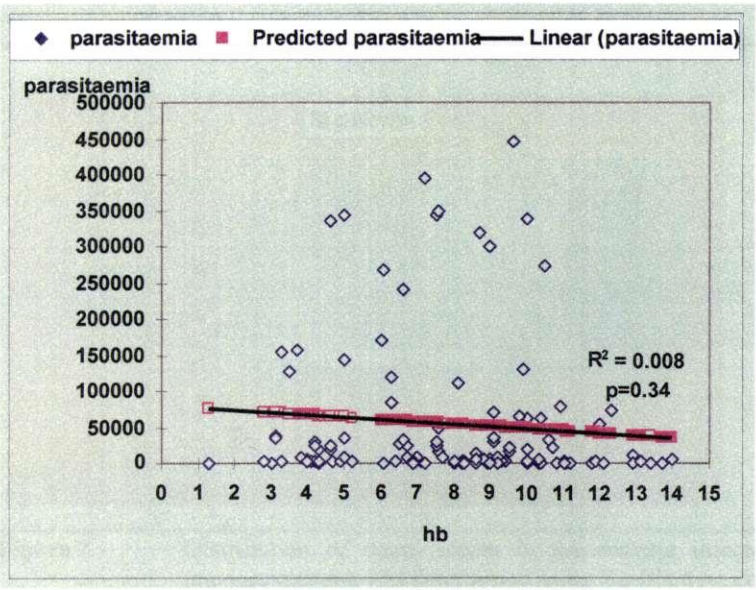

Figure 6 Correlation between parasite densities and Haemoglobin concentrations. Haemoglobin concentrations were determined by the haemoglobin/haematocrit method. Parasitaemia was determined from Giemsa stained thick blood films.

3.3.2 Coma Scores in the Study population

Figure 7 shows that 17 participants out of the 117 malaria-infected participants were either in coma or impaired level of consciousness (coma score < 5) and 100 participants were not. Six of the 17 children with impaired consciousness also had severe anaemia.

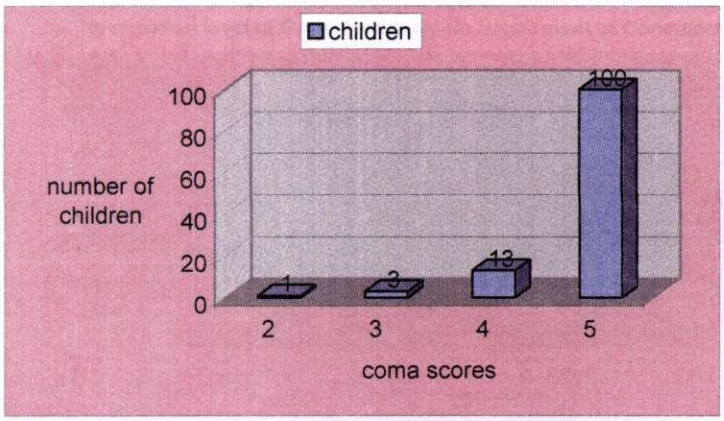

Figure 7 Distribution of coma scores in the malaria infected children. Coma or impaired unconsciousness was determined using the Blantyre coma scale.

Figure 8 shows the coma scores in the different age groups. Coma or impaired consciousness was observed only in children under five years of age and the mean coma scores in children under five years of age (Blantyre coma score = 4.8) were significantly lower than the mean coma scores in children above five years of age (Blantyre coma score = 5) [Z; p=0.037].

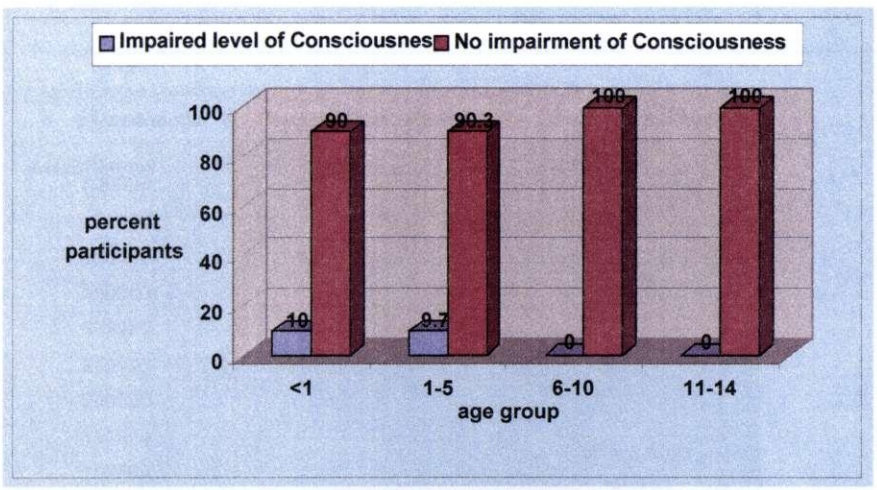

Figure 8 Blantyre coma scores by age group. Coma scores were determined using the Blantyre coma scale. The variable 'coma' represented children with Blantyre coma scores <5 and the variable 'no coma' represented children with Blantyre coma scores =5.

Figure 9 shows that coma or impaired consciousness was mainly observed in children with low parasitaemia. There were only two children (11.8 percent) out of 17 children in coma or impaired consciousness who had high parasitaemia. Peripheral parasitaemia was observed both in comatose children (Blantyre coma scores 2 to 4) and in non-comatose children (Blantyre coma score 5) showing that parasitaemia was not significantly associated (χ^2; p=1.000) nor correlated (R^2; p=0.61) with coma.

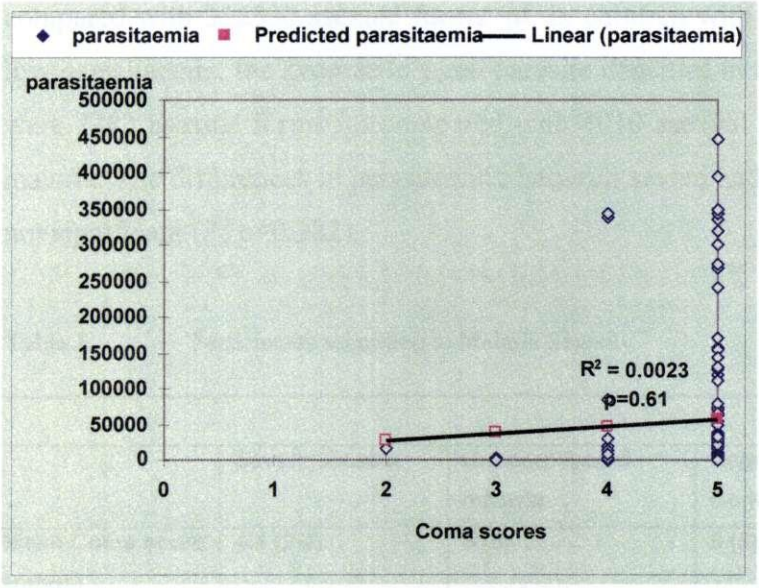

Figure 9 Correlation between parasitaemia and coma scores in the malaria infected children. Coma scores were determined using the Blantyre coma scale. Parasitaemia was determined from Giemsa stained thick blood films.

In our study 117 children had malaria, out of which 37 children (31.6 percent) had severe malaria and 80 children (68.4 percent) had uncomplicated malaria. Of the 37 children with severe malaria, three children (8.1 percent) were hyper parasitaemic, 17 children (45.9 percent) were in coma, 28 children (75.7 percent) were severely anaemic and 6 children (16.2 percent) had a combined diagnosis of coma with severe anaemia.

Severe anaemia was the most common (75.7 percent) form of severe malaria in our study population.

Table 3 shows that the mean haemoglobin concentrations were significantly lower in severe malaria (5.2 g/dl) compared with 9.2 g/dl in the uncomplicated malaria and 11.2g/dl in the controls (Z; $p<0.001$). The mean Blantyre coma scores were also significantly lower in severe malaria (coma score = 4.4) than in uncomplicated malaria and controls with coma score = 5 (Z; $p<0.001$). The geometric mean parasite densities for high parasitaemia, in children with severe malaria were 338114 asexual forms /µl compared with 332330 asexual forms /µl, in children with uncomplicated malaria. For low parasitaemia, the geometric mean parasite densities in children with severe malaria were 7282 asexual forms /µl compared with 4010 asexual forms /µl, in uncomplicated malaria. The differences in parasitaemia between severe and uncomplicated malaria was not significant (Z; $p=0.282$).

Table 3. Participants according to Malaria Severity.

	Severe Malaria	Uncomplicated malaria	Negative Controls	P value
Mean Coma score (range)	4.4 (2-4)	5 (5)	5 (5)	p<0.001
Mean Hb (range)	5.2 (1.3-13.4) g/dl	9.2 (4.6 -14) g/dl	11.2 (6.4-16.1) g/dl	p<0.001
High Para geomean (range)	338114 (334080-342800) as/ul	332330 (268800-447040) as/ul	0	p=0.282
Low Para geomean (range)	7282 (80-155600) as/ul	4010 (120-240000) as/ul	0	p=0.282

Figure 10 shows that coma, coma with severe anaemia and high parasitaemia were observed in children under five years of age. High parasitaemia was only observed in children aged between one to five years. High parasitaemia was not observed in children above five years and in children under one year of age.

Severe anaemia was prevalent in all children up to ten years but not in the older children. Coma and coma with severe anaemia were observed in children aged between three months to five years.

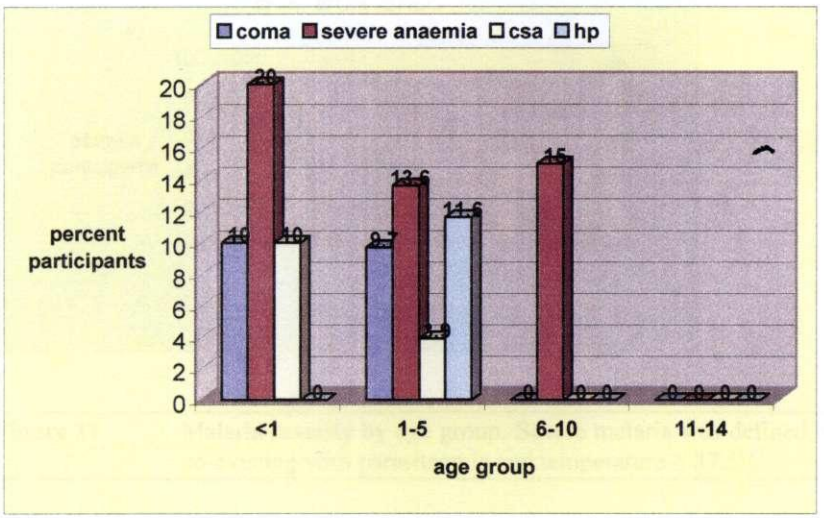

Figure 10 Severe malaria and high parasitaemia in the different age groups. Coma (<5 Blantyre scale); S.A, severe anaemia (≤5g/dl); C/SA, coma/severe anaemia; H.P, high parasitaemia (>250, 000 asexual forms/ µl.

Figure 11 shows that the rate of severe malaria in the study population was highest (20 percent) in children under one year (χ^2; p=0.05). Severe malaria was not observed in the children above 10 years of age. Further analysis showed that the rate of severe malaria was significantly higher (χ^2; p=0.044) in children under five years (19.5 percent) than in children above five years of age (10 percent). Malaria infection rates were also significantly higher (χ^2; p<0.05) in children under five years of age (106 out of 117 malaria infected children; 90.6 percent) compared with children above five years of age (11 out of 117 malaria infected children; 9.4 percent).

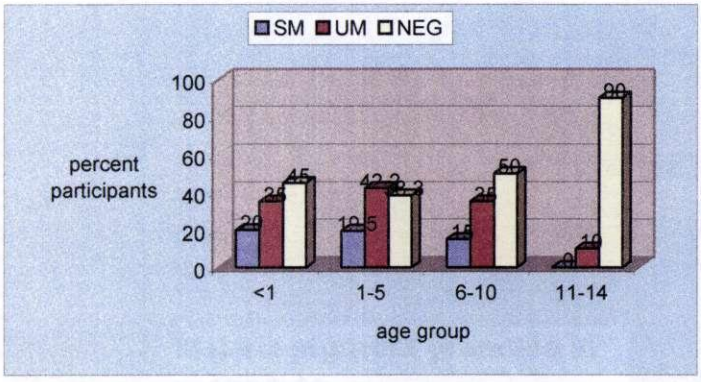

Figure 11 Malaria severity by age group. Severe malaria was defined by coma or severe anaemia co-existing with parasitaemia and temperature ≥ 37.5°C.

3.4 Assessment of Pigment-laden leukocytes

The leukocytes appeared purple in the MayGrunwald-Giemsa stained thin films. The intraleukocytic malaria pigments were recognizable by their amber to black and solid appearance.

Intraleukocytic malaria pigments are illustrated in Plates 1 – 3.

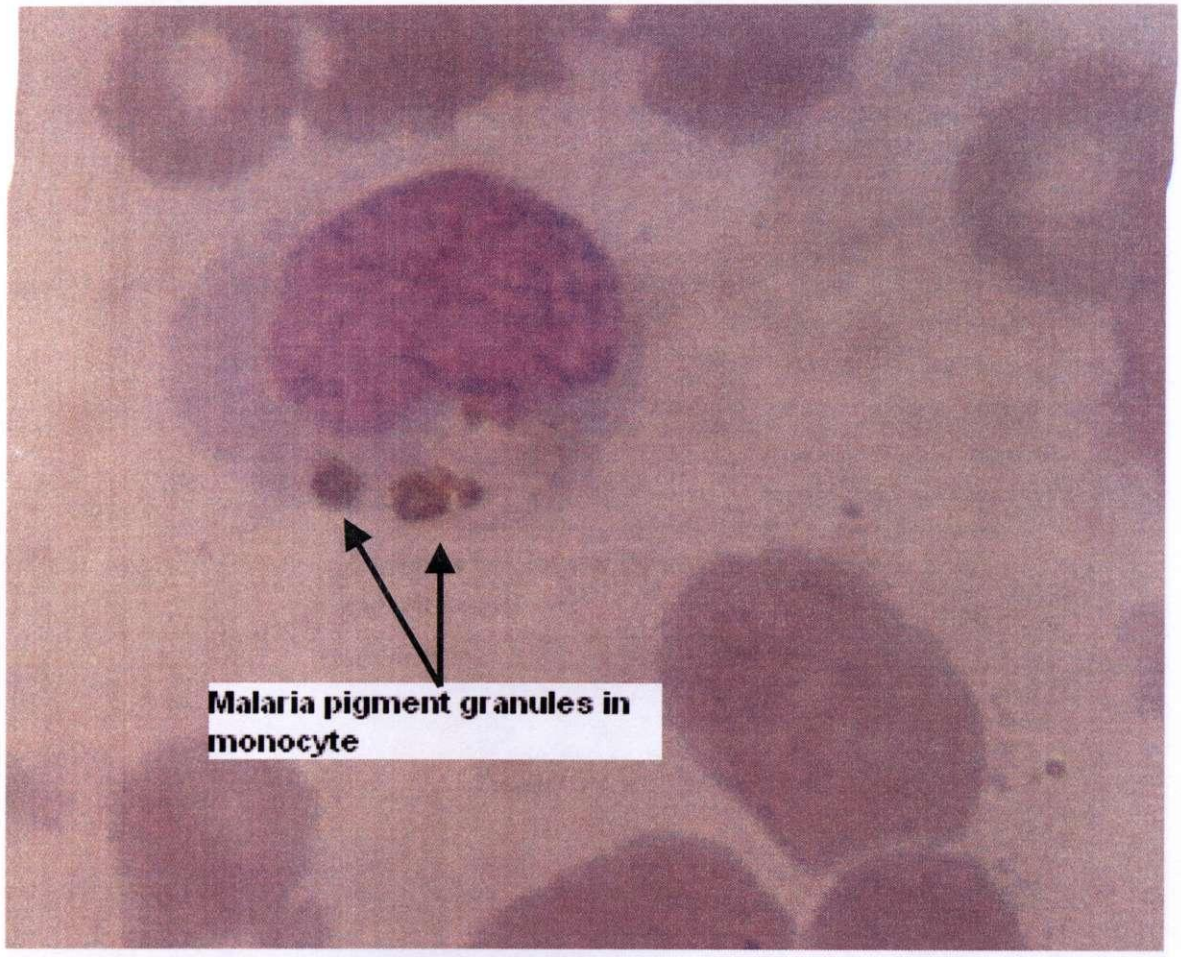

Plate 1 Pigment-laden monocyte in a thin blood film stained using the MayGrunwald-Giemsa stain method. The monocyte, purple in color, is shown containing amber to black solid looking granules of malaria pigment. The red blood cells are numerous and pink in color.

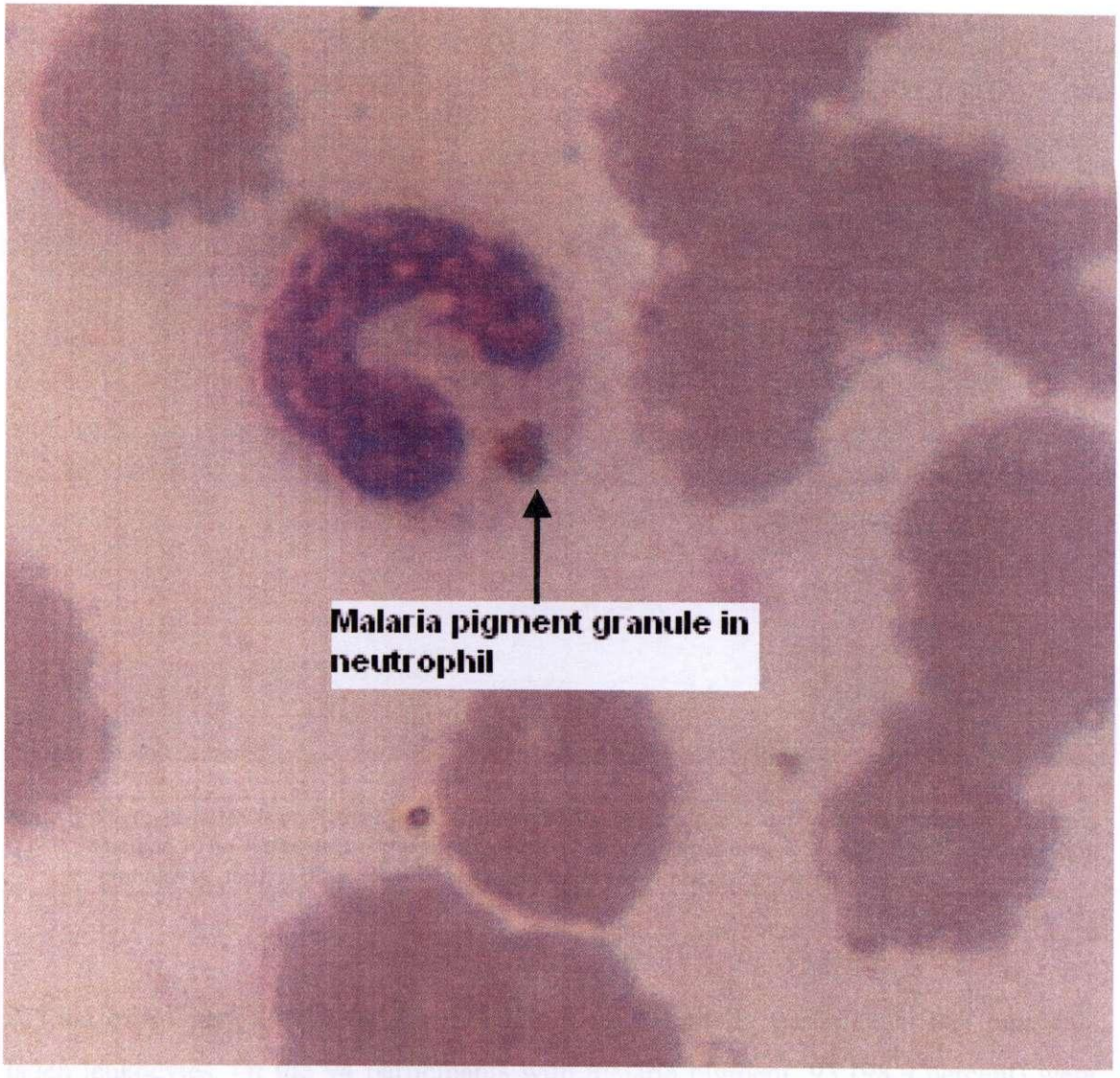

Plate 2 Pigment-laden neutrophil in a thin blood film stained using the MayGrunwald-Giemsa stain method. The neutrophil, purple in color, is shown containing amber to black solid looking granules of malaria pigment. The red blood cells are numerous and pink in color.

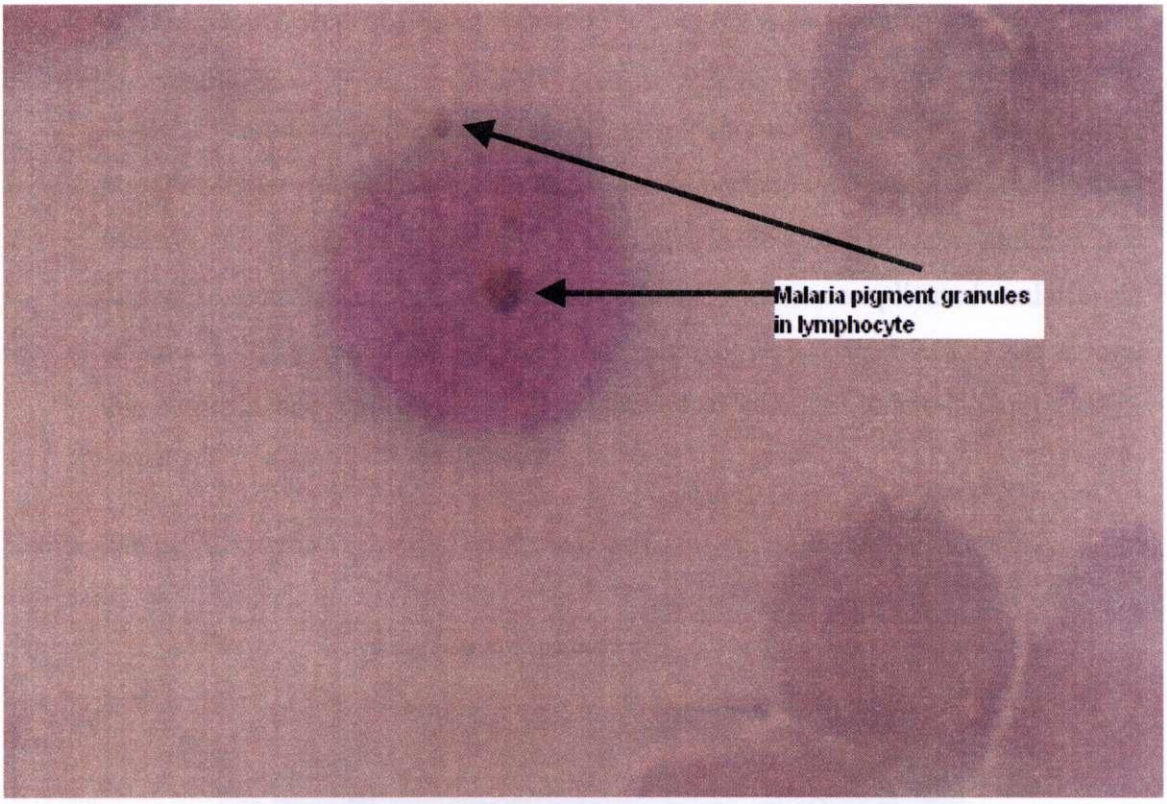

Plate 3 Pigment-laden lymphocyte in a thin blood film stained using the MayGrunwald-Giemsa stain method. The lymphocyte, purple in color, is shown containing amber to black solid looking granules of malaria pigment. The red blood cells are numerous and pink in color.

A total of 94 participants (46.1 percent) out of 204 Study participants had pigment-laden leukocytes. Of the 94 participants with malaria pigment, 93 (98.9 percent) were malaria-infected children and one participant (1.1 percent) was from the controls. The aparasitaemic child with malaria pigment had only pigment-laden monocytes. Sixty-one participants (29.9 percent) had more than one type of pigment-laden leukocytes. Thirty-three participants (16.2 percent) had only one type of pigment-laden leukocytes. Of the 94 participants with pigment-laden leukocytes, 52 participants (55.3 percent) had pigment-laden neutrophils, 46 participants (48.9 percent) had pigment-laden lymphocytes and 86 participants (91.5 percent) had pigment-laden monocytes.

3.5 Association of intraleukocytic malaria pigment with disease severity

3.5.1 Association of pigment-laden leukocytes with malaria severity

Figure 12 shows that there were more participants with severe malaria who had pigment-laden leukocytes compared with participants with uncomplicated malaria. Of the 37 children with severe malaria, 35 (94.6 percent) children had pigment-laden leukocytes, out of 80 children with uncomplicated malaria, 58 children (72.5 percent) had pigment-laden leukocytes (χ^2; p= 0.012) and among the controls one participant (1.1 percent) had pigment-laden leukocytes (χ^2; p<0.001).

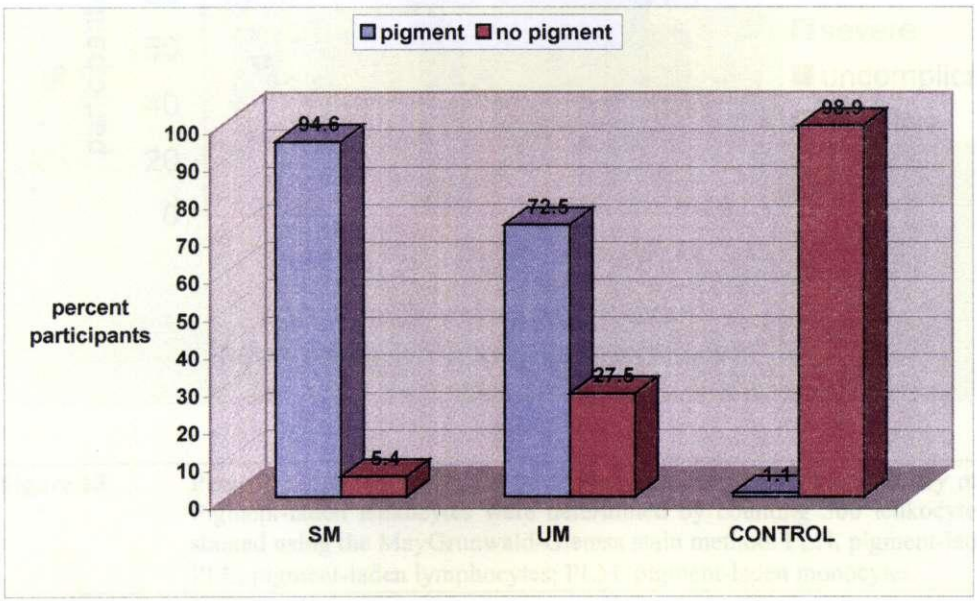

Figure 12 Percent participants with Pigment-laden leukocytes by malaria severity. Pigment-laden leukocytes were determined by counting 500 leukocytes in thin films stained using the Maygrunwald-Giemsa stain method. SM, severe malaria; UM, uncomplicated malaria.

Figure 13 shows that there were significantly more participants with pigment-laden monocytes (χ^2; p=0.025) than participants with either pigment-laden neutrophils (χ^2; p<0.001) or lymphocytes (χ^2; p<0.001). Of the 37 children with severe malaria, 25 children (67.6 percent) had pigment-laden neutrophils, 22 children (59.5 percent) had pigment-laden lymphocytes and 32 children (86.5 percent) had pigment-laden monocytes. In uncomplicated malaria, out of 80 participants, 27 participants (33.8 percent) had pigment-laden neutrophils, 24 children (30 percent) had pigment-laden lymphocytes and 53 children (65 percent) had pigment-laden monocytes.

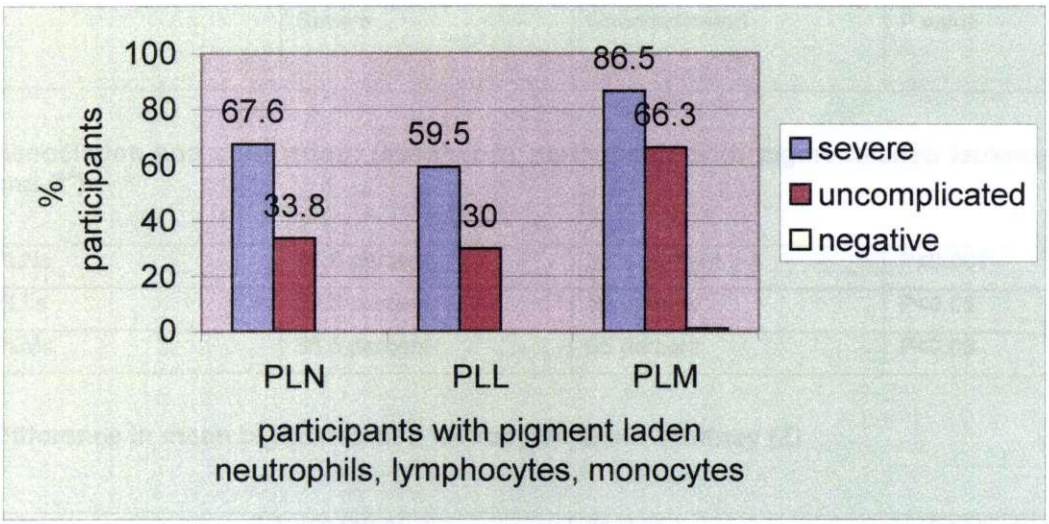

Figure 13 Percent participants with the three Pigment-laden leukocyte types by malaria severity. Pigment-laden leukocytes were determined by counting 500 leukocytes in thin films stained using the MayGrunwald-Giemsa stain method. PLN, pigment-laden neutrophils; PLL, pigment-laden lymphocytes; PLM, pigment-laden monocytes.

Table 4 and Figures 14, 15 and 16 show a significant association and correlation between malaria severity and the presence of pigment-laden neutrophils (χ^2; p=0.001, R^2; p=0.0005), lymphocytes (χ^2; p=0.011, R^2; p<0.001), and monocytes (χ^2; p=0.029, R^2; p<0.001) in peripheral blood. The results show that absolute pigment-laden leukocyte counts of $\geq$ 469189 neutrophils, 324268.5 lymphocytes and 1756778 monocytes per microlitre indicate severe malaria.

Table 4. Association and Correlation of Pigment-laden leukocytes with Malaria Severity.

	Severe	Uncomplicated	P value
Association and correlation: (Number of participants with pigment-laden leukocytes; χ^2 and R^2)			
PLNs	67.6 percent	33.8 percent	**P<0.001**
PLLs	59.5 percent	30 percent	**P<0.05**
PLMs	86.5 percent	65 percent	**P<0.05**
Difference in mean pigment-laden leukocytes Mann-Whitney (Z)			
PLNs	469189.2/ µl	264230 / µl	**p=0.001**
PLLs	324286.5/ µl	195020 / µl	**p=0.003**
PLMs	1756778/ µl	921350 / µl	**p=0.005**

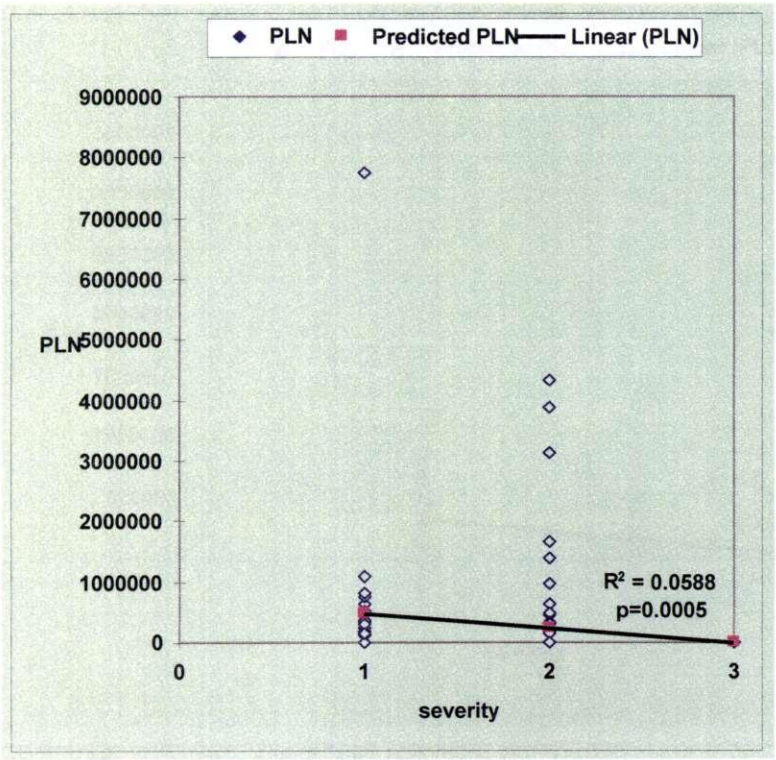

Figure 14: Correlation of pigment-laden neutrophils with malaria severity. Pigment-laden neutrophils were determined by counting 500 neutrophils in thin films stained using the MayGrunwald-Giemsa stain method. PLN, pigment-laden neutrophils. On the x-axis with the variable severity, category 1 represents severe malaria, category 2 represents uncomplicated malaria and category 3 represents controls.

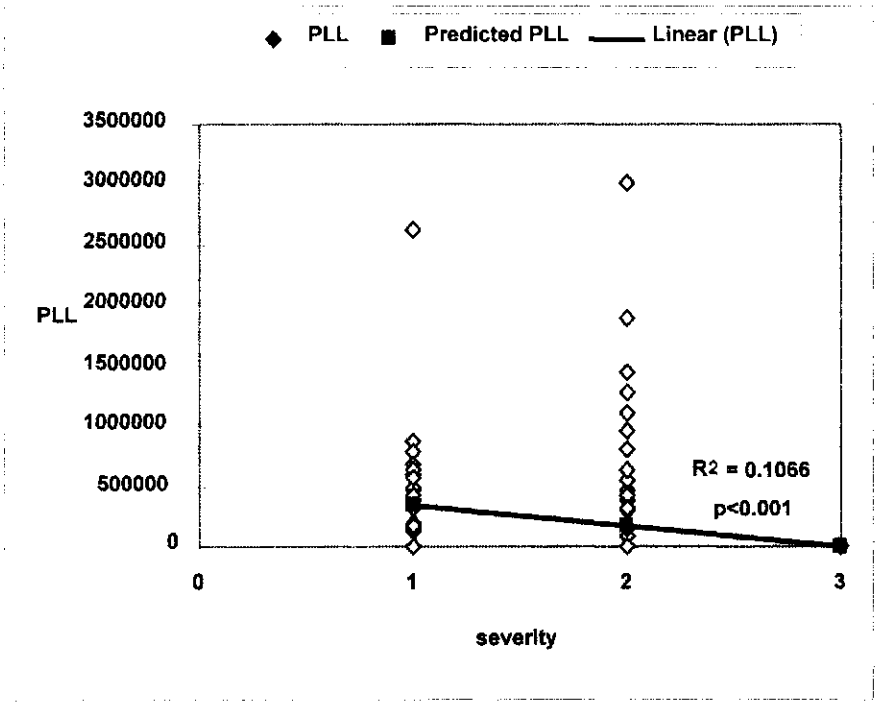

Figure 15: Correlation of pigment-laden lymphocytes with malaria severity. Pigment-laden lymphocytes were determined by counting 500 lymphocytes in thin films stained using the Maygrunwald-Giemsa stain method. PLL, pigment-laden lymphocytes. On the x-axis with the variable severity, category 1 represents severe malaria, category 2 represents uncomplicated malaria and category 3 represents controls.

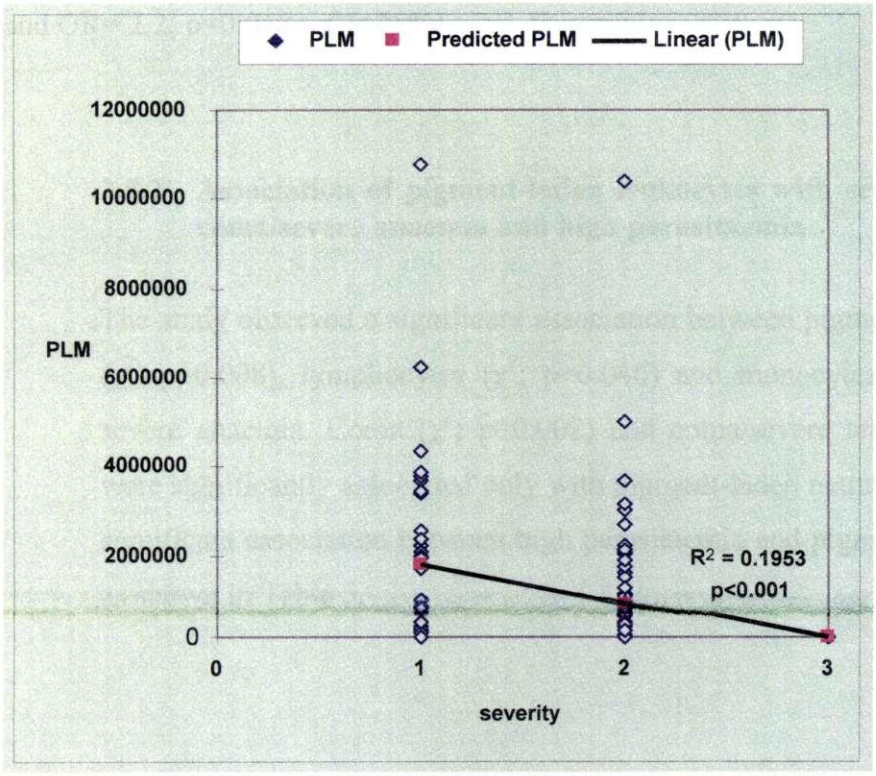

Figure 16: Correlation of pigment-laden monocytes with malaria severity. Pigment-laden monocytes were determined by counting 500 monocytes in thin films stained using the Maygrunwald-Giemsa stain method. PLM, pigment-laden monocytes. On the x-axis with the variable severity, category 1 represents severe malaria, category 2 represents uncomplicated malaria and category 3 represents controls.

Table 4 also shows that the differences in the mean pigment-laden leukocytes in children with severe and children with uncomplicated malaria (Z; $p<0.05$) were statistically significant. Pigment-laden monocytes were significantly more than the pigment-laden neutrophils and lymphocytes in both children with severe malaria and children with uncomplicated malaria (χ^2; $p<0.001$).

The number of children with pigment-laden leukocytes and the quantities of pigment-laden leukocytes increased with malaria severity. The odds of having severe malaria were highest in children with the most quantity of pigment-laden neutrophils and

monocytes (OR= 5.5; p=0.004 and OR= 5.4; p=0.048 – CI=95%) and least in children with the least quantity of pigment-laden neutrophils and monocytes (OR= 2.94; p=0.004 and OR= 2.2; p=0.048 – CI=95%).

3.5.2 Association of pigment-laden leukocytes with severe anemia, coma, coma/severe anaemia and high parasitaemia

The study observed a significant association between pigment-laden neutrophils (χ^2; p=0.008), lymphocytes (χ^2; p=0.046) and monocytes (χ^2; p=0.043) with severe anaemia. Coma (χ^2; p=0.002) and coma/severe anaemia (χ^2; p=0.007) were significantly associated only with pigment-laden neutrophils. There was no significant association between high parasitaemia and pigment-laden leukocytes as shown in Table 5.

Table 5 Association of pigment-laden leukocytes with severe malaria and high parasitaemia. Chi-square and Mann-Whitney U test were used to determine the association and the difference in mean pigment-laden leukocytes. PLNs, pigment-laden neutrophils; PLLs, pigment-laden lymphocytes; PLMs, pigment-laden monocytes.

	Association: (Number of participants with PLNs, PLLs, PLMs) Chi-square (χ^2)	Difference in mean geometric pigment-laden leukocytes Mann-Whitney (Z)
Severe anaemia vs. no severe anaemia		
PLNs	P= **0.008**	265171.4 vs. 349142.2 /µl; p=0.010
PLLs	P= **0.046**	355321.9 vs. 198328.8 /µl; **p=0.015**
PLMs	P= **0.043**	1928543 vs. 951793.6 /µl; **p=0.005**
Coma vs. no coma		
PLNs	P= **0.002**	762588.2 vs. 255344.6 /µl; **p**=0.001
PLLs	P=0.130	353270.6 vs. 215946.6 /µl; p=0.130
PLMs	P=0.149	1382588 vs. 1152048 /µl; p=0.149
Coma/Severe anaemia vs. no coma/severe anaemia		
PLNs	P= **0.007**	446000 vs. 322724.9 /µl; **p<0.001**
PLLs	P=0.210	659333.3 vs. 213011.4 /µl; p=0.210
PLMs	P=0.187	1384400 vs. 1174797 /µl; p=0.187
High vs. low parasitaemia		
PLNs	P= 0.537	3094400 vs. 237553.3 /µl; **p=0.008**
PLLs	P=1.000	360533.7 vs. 321088.6 /µl; p=0.645
PLMs	P=1.000	1538400 vs. 1776047.2 /µl; p=0.956

Table 5 and Figures 17 and 18 also show that the mean pigment-laden leukocytes were significantly higher in severe anaemia compared with cases of haemoglobin concentrations above 5g/dl. PLNs were 265171.4 against 349142.2 PLNs/µl, Z; p=0.010, PLLs were 355321.9 against 198328.8 PLLs/µl, Z; p=0.015 and PLMs were 1928543 against 951793.6 PLMs/µl, Z; p=0.005.

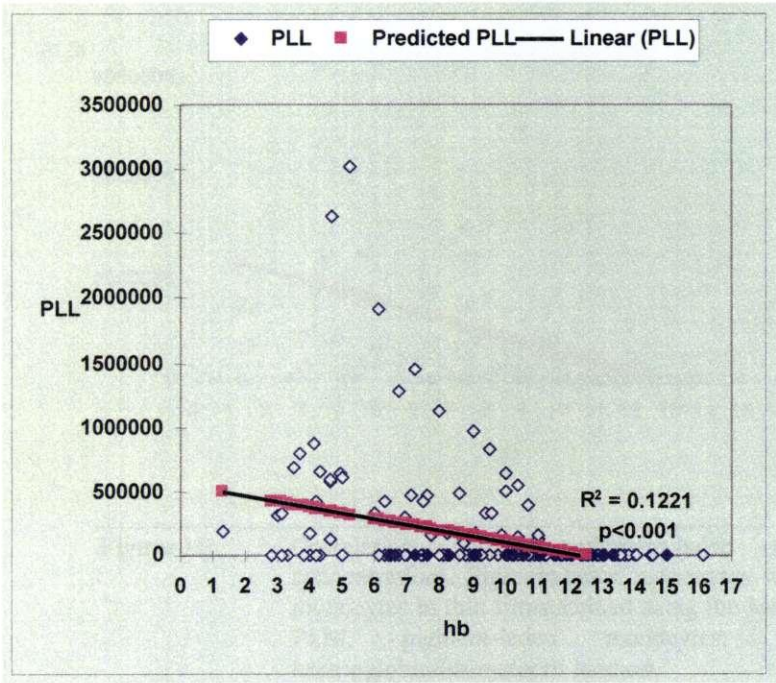

Figure 17: Correlation between pigment-laden lymphocytes and haemoglobin concentrations. Pigment-laden lymphocytes were determined by counting 500 lymphocytes in thin films stained using the Maygrunwald-Giemsa stain method. PLL, pigment-laden lymphocytes. Hb was determined by haemoglobin/haematocrit method.

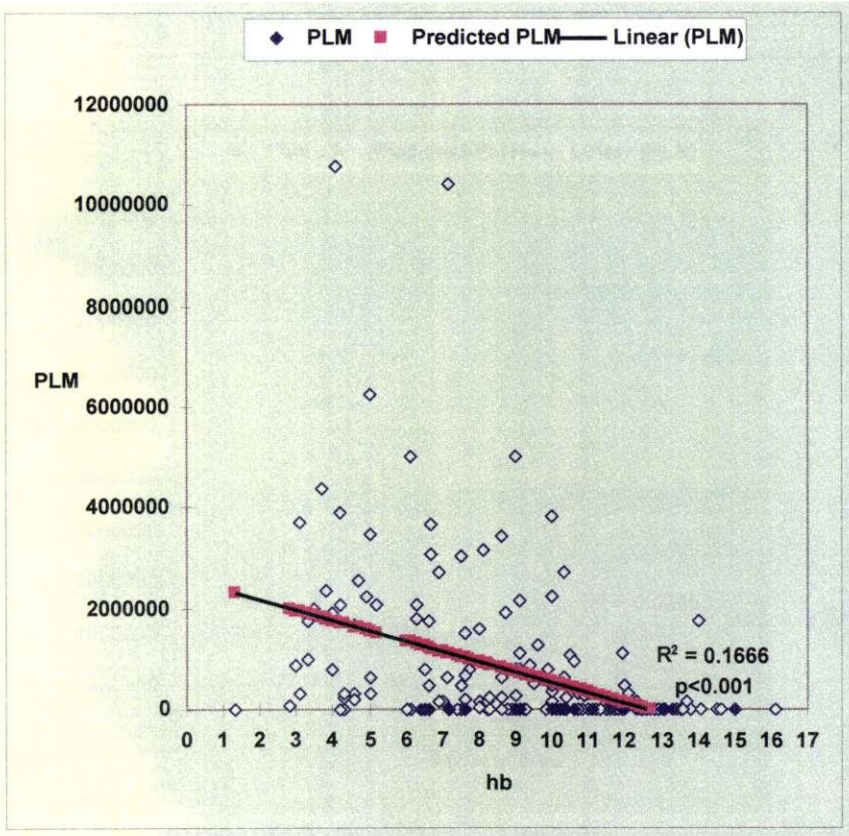

Figure 18: Correlation between pigment-laden monocytes and haemoglobin concentrations. Pigment-laden monocytes were determined by counting 500 monocytes in thin films stained using the Maygrunwald-Giemsa stain method. PLM, pigment-laden monocytes. Hb was determined by haemoglobin/haematocrit method.

Comparing coma with non-coma cases and coma/severe anaemia with non coma/severe anaemia cases, pigment-laden neutrophils were 762588.2 against 255344.6 PLNs/μl, Z; $p=0.001$ and 446000 against 322724.9 PLNs/μl, Z; $p<0.001$ respectively. Only pigment-laden neutrophils were significantly associated with coma and coma/severe anaemia.

Figure 19 shows correlation between pigment-laden neutrophils and coma.

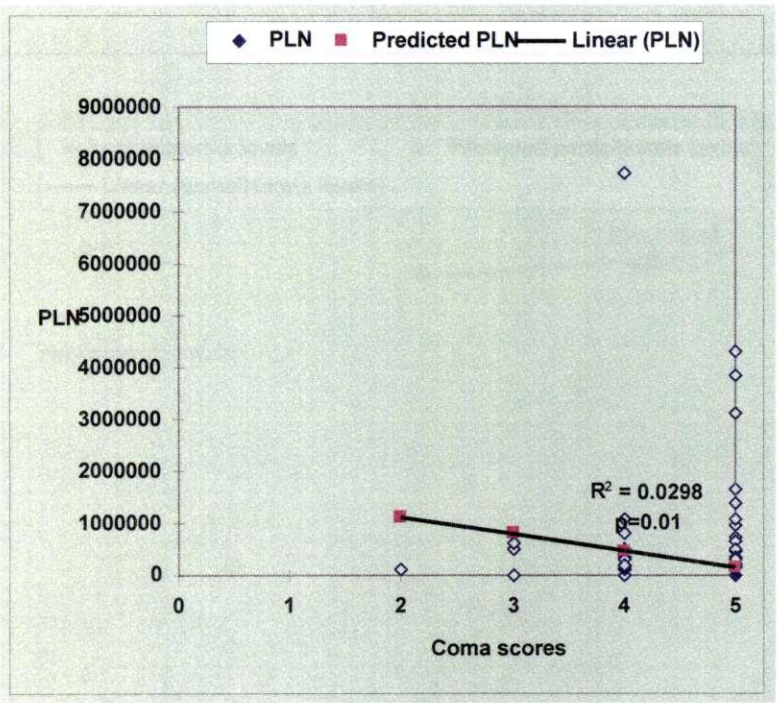

Figure 19: Correlation between pigment-laden neutrophils with coma. Pigment-laden neutrophils were determined by counting 500 neutrophils in thin films stained using the Maygrunwald-Giemsa stain method. PLN, pigment-laden neutrophils. Coma was determined using the Blantyre coma scale.

There was neither significant association nor correlation between pigment-laden leukocytes and high parasitaemia as shown in Figure 20.

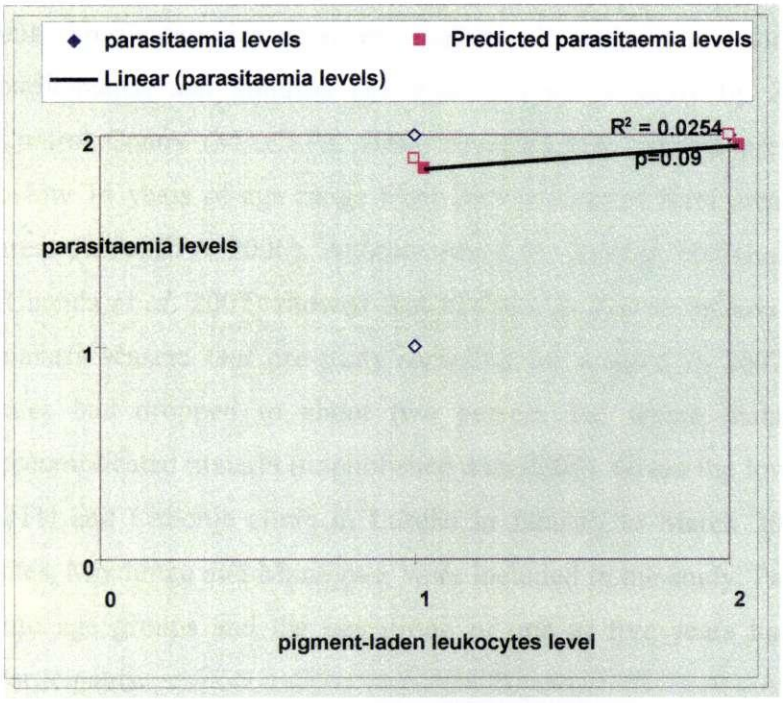

Figure 20: Correlation between parasitaemia levels and pigment-laden leukocytes. Pigment-laden leukocytes were determined by counting 500 leukocytes in thin films stained using the Maygrunwald-Giemsa stain method. On y-axis, category 1 represents high parasitaemia and category 2 represents low parasitaemia. On x-axis, category 1 represents presence of pigment-laden leukocytes and category 2 represents absence of pigment-laden leukocytes. Parasitaemia was determined from thick giemsa stained blood smears.

Table 5 further shows that mean pigment-laden neutrophils in coma/severe anaemia, 446000PLNs/ul, were significantly higher than pigment-laden neutrophils in severe anaemia alone 265171.4 PLNs/μl, Z; $p = 0.017$.

Chapter four Discussion

4.1 Study Population

This study was a hospital based case-control study designed to study the intraleukocytic malaria pigment in relation to severe malarial disease. Study participants were recruited into three categories of severe malaria (37 participants), uncomplicated malaria (80 participants) and controls (87 participants). A study by Zambia National Malaria Control Centre (MACEPA 2006) revealed that parasite prevalence rates in children below 14 years of age range from 28.9 percent in rural areas to 4.9 percent in urban areas (MACEPA 2006). Another study by Zambia National Malaria Control Centre (Chanda *et al.* 2007) showed that admissions due to malaria were rare in 2005 /2006 malaria season. Our pre-study sampling for malaria in 2007 showed that prevalence rates had dropped to about two percent for severe malaria and six percent for uncomplicated malaria (unpublished data 2007). Given the low rates of malaria cases at UTH and Chilenje clinic in Lusaka in January to March 2007, two additional study sites, Mpulungu and Mpongwe, were included in the study. Participants were organized into age groups and the age group of one to five years had the highest number of participants.

4.2 Peripheral Malaria Parasite densities

A total of 115 out of 117 malaria infected children (98.3 percent) were exclusively infected with *P. falciparum* and two children had a *P. falciparum/P. malariae* co-infection. Literature (McKenzie *et al.* 2002) shows that in co-infections of malaria, the *P. falciparum/P. malariae* is the combination common to Africa and Asia. Malaria rates were significantly higher (χ^2; P=0.023) in children under five years of age (60.9 percent) than in children above five years of age (36.7 percent).

Parasite densities in the study population ranged from 80 to 450 000 asexual forms /µl. High parasitaemia was observed only in children between one to five years old (Figure 2). Our study shows that the prevalence of infection and parasite density decreased with increasing age. Descriptive epidemiological studies of malaria infection in areas of stable transmission have revealed distinct age-specific pattern of parasite prevalence and density as detected by positive blood slide smears (Odongo-Aginya *et al.* 2005). These patterns show that prevalence of malaria infection and parasitaemia decreases in children above the age of five years. Data from our study is consistent with these age-related patterns of malaria infection prevalence. The higher prevalence and parasite density levels in children under five years of age than in older children are a result of a developed immunity in the latter (WHO/UNICEF, 2003; Kakkilaya 2006; Gebre and Negash 2002). This shows that malaria infection and parasitaemia levels are high and common in the younger age groups especially in children under five years old. The 2003 WHO/UNICEF report states that most children experience their first malaria infections during the first two years of life, when they have not acquired natural immunity (WHO/UNICEF 2003), making them vulnerable to malaria.

This study observed that children between one to five years of age had high parasitaemia. Children under one year and those above five years of age did not have high parasitaemia. Malaria infections in infants are rare and usually with low parasitaemia (Afolabib *et al.* 2001; Mockenhaupt *et al.* 2000; Kakkilaya 2006). In the first two months of life, children may not contract malaria or the manifestations may be mild with low-grade parasitemia, due to the passive immunity offered by the maternal antibodies (Kakkilaya 2006). However, when infants and children under the age of five years get malaria infection, the disease tends to be atypical and more severe. The observed decline (Figure 2) in parasite density levels in children above five years is most likely due to the development of immunity over time (Odongo-Aginya et al. 2005). This immunity regulates infection and is usually pronounced in children above 15 years and in adults (Odongo-Aginya et al. 2005; Kakkilaya 2006). These are people who have been exposed to infective mosquito bites and their systems challenged over the years, hence the acquired immunity.

Of the children with high parasitaemia, 27.3 percent had severe malaria and 72.7 percent had uncomplicated malaria showing that (Table 2) parasitaemia levels were not associated with malaria severity (χ^2; P>0.05). Parasitaemia alone is not a reliable indicator of malaria severity.

Furthermore, the odds of having severe malaria were highest in children with the least parasite densities (OR= 3.02; p<0.05; 95%CI). This implies that malaria severity increases with low parasite densities and further substantiates the fact that high parasitaemia cannot be an indicator of severe malaria. Previous findings (Lyke *et al.* 2003) showed that parasitaemia, is usually low in severe malaria compared with uncomplicated malaria. Other authors (Casals-Pascual *et al.* 2006; Lell *et al.* 2005) have shown that where parasitaemia has been associated with the severity of malaria, the relationship is weak and the association of parasite density with specific syndromes of severe disease is less clear. Yet another study, (Missinou 2005) showed that in Malawi, patients with high parasitaemia were less severely ill.

4.3 Severity of Malaria

Our study (Figure 11) shows that the rate of severe malaria was highest (20 percent) in children under one year (χ^2; P= 0.05). In our study the rate of severe malaria was also significantly higher (χ^2; P= 0.044) in children under five years (19.5 percent) than in children above five years of age (10 percent). We discuss severity of malaria in our study based on haemoglobin concentrations and coma scores.

4.3.1 Haemoglobin concentrations in the study participants

Haemoglobin concentrations ranged from 1 g/dl to 16 g/dl in the study participants. Figure 11 shows that severe anaemia was observed in children aged between three months to 10 years although it was highest in children under one year (20 percent).

The impact of malaria on haemoglobin concentrations of children is more pronounced in children under one year. Severe anaemia is considered more likely in this age group considering the rapid physiological changes, at this stage of development, which necessitate a huge increase in the daily iron requirements of children (Kakkilaya 2006; Alumanah and Nwanguma 2007). During this stage of life, malaria infection rapidly results in the further depletion of any iron deposits, leading to severe anaemia. Severe anaemia may also be observed when other parasitic or bacterial infections affecting the gastrointestinal tract run concurrently with malaria. The risk of exposure to these infections is increased at the 7-12 months stage by the introduction of bottle-feeding and the consumption of other food (WHO RBM; Alumanah and Nwanguma 2007). Another factor is that many of the children would have suffered previous attacks of malaria or helminthiasis in early life, and these may have depleted their iron status, leaving them clearly or marginally anaemic (Alumanah and Nwanguma 2007; Kakkilaya 2006). Typically, these children are often not allowed enough time to re-build their iron deposits before a second infection strikes to further deplete the iron stores.

In our study, the mean haemoglobin concentrations in children under five years of age (9g/dl) were significantly lower than the mean haemoglobin concentrations in children above five years of age (10.5g/dl) [Z; p=0.001]. Figure 11 also shows that severe anaemia was higher in children under five years five years of age than in older children. This is probably due to variances in immunity development in the two groups. Two studies (Biemba *et al.* 2000) and (Bjorkman *et al.* 2004) conducted in Macha and in Kenya respectively, illustrated that the highest rates of Severe Malaria Anaemia -associated morbidity and mortality occur in children under five years of age. Bjorkman and colleagues (2004) showed that 70 percent of all patients with severe malarial anaemia were children under the age of five years (Bjorkman *et al.* 2004).

The presence of parasitaemia (χ^2; $p<0.001$) and not the level of parasitaemia (χ^2; $p=1.000$) was significantly associated with haemoglobin concentrations. From Figure 6, there was no correlation between parasitaemia and haemoglobin concentrations (R^2; $p=1.000$). Our study concludes that malaria infection, and not the level of parasitaemia, impact on haemoglobin concentrations in patients.

4.3.2 Coma rates in the study participants

In our study, coma and or impaired consciousness and a combination of coma or impaired consciousness with severe anaemia were observed in children aged between three months to 5 years (Figure 8). Literature shows that coma is common in children aged between 6 months to five years (Kakkilaya 2006). This is the stage of life when malaria rates are high and severe due to less developed immune systems. Parasitaemia was not significantly associated (χ^2; $p=1.000$) nor correlated (R^2; $p=0.61$) with coma (Figure 9). From our study we conclude that coma or impaired consciousness may occur in children with malaria regardless of the parasite density levels involved. Coma alone indicates increased risk of dying and does not depend on the presence or absence of anaemia or convulsion (Gebre and Negash 2002) or indeed parasitaemia levels. Coma is a poor prognostic indicator confirming that in severe malaria neurological involvement is mostly associated with poor outcomes (Oduro *et al.* 2007). Any level of impaired consciousness, regardless of coma score, warrants prompt intervention as a case of severe malaria.

4.4 Association of Intraleukocytic Malaria Pigment with Disease Severity

Malaria pigment, the metabolic end product of haemoglobin, once released into host circulation, is engulfed by scavenger leukocytes as they help protect the body from disease and infection. This pigment is visible and was seen in leukocytes as amber to

black solid granules. Our study did not observe phagocytosis of any malaria-infected red blood cells by leukocytes in peripheral blood smears. Studies (Hepeng 2006) have shown that the parasites avoid being engulfed by the leukocytes of the immune system by accumulating calcium ions released from within the host cell. The accumulation of calcium ions by the parasites blocks the outer surface of the merosomes, creating the appearance of a protein that would normally act as an inviting signal for the immune cells (Hepeng 2006). Secondly, such parasitised red blood cells may only be observed in vivo because in vivo they sequester in very deep tissues. Other studies (Martiney *et al.* 2000) have shown that deposition of extensive pigment and parasitised red blood cells occurs in tissues like the spleen and bone marrow. Further, erythrocytes have half the lifespan of monocytes (Adrianus *et al.* 2006), implying that they get cleared from peripheral blood at a faster rate compared to leukocytes.

4.4.1 Association of Pigment-Laden Leukocytes with Malaria Severity

Among the 37 children with severe malaria in the study, Figure 13 shows that the percentage of children with malaria pigment was significantly higher (χ^2; $P<0.05$) than in the children with uncomplicated malaria. From our study and previous studies (Casals-Pascual *et al.* 2006), malaria pigment is evident in peripheral blood leukocytes in greater than 90 percent of patients with severe malaria and therefore can be used as a surrogate marker for acute or chronic parasite load. The mean pigment-laden leukocytes were also significantly higher (Z; $P<0.05$) in the children with severe malaria than in the children with uncomplicated malaria. This finding is similar to previous findings (Nguyen *et al.* 1995 and Missinou 2005) where high quantities of pigment-laden leukocytes in children with severe malaria were observed. In another study (Lyke *et al.* 2003), the proportions of neutrophils and monocytes with malaria pigment were significantly higher in patients with severe malaria than in patients with uncomplicated malaria. Our study shows that absolute pigment-laden leukocyte

counts of ≥ 469189 neutrophils, 324268.5 lymphocytes and 1756778 monocytes per microlitre indicate severe malaria.

The percentage of participants with pigment-laden monocytes was significantly higher (χ^2; $P<0.001$) than the percentage of participants with pigment-laden neutrophils and lymphocytes (Figure 14). The mean pigment-laden monocytes were also significantly high (Z; $P<0.05$) over the other leukocyte types. The proportion of neutrophils and monocytes, containing malaria pigment is affected by total parasite burden and synchronicity of the parasite life cycle, and the clearance kinetics of these pigmented cells may be inherently different (Lyke *et al.* 2003). Cellular clearance kinetic studies performed by Day and others (Day *et al.* 1996) have shown peripheral pigment-laden neutrophil clearance times of 72 hours (range = 49–95 hours) and peripheral pigment-laden monocyte clearance of 216 hours (range = 180–240 hours). While clearance of pigment-laden monocytes appears to follow first-order kinetics, that of pigment-laden neutrophils departs from first-order kinetics, with increased rates of clearance at a lower cell density (Day *et al.* 1996; Awandare *et al.* 2007). The presence of pigment-laden neutrophils, with the rapid turnover of neutrophils, may indicate a recent heavy parasitic burden and provide prognostic indications of disease, while longer-lived pigmented monocytes with longer clearance rates may reflect a more protracted infection or repeated infections (Lyke *et al.* 2003) considering that the typical half-life of a neutrophil is 6–8 hours and that of a monocyte is several days (Lyke *et al.* 2003). Microscopy for intraleukocytic pigment is valuable in the differential diagnosis of severe febrile illnesses in malarious areas where uncontrolled use of antimalarial drugs is widespread (Day *et al.* 1996).

In our study, none of the controls, except one, possessed pigment-laden leukocytes and monocytes particularly. This contrasts the results of a study where a number of pigment-laden neutrophils and monocytes were observed in controls (Lyke *et al.* 2003). The reason for the presence of pigment-laden

monocytes in the uninfected patient could be the extended time in the clearance rates of monocytes as compared with the other leukocyte types. According to Casals-Pascual and others (2006), it is possible that the different clearance times of pigment containing monocytes and neutrophils may partially explain these differences. The presence of intra-monocytic malaria pigment is evidence that this patient was infected with malaria but probably the parasitaemia was too low to be detected by microscopy or parasitaemia could have cleared earlier than the time of examination. Studies (Adrianus *et al.* 2006) have shown that because of sequestration (which is common in *P. falciparum* malaria) or a parasite load below the detection limit of microscopy, low levels of parasitaemia can result in a negative malaria smear, but with continued circulation of haemozoin. Apart from the long lifespan of (pigment-containing) monocytes, newly formed monocytes keep phagocytising circulating haemozoin (Adrianus *et al.* 2006) and hence result in pigment-laden monocytes in the negative blood smears.

Furthermore, the odds of having severe malaria were highest in children with the highest quantity of pigment-laden neutrophils and monocytes (CI=95). This implies that pigment-laden leukocytes increase with malaria severity.

4.4.2 Association of pigment-laden leukocytes with severe anaemia, coma, coma/severe anaemia and high parasitaemia

The study, as shown in Table 5, observed a significant association between all three pigment-laden leukocyte types with severe anaemia (χ^2; $P<0.05$) and between pigment-laden neutrophils with coma and with coma/severe anaemia (χ^2; $P<0.05$).

The mean pigment-laden leukocytes were significantly higher (Z; $P<0.05$) in the severely anaemic compared with the non-severely anaemic children. For the children in coma and those with coma/severe anaemia, the mean pigment-laden neutrophils were significantly higher (Z; $P<0.05$) than in the non-comatose

children and in children without coma/severe anaemia respectively. Our study shows that pigment-laden neutrophils are better markers for all categories of malaria severity while pigment-laden lymphocytes and monocytes are better markers for severe anaemia only. While all leukocyte types laden with pigment were associated with the children in the severe malaria group, pigment-laden neutrophils were particularly associated with all determinants of severity. Given this finding, we recommend that absolute pigment-laden leukocyte counts of $\geq$ 469189 neutrophils be taken to represent severe malaria.

In our study, we show the association of severe anaemia with pigment-laden neutrophils and monocytes, one study (Casals-Pascual *et al.* 2006), observed the association of severe malarial anaemia with pigment-laden monocytes only. Another study, (Lell *et al.* 2005), in contrast to our study which showed that coma is only associated with pigment-laden neutrophils, observed that monocytes are better markers of coma. The association of severe anaemia with pigment-laden monocytes was also observed in other studies (Bojang 2005; Lell *et al.* 2005; Lyke *et al.* 2003).

Further, Table 5 shows that mean pigment-laden neutrophils were significantly higher (Z; $P<0.05$) in children with a combination of coma or impaired consciousness with severe anaemia than in children with severe anaemia alone. These findings agree with a study (Lyke *et al.* 2003) where mean pigment-laden leukocytes were more in coma/severe anaemia than in severe anaemia alone. The high mean pigment-laden leukocytes in the combination of coma or impaired consciousness with severe anaemia and not in severe anaemia alone could be due to the fact that the pathophysiology of acute cerebral malaria and severe anaemia may have properties creating a milieu for increased pigment formation unlike in severe anaemia alone (Lyke *et al.* 2003). Any degree of anaemia, combined with malaria severity indicators, should be regarded as a sign of life-threatening malaria and deserves appropriate management (Gebre and Negash 2002).

Our study found no association (χ^2; P<0.05) between pigment-laden leukocytes and peripheral parasitaemia levels. We have demonstrated that the level of parasitaemia is not significantly associated with pigment-laden leukocytes, neither is it associated with malaria severity (χ^2; P<0.05). Studies have shown (Lyke *et al.* 2003; Awandare et al. 2007) that the presence of pigment unlike parasitaemia reflects overall sequestered parasite burden within micro vascular networks and or the duration of infection.

These findings are also in agreement with the study, (Lyke *et al.* 2003) where no significant association for both pigment-laden neutrophils and pigment-laden monocytes with parasitaemia was observed. Unlike the findings from previous studies, (Lyke *et al.* 2003; Lell *et al.* 2005; Casals-Pascual *et al.* 2006; Bojang 2005) and our findings, other authors found an association between pigment-laden neutrophils and pigment-laden monocytes with hyper parasitaemia, shock and hypoglycemia although the relationship was weak (Nguyen *et al.* 1995).

Our results show that there is justification to reject the hypothesis for no association since pigment-laden neutrophils, lymphocytes and monocytes, showed significant association and correlation with malaria severity.

Chapter five Conclusion and Recommendations

5.1 Conclusion

Major conclusions can be drawn from this study. The prevalence of malaria infections and parasite density is high in children under five years of age and decreases with increasing age. Parasite density levels in children above five years are lower than in children under five years of age due to the development of immunity over time. Malaria infections in infants are rare and usually with low parasitaemia due to the passive immunity offered by the maternal antibodies. Parasitaemia levels are not associated nor correlated with malaria severity and therefore parasitaemia alone is not a reliable measure of malaria severity.

Secondly, the rate of severe malaria was significantly higher in children under five years with less developed immune systems than in children above five years of age whose immune systems are much more developed. Severe anaemia was the most common form of severe malaria in our study population. The impact of malaria on haemoglobin concentrations of children was more pronounced in children under one year considering the rapid physiological changes, at this stage of development. Although parasitaemia is not associated with malaria severity, it is related to low haemoglobin concentrations and coma scores in patients.

Thirdly, malaria pigment is significantly associated with severe malaria as opposed to uncomplicated malaria. Further, since malaria pigment is evident in peripheral blood leukocytes in greater than 90 percent of patients with severe malaria, it may be used as a surrogate marker for acute or chronic parasite load. From our study, neutrophils were associated with all determinants of severity; therefore, we conclude that an absolute pigment-laden neutrophil count of ≥ 469189 per microlitre will be the best indicator of severe malaria.

Among the three leukocytes types, monocytes were most active and high in quantity. The clearance kinetics of the pigment-laden leukocytes vary considering their life spans of 6-8 hours for neutrophils and several days for monocytes. The presence of intra-monocytic malaria pigment in aparasitaemic patients is evidence of malaria infection and such patients with an illness consistent clinically with severe malaria but with negative blood smears, must be managed as patients with severe malaria.

Our study determined an association between all three pigment-laden leukocyte types with severe anaemia but only pigment-laden neutrophils were associated with coma and with coma/severe anaemia. Therefore, pigment-laden neutrophils are better markers for all categories of malaria severity while pigment-laden lymphocytes and monocytes are better markers for severe anaemia only. Pigment-laden leukocytes were significantly higher in children with a combined diagnosis of coma with severe anaemia than in children with severe anaemia alone. Therefore, we conclude that severe anaemia coupled with any other indicator of malaria severity, in this case, coma, is characteristic of severe disease compared with severe anaemia alone. Our study found no association between pigment-laden leukocytes and parasitaemia levels.

Our results show that there is justification to reject the hypothesis for no association since pigment-laden neutrophils, lymphocytes and monocytes, showed significant association with malaria severity. Based on these results, the assessment of intraleukocytic pigment appears to be valuable for more detailed characterization of children who present with *P. falciparum* parasitaemia and clinical symptoms consistent with a malarial illness.

5.1 Recommendations

This study validates the presence of malaria pigment in neutrophils and monocytes, as a marker for disease severity. Therefore, this diagnostic indicator may be used as a basis to stratify patients for appropriate treatment and medical attention according to severity

of the disease. We recommend that while parasitaemia is important in diagnosis of malaria, it should not be used alone as an indicator of malaria severity. We further recommend that the presence of pigment in leukocytes particularly neutrophils and monocytes, which are better indicators of severe malaria, be reported by microscopists alongside malaria parasite results. An absolute pigment-laden neutrophil count $\geq$ 469189 per microlitre may be used as an indicator for severe malaria.

List of References

Adrianus, J.,van Dillen, J., de Witte1, P., Mucheto, S., Nagelkerke, N. and Kager1, P. 2006. Automated detection of malaria pigment: feasibility for malaria diagnosing in an area with seasonal malaria in northern Namibia. *Tropical Medicine and International Health* doi:10.1111/j.1365-3156.2006.01634.x. **11**(6) 809–816. Blackwell Publishing Ltd 809

Afolabi, B. M., Salako, L. A., Mafe, A. G., Ovwigho, U. B., Rabiu, K. A., Sanyaolu, N. O. and Ibrahim, M. M., 2001. Malaria in the first 6 months of life in urban African infants with anemia. *Am. J. Trop. Med. Hyg.*, **65**(6): 822-827

Alumanah, E. O. and Nwanguma, B. C., 2007. Severity of anaemia in Nigerian children diagnosed with *Plasmodium falciparum* malaria in the first year of life *JRTPH* 6: 11-15.

Amodu, O. K., Adeyemo, A. A., Olumese, P. E. and Gbadegesin R. A., 1998. Intraleukocytic malaria pigment and clinical severity of malaria in children. *Trans R Soc Trop Med Hyg.*, **92**(1): 54-6.

Anstey, N. M., Weinberg, J. B., Hassanali, M. Y., Mwaikambo, E. D., Manyenga, D., Misukonis, M. A., Arnelle, D. R., Hollis, D., McDonald, M. I. and Granger, D. L., 1996. Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type2 expression. *J Exp Med.*, **184**(2): 557-67.

Awandare, G. A., Ouma,Y., Ouma,C., Were,T., Otieno, R., Keller, C. C. Davenport, G. C., Hittner, J. B., Vulule, J., Ferrell, R., Ong'echa, J. M. and Perkins, D. J. 2007. Role of Monocyte-Acquired Hemozoin in Suppression of Macrophage Migration Inhibitory Factor in Children with Severe Malarial Anemia. *Infect Immun.* **75**(1):201-210.

Biemba, G., Gordeuk, V. R., Thuma, P. and Weiss, G., 2000. Markers of inflammation in children with severe malarial anemia. *Tropical Medicine & International Health.* **5**(4): 256.

Bjorkman, A., Kaneko, A., Kobayakawa, T., Lum, J. K., Eto, H., Obonyo, C. and Akhwale W. S., 2004. Anemia and malaria at different altitudes in Western highlands of Kenya. *Acta Tropica.* **91**(2) 167-175.

Bojang, K., 2005. Progress and challenges in the diagnosis of severe malaria. MIM 2005 Conference PPT presentation. *MIM website mim.su.se/conference2005/eng/presentations/16%20November-Wednesday/81%20Bojang.ppt.*

Casals-Pascual, C., Kai, O., Lowe, B., English, M., Williams, T. N., Maitland, K., Newton, C. R. C. J., Peshu, N. and Roberts, D. J., 2006. Lactate levels in severe malarial anemia are associated with hemozoin-containing neutrophils and low levels of IL-12. *Malar J.,* 5: 101.

Central Statistical Office (CSO) [Zambia]., 2002. *2000 Population and Housing Census of Zambia, Core Indicators handout.* Lusaka, Zambia: Central Statistical Office.

Chanda, P., Masiye F., Chitah, B. M., Sipilanyambe, N., Moonga, H., Banda, P. and Okorusobo, T., 2007. A Cost-effectiveness analysis of artemether-lumefantrine for treatment of uncomplicated malaria in Zambia. *Malar J.*, 6:21.

Coban, C., Ishii, K. J., Sullivan, D. J. and Kumar, N., 2002. Purified Malaria Pigment (Hemozoin) Enhances Dendritic Cell Maturation and Modulates the Isotype of Antibodies Induced by a DNA Vaccine. *Infect Immun.*, **70**(7)3939-3943.

Day, P. N., Pham, T. D., Phan, T. L., Dinh, X. S., Pham, P. L., Ly, V. C., Tran, T. H., Bethel, D. B., Phu, N. H., Nguyen, T. H., and White, N. J., 1996. Clearance kinetics of parasites and pigment-containing leukocytes in severe malaria. *Blood.* **88**(12):4694-700.

Demirev, P. A., Feldman, A. B., Kongkasuriyachai, D., Scholl, P., Sullivan, D. Jr., and Kumar, N., 2002. Detection of Malaria Parasites in Blood By Laser Desorption Mass Spectrometry. *Anal Chem.*, **74** (14): 3262-6.

Gebre, B. and Negash, Y. 2002. Malaria among children in Gambella, western Ethiopia. *Ethiop. J. Health Dev.* **16**(1):61-70.

Hempelmann, E. and Egan, T. J., 2002. Pigment biocrystallisation in *Plasmodium Falciparum. TRENDS in Parasitology* **18**(1).

Hepeng, J., 2006. Malaria hijacks liver cells to avoid immune system. *SciDev Network Journal*, Science doi 10.1126/science.1129720.

Howen, B. 2000. Blood Film Preparation and Staining procedures. *Lab Hematol* 6:1-7.

Johnson, S. C., ed. *Netmark Regional Africa Program Briefing Book.* 2000. Insecticide Treated Materials in Zambia September, 2000. Academy for Educational Development, Group Africa, Inc., Johns Hopkins University.

Kakkilaya B. S. 2006. Malaria in Children. *B. S. Kakkilaya 2006-2008 malariasite.com 2006-2008.*

Kaona, F. A. D. and Tuba, M., 2005. A qualitative study to identify community structures for management of severe malaria: a basis for introducing rectal artesunate in the under five years children in Nakonde District of Zambia. *BMC Public Health.* 5: 28.

Lell, B., Missinou, M. A., Issifou, S., Matsiegui, P. B., Olola, C. H., Taylor, T. E. and Kremsner, P. G., 2005. Assessment of a simplified method for counting leukocytic malaria pigment. *Am J Trop Med Hyg.*, **73**(3):588-92.

Lyke, K. E., Diallo, D. A., Dicko, A., Kone, A., Coulibaly, D., Guindo, A., Cissoko, Y., Sangare, L., Coulibaly, S., Dakouo, B., Taylor, T. E., Doumbo, O. K., and Plowe, C. V., 2003. Association of intraleukocytic *plasmodium falciparum* malaria pigment with disease severity, clinical manifestations, and prognosis in severe malaria *Am. J. Trop. Med. Hyg.*, **69**(3): 253-259.

MACEPA 2006. *Zambia 2006 National Malaria Indicator Survey.* Zambia Ministry of Health.

Marsh, K. and Forster, D., 1995. Indicators of life-threatening malaria in African children. *N Engl J Med.*, 332: 1399-1404.

Martiney, J. A., Sherry, B., Metz, C. N., Espinoza, M., Ferrer, A.S., Calandra, T., Broxmeyer, H. E. Bucala, R. 2000. Macrophage Migration Inhibitory Factor Release by Macrophages after Ingestion of *Plasmodium chabaudi*-Infected Erythrocytes: Possible Role in the Pathogenesis of Malarial Anemia. *Infect Immun.* **68**(4): 2259–2267.

McKenzie, F. E, Jeffery, M. G. and Collins, W. E., 2002. *Plasmodium malariae* infection boosts *plasmodium falciparum* gametocyte production. *Am. J. Trop. Med.* Hyg., **67**(4) 411-414.

Mharakurwa, S., M., Simoloka C., Thuma P. E., Shiff C. J. and Sullivan D. J. 2006. PCR detection of *Plasmodium falciparum* in human urine and saliva samples. *Malaria Journal* **5**:103doi:10.1186/1475-2875-5-103

Missinou, M., 2005. MIM 2005 Conference PPT presentation. *MIM website mim.su.se/conference2005/eng/presentations/16%20November-Wednesday/83%20Missinou.ppt.*

Mockenhaupt, F. P., May, J., Bergqvist, Y., Ademowo, O. G., Olumese, P. E., Falusi, A. G., Großterlinden, L., Meyer, C. G. and Bienzle, U. 2000. Concentrations of Chloroquine and Malaria Parasites in Blood in Nigerian Children. *Antimicrob Agents Chemother.* **44**(4): 835–839.

Molyneux, M. E., Taylor, T. E., Wirima, J. J. and Borgstein, A., 1989. Clinical features and prognostic indicators in pediatric cerebral malaria: a study of 131 comatose Malawian children. *Q J Med.*, **71**(265): 441-59.

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**(1–2 Suppl.): 57–67.
Newton, C. R. J. C. and Krishna, S., 1998. Severe Falciparum Malaria in Children: Current Understanding of Pathophysiology and Supportive Treatment. *Pharmacol. Ther.*, **79**(1) 1–53.

Nguyen, P. H., Day, N., Pram, T. D., Ferguson, D. J. and White, N. J., 1995. Intraleukocytic malaria pigment and prognosis in severe malaria. *Trans R Soc Trop Med Hyg.*, **89**(2): 200-204.

Odongo-Aginya, E., Ssegwanyi, G., Kategere, P. and Vuzi, P. C. 2005. Relationship between malaria infection intensity and rainfall pattern in Entebbe peninsula, Uganda. *Afr Health Sci.* **5**(3): 238–245.

Oduro, A. R., Koram, K. A., Rogers, W., Atuguba, F., Ansah, P., Anyorigiya, T., Ansah, A., Anto, F., Mensah, N., Hodgson, A. and Nkrumah, F. 2007. Severe falciparum malaria in young children of the Kassena-Nankana district of northern Ghana. *Malaria Journal* 6:96doi:10.1186/1475-2875-6-96.

Petit P. L. and van Ginneken J. K. 1995. Analysis of hospital records in four African countries, 1975-1990, with emphasis on infectious diseases. *Am J Trop Med Hyg.* **98**(4):217-27.

Ralph, B. D., Holleran, S. and Ramakrishnan R., 2002. Sample size determination. *Ilar Journal.* 43(4)

Robergs R. 2007. Hematology A: Hematocrit, Hemoglobin, Relative Change in Plasma Volume. *Website. http://www.unm.edu/~rrobergs/LabVIEW%20Programs.htm*

Scholl, P. F., Kongkasuriyachai, D., Demirev, P. A., Feldman, A. B., Lin, J. S., Sullivan, D. J. jr. and Kumar, N. J., 2004. Rapid detection of malaria infection in vivo by laser desorption mass spectrometry. *Am. J. Trop. Med. Hyg.*, **71**(5) 546-551.

Shute, G. T., 1988. The microscopic diagnosis of malaria. In *Malaria;Principles and practice of malariology* (Wernsdorfer W.H., McGregor I eds)., 781-814

Snow, W. R., Craig, M. H., Newton, C. R. J. C and Steketee, R.W., 2003. *The public health burden of Plasmodium falciparum malaria in Africa: Deriving the numbers.* Working Paper No. 11, Disease Control Priorities Project. Bethesda, Maryland: Fogarty International Center, National Institutes of Health.

Statistical Package for Social Sciences (SPSS) for Windows version 11.5 (Computer Software). 2003. Chicago, IL: SPSS Inc.

Steketee, R. W., Nahlen, B. L., Parise, M. E. and Menendez, C., 2001. The burden of malaria in pregnancy in malaria-endemic areas. *Am. J. Trop. Med. Hyg.*, **64**(1–2 Suppl.): 28–35.

Sturchler, D., 1989. How much malaria is there worldwide? *Parasitology Today*. 5:39-40.

Unpublished 2007. Malaria prevalence rates in Lusaka 2006/2007 season.

Waller, D. and Krishna, S., 1995. Clinical features and outcome of severe malaria in Gambian children. *Clin Infect Dis.*, 21: 577-587.

Watts, T. E., Wray, J. R., Ng'andu, N. H. and Draper, C. C., 1990. Malaria in an Urban and a Rural area in Zambia. *Trans R Soc Trop Med Hyg.* 84 (2): 196-200.

Williams, H. E. 1989. "*Method for measuring hemoglobin*". Inventors: Levine, Robert A. Guilford, CT, US. Wardlaw, Stephen C. Old Saybrook, CT, US. 5 Apr Appl: 24 Oct 1989. 73/61.4, 61.1 R, 149, 53 128/637, 638, 771 422/68, 55, 58, 59 210/927, 787 436/70, 63, 177 494/10, 16 United States Patent 4875364.

World Health Organisation., 2006. W. H. O. Criteria on Severe Falciparum Malaria in - *The Medical Algorithms Project*. Chapter 24. Institute for Algorithmic Medicine, Houston, TX, USA.

World Health Organization/ UNICEF., 2003. *The Africa Malaria Report 2003*. WHO/UNICEF 2003.

Appendix A: W. H. O. Criteria For Severe Malaria

WHO Criteria for Severe Falciparum Malaria

Severe falciparum malaria is defined as:

(1) one or more of the defining criteria below

(2) asexual parasitemia with Plasmodium falciparum (although smear-negative cerebral malaria may occur)

Defining Criteria	**Finding**
cerebral malaria (unrousable coma)	unrousable coma not attributable to any other cause in a patient with falciparum malaria. Coma should persist at least 30 minutes after a generalized convulsion to make the distinction from transient post-ictal coma.
Severe normocytic anemia	normocytic anemia with hematocrit < 15% or hemoglobin < 5 g/dL in the presence of parasitemia > 10 0 parasites per µL. If microcytic indices seen need to consider iron deficiency anemia thalassemia and hemoglobinopathy.
Renal failure	urine output < 400 mL in 24 hours in adults or 12 mL per kg in children failing to improve after rehydration and with serum creatinine > 265 µmol/L (3 mg/dL)
pulmonary edema ARDS	
Hypoglycemia	whole blood glucose < 2.2 mmol/L (< 40 mg/dL)
circulatory collapse shock	hypotension (systolic blood pressure < 50 mm Hg in children 1-5 years old; < 70 mm Hg in adults) with cold clammy skin or a core-to-skin temperature difference > 10 °C
spontaneous bleeding. DIC	spontaneous bleeding from gums nose GI tract or other sites with laboratory evidence of DIC
Repeated generalized seizures	more than 2 observed seizures (>=3) within 24 hours despite cooling
Academia or acidosis	arterial pH < 7.25 plasma bicarbonate < 15 mmol/L
malarial hemoglobinuria	need to exclude hemoglobinuria due to antimalarial medications and to G6PD deficiency
Additional Criteria	**Finding**
Impaired consciousness but rousable	impaired consciousness less marked than unrousable coma can localize a painful stimulus
prostration and extreme weakness	patient unable to sit or walk with no other obvious neurological explanation
Hyperparasitemia	very high parasite densities are associated with increased risk of severe disease but is affected by the immune status (more than 5% parasitemia in non-immune is serious but may be well tolerated in semi-immune children); > 500 0 per µL
Jaundice	total bilirubin > 50 µmol/L (> 3 mg/dL)
Hyperpyrexia	rectal temperature > 40 °C
Post-mortem evidence of severe malaria	neuropathologic evidence of venules and capillaries packed with erythrocytes containing malarial parasites

Risk factors for development of severe falciparum malaria:

(1) splenectomy

(2) pregnancy especially primigravid

(3) immunosuppression

(4) low immunity states: non-immune (lack of previous exposure) especially in small children or lapsed immunity (due to living away from malarious area for several years) (Waller et al, 1995; Warrell et al, 1990; White et al, 1983; White et al, 1987)

Prognostic Indicators for Severe Falciparum Malaria in Children

Poor prognosis - Differences between children and adults with severe malaria:

(1) Common causes of death in adults are acute renal failure and acute pulmonary edema but these are rare in children.

(2) Common causes of death in children are severe anemia and lactic acidosis.

Indicator	Findings indicating a poor prognosis
neurological status	combination of cerebral malaria with coma and extensor posturing
Hypoglycemia	blood glucose < 2.2 mmol/L
Heart rate (tachycardia)	> 150 beats/minute
respiratory rate (tachypnea)	> 50 breaths per minute
Hyperlactatemia	plasma lactate > 5 mmol/L
malarial parasites in peripheral blood	parasite count > 500 0 per µL
	> 40% trophozoites (pigment evident in asexual stage)

where:

• Coma is indicated by a Blantyre coma score of 0 1 or 2.

Additional Laboratory Indicators	Findings indicating poor prognosis
serum urea nitrogen	> 6.4 mmol/L
"corrected" serum calcium	> 2.2 mmol/L
serum potassium	> 5 mmol/L
serum albumin	normal
Liver function tests	elevations of total bilirubin GGT AST

where:

• Serum albumin in survivors is usually mildly decreased.

• How the serum calcium is "corrected" is not stated but is assumed to be for low serum albumin [approximate total serum calcium in mg/dL with normal albumin = (current total serum calcium in

mg/dL) + (0.8 * (4 - (albumin in g/dL)); approximate total serum calcium in mmol/L with normal albumin = (current total serum calcium in mmol/L) + (0.8 * (1 - ((albumin in g/L) /40))] (Kain and Keystone, 1998; Marsh et al, 1995).

Intraleukocytic Malaria Pigment as a Prognostic Marker for Severe Plasmodium falciparum Infection

The presence of malaria pigment within neutrophils and monocytes is a prognostic marker for patients with Plasmodium falciparum infection. This can help identify patients with severe malaria who have an increased risk of death.

Measures of intraleukocytic malaria pigment:

(1) percent neutrophils and monocytes with cytoplasmic pigment

(2) absolute count of neutrophils and monocytes with cytoplasmic pigment

percent neutrophils with cytoplasmic pigment =

= (number of neutrophils with cytoplasmic pigment) / (number of neutrophils counted) * 100%

total number pigmented neutrophils with cytoplasmic pigment per μL =

= (percent neutrophils with cytoplasmic pigment) * (WBC count per μL) * (percent neutrophils in differential count of peripheral blood)

total number pigmented monocytes with cytoplasmic pigment per μL =

= (percent monocytes with cytoplasmic pigment) * (WBC count per μL) * (percent monocytes in differential count of peripheral blood)

where:

- If the percent of monocytes with pigment was > 0 and < 1% then a value of 0.5% was used by Lell et al, 2005 and Lyke et al, 2003. I will not do this in the implementation.

According to Phu et al, 1995, a percent neutrophils with cytoplasmic pigment >= 5% was associated with a fatal outcome with a sensitivity of 73% and specificity of 77%.

According to Lyke et al, 2003, an absolute pigment laden neutrophil count > 324 per μL was associated with cerebral malaria in children in Mali

Appendix C: Clinical Data Form

ASSOCIATION OF INTRALEUCOCYTIC MALARIA PIGMENT AND DISEASE SEVERITY IN CHILDREN STUDY: PATIENT SCREENING/RECRUITMENT CLINICAL DATA FORM A (To be filled in by Study Investigator)

Study ID Number									Investigator's Code:.....
I	M	P				DD	MM	YY	

1.1 Health Facility .....

1.2 Date and Time of Presentation .....

PATIENT DETAILS

2.1 Age [ ] 2.2 Sex [ ] 2.3 Medical File No. [ ] 2.4 Religion/Church [ ]

2.5 Body Weight on Admission [ ] 2.6 Body Temperature [ ]

2.7 Height [ ] 2.8 Z-Score <Weight for Height> [ ] 2.9 If >5 Years old MAC [ ]

PRESENTING SYMPTOMS

3.1 Fever within Seven Days? [ ] 3.2 Fever more than 1 week? [ ]

4.1 Headache within seven days? [ ] 4.2 Headache for more than 1 week? [ ]

5.1 Vomiting within seven days? [ ] 5.2 Vomiting for more than 1 week? [ ]

6.1 Diarrhoea within seven days? [ ] 6.2 Diarrhoea for more than 1 week? [ ]

6.3 Specify type of Diarrhoea [ ]

7.1 Cough within Seven days? [ ] 7.2 Cough for more than 1 week? [ ]

8.1 Single episode of convulsions? [ ] 8.2 Multiple convulsions? [ ]

9.1 Other main complaints? 1 2 3

PRESENTING SIGNS

10.0 Pallor [ ] 11.0Pyrexia [ ] 12.0 Jaundice [ ] 13.0 Respiratory distress [ ]

14.0 Haemoglobinuria [ ] 15.0 Level of Consciousness /Blantyre Coma Scale [ ]

16.0 Splenomegaly [ ] 17.0 Hepatomegaly [ ] 18.0 Generalised Lymphadenopathy [ ]

19.0 Other Presenting Signs 1 2

20.0 Provisional Diagnosis? [ ]

20.1 Severity of Malaria (If malaria is confirmed, state severity; uncomplicated malaria-UM; severe malaria-SM [ ]

20.2 Treatment for Malaria in past 2 weeks? [ ]

Appendix C: Clinical Data Form

ASSOCIATION OF INTRALEUCOCYTIC MALARIA PIGMENT AND DISEASE SEVERITY IN CHILDREN STUDY: PATIENT SCREENING/RECRUITMENT CLINICAL DATA FORM A (To be filled in by Study Investigator)

GUIDE TO THE INVESTIGATORS AND RESEARCH ASSISTANTS

A. STUDY SCREENING CRITERIA

Any Child of between 3 months and 14 Years with;

- Fever of 37.5°C and clinically suggestive of malaria (No foci of URTI, OM nor UTI)

B. STUDY RECRUITMENT CRITERIA

Recruitment inclusion and exclusion criteria

Any Child admitted with a provisional diagnosis of malaria and has;

- Confirmed Positive MP-slide (Cases)
- Negative MP-slide (Controls)

Patient Sampling guide of recruited patients

Closely and randomly match the study groups (control and Case) in

- Age
- Sex

C. KEY FOR CODES (See Table Below)

TERM	CODES/DESCRIPTION
INVESTIGATOR/RESEARCH ASSISTANT'S CODES	
• Mrs Nzooma Shimaponda Mataa- Research Principal Investigator	**PI**
• Dr. Cecilia J Shinondo- Research Supervisor	**RS**
• Dr. James Chipeta- Research Co-Supervisor	**Co- RS**
• Mr. Makasa-Clinical Research Assistant	**CRA**
• Mr. Mbuzi -Study Independent Microscopist	**SIM1**
• Ms Jane C Kabwe -Study Independent Microscopist	**SIM2**
PATIENT PARTICULARS	
• **Age**	**In years and Months**
• **Sex**	**Female (F) and Male (M)**
• **Religion**	**CC(Catholic),PC(Protestant Christian),M(Moslem) or O(Others)**
• **Weight**	**In Kilograms**
• **Height**	**In Centimetres**
• **Temperature**	**In Degrees Centigrade**
• **Mid-Arm Circumference (MAC)**	**In Centimetres of Left MAC**
• **Blantyre Coma scale**	**Score Out of 5 Stages 1,2,3, or 4**

Appendix D: Laboratory Data Form

ASSOCIATION OF INTRALEUCOCYTIC MALARIA PIGMENT AND DISEASE SEVERITY IN CHILDREN STUDY: PATIENT LABORATORY DATA FORM B (To be filled in by Study Investigator and Laboratory Technologist)

Study ID Number	DD MM YY	Investigator: Technologist:
I M P		

1.1 Patient Hospital File:.....

1.2 Date and Time Sample Collected: Date.....Time.....

1.2 Date and Time Sample Received: Date.....Time.....

HAEMATOLOGY (FBC AND DIFFERENTIALS)

2.0 WBC [ ] 2.1 HB [ ] 2.2 HCT [ ] 2.3 RBC? [ ] 2.4 PLT? [ ] 2.5 PCT [ ]

2.6 MCV? [ ] 2.7 MCH? [ ] 2.8 MCHC? [ ] 2.9 RDW? [ ] 2.10 MPV? [ ]

2.11 PDW? [ ] 3.1 ESR? [ ] 3.2 Sickling Test? [ ]

PARASITOLOGY RESULTS

4.0 Positive or negative? [ ] 4.1 If positive which Plasmodium? 4.1a P. falciparum? [ ]

4.1b P.Malariae? [ ] 4.1c P. ovale? [ ] 4.1d P. vivax? [ ]

5.0 Parasitaemia (Parasites/μL)? [ ]

IMMUNOLOGICAL PROFILES

7.1 Total WBC? [ ] 7.2 Lymphocytes%? [ ] 7.3 Monocytes%? [ ]

7.4 Neutrophils%? [ ] 7.5 Basophils? [ ] 7.6 Oesinophils? [ ]

Appendix D: Laboratory Data Form

ASSOCIATION OF INTRALEUCOCYTIC MALARIA PIGMENT AND DISEASE SEVERITY IN CHILDREN STUDY: PATIENT LABORATORY DATA FORM B (To be filled in by Study Investigator and Laboratory Technologist)

HEMOZOIN CHARACTERISTICS

8.1 Pigment containing PMNs %	**8.1a**		**8.2 Pigment containing Monocytes %**	**8.2a**	
	8.1b			**8.2b**	
	8.1c			**8.2c**	
Mean PMNs %			**Mean Monocytes %**		

Total pigmented PMN/mm^3 = (number of pigmented PMNs/500 WBCs) x (absolute WBCs x percent of PMNs). Similarly, total pigmented monocytes/mm^3 = (number of pigmented monocytes/500WBCs) x (absolute WBCs x percent of monocytes).

Appendix E: Research Ethics Committee Approval

THE UNIVERSITY OF ZAMBIA

RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@zamtel.zm

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

3 August, 2006
Ref.: 015-05-06

Ms Nzooma M. S. Mataa
C/O Mr W. Mataa
Stanbic Bank Zambia Ltd
P.O. Box 31955
LUSAKA

Dear Ms Mataa,

RE: RESEARCH PROPOSAL ENTITLED: **"ASSOCIATION OF INTRALEUKOCYTIC MALARIA PIGMENT WITH DISEASE SEVERITY AND OUTCOME IN CHILDREN WITH SEVERE *PLASMODIUM FALCIPARUM* MALARIA"**

The above research proposal was presented to the Research Ethics Committee meeting on 5 July, 2006 where changes were recommended. We would like to acknowledge receipt of the corrected version with clarifications. The proposal has now been approved. Congratulations!

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).

Yours sincerely,

Prof. J. T. Karashani, MB, ChB, PhD
CHAIRMAN

Date of approval: 3 August, 2006 **Date of expiry:** 2 August, 2007

Appendix F: Board of Graduate Studies Approval

THE UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE

Telephone: 252641
Cell: 097 849302

Assistant Dean's Office (PG)
P.O. Box 50110
LUSAKA

3rd May, 2007

Mrs. Nzooma Munkwangu Shimaponda Mataa
Department of Biomedical Sciences
LUSAKA

Dear Mrs. Mataa

Re: GRADUATE PROPOSAL ADJUSTMENTS

Following the Graduate proposal presentation (GPPF) of 31st August, 2006, we wish to inform you that we are now happy that all the recommended corrections to your research proposal have been done. The proposal meets the standard as laid down by the Board of Graduate Studies.

You can proceed and present to the Research Ethics.

Yours faithfully

Mr. K. Bowa
ASSISTANT DEAN POSTGRADUATE

CC: Director, Graduate studies
Dean, School of Medicine
Head of Department, Biomedical Sciences

Appendix G: University Teaching Hospital Approval

The University of Zambia
School of Medicine
Biomedical Sciences Dept
P.O.BOX
LUSAKA

3 August, 2006

REPUBLIC OF ZAMBIA MANAGING DIRECTOR

Approved

The Managing Director
UTH Board of Managemet
LUSAKA

Dear Sir/Madam,

RE: MSC RESEARCH WORK FOR NZOOMA MUNKWANGU SHIMAPONDA MATAA

The above named is a Master of Science student in Medical Parasitology at the University of Zambia in the School of medicine. She is now beginning the research part of her programme. Her research involves studying the intraleucocytic malarial pigment as a marker of disease severity.

I therefore write to request that you grant her admission to all departments of the Children's Wing in the UTH necessary for her work.

Thank you

Dr. C.J. Shinondo
Head – Biomedical Sciences/ Supervisor

UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE
- 3 AUG 2006
HEAD OF DEPARTMENT
DEPARTMENT
OF BIOMEDICAL SCIENCES

c.c. Head – Paediatrics
Assistant Dean - PG

No problem.
To link Dr Schipeta
for support

DEPARTMENT OF PAEDIATRICS
AND CHILD HEALTH
UNIVERSITY TEACHING HOSPITAL
HEAD OF DEPARTMENT

Appendix H: **Lusaka Distirct Management Health Team Approval**

P.O. Box 50827
Lusaka
Tel: +260-1-235554
Fax: +260-1-236429

In reply please quote
No.....

Republic of Zambia

MH/DHMB/.....

MINISTRY OF HEALTH

LUSAKA DISTRICT HEALTH MANAGEMENT TEAM

Date : 26th January 2007

The Health Centre In-Charge
Chilenje ..... Health Centre
P.O. Box 50827
LUSAKA.

Dear Sir,

RE: PRACTICAL ATTACHMENT ~~MR~~/MS ..... NZOOMA MUNKWANGU

Be informed that permission has been granted for the above named student to be attached to your Health Centre for practical/research.

However, this should be done with minimal disruption to the day-to-day activities at the Health Centre.

Attached hereto is the letter from the College for your action.

Your usual cooperation will be highly appreciated.

Yours faithfully,

DR. M. KABASO
CLINICAL CARE MANAGER
FOR/DISTRICT DIRECTOR OF HEALTH-LDHMT

Appendix I: Ministry of Health Approval

All Correspondence should be addressed to the
Permanent Secretary
Telephone: +260 1 253040/5
Fax: +260 1 253344

In reply please quote:

No.....

REPUBLIC OF ZAMBIA

MINISTRY OF HEALTH

NDEKE HOUSE
P. O. BOX 30205
LUSAKA

2nd March, 2007

The Dean – School of Medicine
University of Zambia
LUSAKA

Dear Dr. Shinondo,

RE. AUTHORITY TO CONDUCT MALARIA FIELD STUDY; MASTER OF SCIENCE MEDICAL PARASITOLOGY STUDENTS – UNZA.

We acknowledge receipt of your letter on the above subject.

The Ministry of Health has no objection to your request. You may therefore go a head and conduct your research study. However, the following guidelines need to be adhered to;

1. Consent from patients / clients obtained and ethical consideration taken into account.
2. Picture / reports need to be cleared and seen by Ministry of health prior to use outside the country.
3. Students need to report to Mpulungu and Mpongwe District Directors of Health respectively and work closely with them.
4. Also ensure that all required formalities with the Ethics Committee are followed.

Dr. Victor M. Mukonka
DIRECTOR OF PUBLIC HEALTH AND RESEARCH

Cc. The District Director of Health – Mpulungu
The District Director of Health - Mpongwe