DETERMINATION OF TUMOR NECROSIS FACTOR-α, INTERLUEKIN-10, INTERLUEKIN-12 AND INTERCELLULAR ADHESION MOLECULE -1 LEVELS IN RELATION TO MALARIA SEVERITY IN CHILDREN

by

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A dissertation submitted to the University of Zambia in partial fulfillment of the requirement for the Degree of Masters of Science in Medical Parasitology.

The University of Zambia

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DECLARATION

This dissertation is the original work of **LUNGOWE SITALI**

It has been done in accordance with the guidelines for MSc. in Medical Parasitology dissertations of the University of Zambia. It has not been submitted elsewhere for a degree at this or another University.

Signature.....

Date.....02/07/08.....

CERTIFICATE OF COMPLETION OF DESERTATION

I, LUNGOWE SITALI

Hereby certify that this dissertation is the product of my own work and, in submitting it for my Master of Science in Medical Parasitology programme, further attest that it has not been submitted to another University in part or whole for the award of any programme.

Signature.....

Date 02/07/08

I, DR.....

Having supervised and read this dissertation is satisfied that this is the original work of the author under whose name it is being presented.

I confirm that the work has been completed satisfactorily and is ready for presentation to the examiners.

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CERTIFICATE OF APPROVAL

The University of Zambia approves this dissertation of Lungowe Sitali in partial fulfillment for the requirements for the award of the degree in Master of Medical Parasitology.

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ABSTRACT

Malaria is highly prevalent in sub-Sahara Africa and is the leading cause of morbidity and mortality accounting for approximately one million deaths per annum. Disease severity and parasitaemia have been linked to cytokine levels, but their relationship requires further elucidation. Better understanding of these relationships would bear on the management of malaria.

Potential associations between TNF-α, IL-10, IL-12 and ICAM-1 and malaria disease severity were investigated in the study. Children of ages 12 to 144 months presenting with severe or uncomplicated malaria were assessed as regards to the respective serum cytokine levels at enrollment and 3 days after treatment. There were 102 participants recruited into the study, of which 20 had severe malaria, 55 had uncomplicated malaria and 27 were healthy controls. Parasite densities were also determined using geimsa stained blood thick films. The study was conducted at UTH, Chongwe Clinic, Mpongwe Mission and Mpulungu Hospitals.

The levels of parasitaemia were found to be relatively similar in children with severe malaria and those with uncomplicated malaria. The parasite densities were zero in all the patients after treatment. This study concluded that parasite densities did not correlate to disease severity.

The levels of TNF-α (picogram per milliliter), were measured by ELISA and were found to be higher in patients than in the healthy controls (31.7±3.4/17.41±16-severe/uncomplicated malaria and 8.9±6.67 in the controls). No significant reduction in the levels was observed after three days of treatment. Levels of IL-10 in patients with uncomplicated malaria were not significantly higher as compared to those with severe malaria (602.56±1,032.89 and 409.59±627.39 respectively). When compared to the healthy controls, the levels of IL-10 were significantly higher in the patients (602.56±1,032.89 and 409.59±627.39 (Uncomplicated /severe malaria) and 11.21±28.16 for the controls; P value <0.001). Furthermore, the levels of IL-10, after three days of treatment, significantly reduced. The levels of IL-12 were relatively similar in severe and uncomplicated malaria, and there was no significant difference when compared to the levels in the healthy controls. After treatment, the reduction in the levels of IL-12 was not significant. Finally, ICAM-1(nanograms per milliliter) levels were significantly higher in the patients (447.43 ± 380.53 and 392.60 ±167.38) than in the healthy controls (196.06±125.19), P value < 0.001 and the levels in severe and uncomplicated malaria groups were similar, P value was 0.88. After treatment, the levels of ICAM-1 remained unchanged in uncomplicated malaria, but not significantly reduced in severe malaria.

In addition, there were correlations observed between serum cytokine (TNF-α, IL-10 and ICAM-1) levels and temperature as well as parasite density implying that cytokines may play an important role in the pathogenesis and diagnosis of malaria. These results also showed a significant association between parasite density and TNF-

α (rho=0.23, p< 0.001), IL-10 (rho= 0.59, p< 0.05), IL-12 (rho= 0.21, p <0.05), ICAM-1 and parasite density (rho= 0.45, p=0.001), implying that when the parasite density was high, the mentioned cytokines were high. There was a significant association between TNF-α and Hb (rho= -024, p< 0.05), IL-10 (-0.43, p<0.001), showing that when Hb is low, the named cytokines were high.

In conclusion, the null hypothesis of the study was rejected. This study has demonstrated relatively similar parasite densities in children with severe and uncomplicated malaria. The levels of TNF-α, IL-10, IL-12 and ICAM-1 were significantly raised in malaria as compared to the healthy controls. Finally, the observed correlations between cytokines and parasitaemia as well as temperature are suggestive of the pathogenetic role of cytokines in malaria.

DEDICATION

This dissertation is dedicated to Dad and Mum (Dr and Mrs. Sitali) who have been and continue to be my source of strength and role models, and to my sisters and brothers Liseli, Mubita, Mwangala, Namukolo and Etambuyu who encourage me in all my endeavors.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	Degree Celcius
DHMT	District Health Management Team
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBC	Full Blood Count
g/dl	Grams per deciliter
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
ICAM-1	Intercellular Adhesion Molecule-1
ie	That is
IFN-γ	Interferon gamma
IRBC	Infected Red Blood Cells
IL	Interleukin
LUDHT	Lusaka Urban District Health Team
MIS	Malaria Information System
ml	Millielitre
MOH	Ministry of Health
No	Number
nm/µl	Nanometer per microilitre
NMCC	National Malaria Control Center
P	Plasmodium
Pg/µl	Picograms per microlitre
P	Probability
SD	Standard Deviation
SM	Severe malaria
SMUTH-MRU	School of Medicine-University Teaching Hospital-Malaria Research Unit

TNF-α	Tumor Necrosis Factor- alpha
UNZA	University of Zambia
UM	Uncomplicated Malaria
UTH	University Teaching Hospital
WHO	World Health Organization
%	Percentage
α	Alpha
γ	Gamma
µl	Microliter
Rho	Spearman's correlation coefficient
≥	Greater than
≤	Less than
±	plus or minus

DEFINATIONS

T-helper cells- These are a subset of lymphocytes that play a role in establishing and maximizing the capacity of the immune system.

T-helper type 1 cell (Th1) – T Cell subsets that produce IFN-γ, TNF-α, IL-12 which promote cellular immune system. They maximize the killing efficacy of the macrophages and are involved in the proliferation of cytotoxic CD8 T cells. Interferon gamma promotes the production IL-12 by dendritic cells and macrophages via positive feedback mechanism; IL-12 stimulates the production of IFN-γ in helper T cells, thereby promoting the Th 1 profile.

T-helper type 2 cell(Th2)- T Cell subsets that produce IL-4, IL-5, IL-6, IL-10 and IL-13 that promote humoral immune system by stimulating B-cell into proliferation to induce B-cell antibody class switching and to increase antibody production. Th2 responses promote its own profile by IL 10 inhibiting a variety of cytokines including IL-12 and IFN-γ.

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria infection is highly prevalent in sub-Sahara Africa and is of great public health concern Of the 300 – 500 million annual cases of malaria infection occurring worldwide, about 90% caused by *Plasmodium falciparum* infection occur in sub-Saharan Africa resulting in approximately 1 million deaths mostly in children under five years of age (Obonyo *et al* 2004). As mortality from poorly managed infections increases there is little hope for countries reaching the targets outlined in the Abuja declarations (WHO 2000) Roll Back Malaria.

In Zambia, malaria is a major public health problem accounting for about 45% of all outpatient attendance and 50% of these cases are among children below the age of five years. The National Malaria Control Centre (NMCC) estimates that malaria is responsible for nearly 4.3 million clinical cases and an estimated 50,000 deaths per year, including up to 20% of maternal mortality (Ministry of health, HIMS, 2005).

Malaria predominantly affects pregnant women and children below the age of five. Infant mortality rate is 95/1000 of which 40% is attributable to malaria and maternal mortality rate is 729/100,000 of which 20% is due to malaria (Ministry of health, HIMS, 2005).

The most common parasite species in Zambia is *P. falciparum*, which accounts for 95-98% of all malaria infections (Ministry of health, HIMS, 2005).

The pathogenesis of malaria is complex; it involves cell mediated immunity which is also involved in protection of the infected individual. *P.falciparum* parasites can

induce the production of several cytokines regulating the immune response and possibly influencing the mechanism of clinical outcome (Baptista *et al* 1997). One of the most prominent pathological features of falciparum malaria is the sequestration of infected red blood cells (IRBCs) in small blood vessels, which is considered to be a major cause of Cerebral Malaria. It is clear that several different host molecules, including Intercellular Adhesion Molecule-1(ICAM-1) act as receptors for IRBCs (Matsumotol *et al* 1998)

ICAM-1 is a siaylated glycoprotein with tissue specific differential glycosylation and has a molecular weight ranging between 70 and 120 Kilo Daltons. It is readily induced in various cell types in response to cytokines such as Interferon γ (IFN-γ), Tumour Necrosis Factor-α (TNF-α) and interleukin-1. The latter two cytokines have now been established to be upregulated by exogenous heat stable malaria antigens (Graninger *et al* 1994).

IL-12 is a proinflammatory cytokine that plays an important role in protection. Studies conducted in rodents have shown that administration of IL-12 before infection of mice with *Plasmodium yoelii* of Rhesus monkeys with *P. cynomolgi* provide protection (Hoffman *et al* 1997, Sedegah *et al* 1994).Furthermore, in another study by Stevenson, it was demonstrated that IL-12 induces protection against blood stages of *P. chabaudi* and this is mediated by IFN-γ and TNF-α. (Stevenson *et al* 1995).

On the other hand, TNF-α is an inflammatory cytokine which has been implicated in several cellular and biological changes associated with malaria, including enhancement of the production of cytokines (Kremner *et al* 1995).

It has been established that the levels of certain cytokines and adhesion molecules are high in patients with severe malaria (Gray and Craig 2006, Othoro *et al* 1999, Lyke *et al* 2004), but the relation between these levels and parasitaemia is unclear

1.2 Statement of the problem.

There are a number of aspects of the pathogenesis of malaria that are not clearly understood. Although there are a lot of reports implicating cytokines and adhesion molecules in the pathogenesis, their role is poorly defined. For instance, it is not clear how their levels in malaria infection correlate to malaria severity and parasitaemia. It is also not well elucidated if whether there is any correlation between the levels of ICAM-1 and uncomplicated as well as malarial anaemia. Elucidation and better understanding of these issues will help in understanding the disease pathogenesis and thereby coming up with ways of effectively managing malaria.

1.3 Justification of the study

Understanding the role of cytokines and adhesion molecules in malaria pathogenesis will help in determining malaria disease prognosis. It will further provide insights into the pathology of severe falciparum malaria and herald new therapeutic and diagnostic modalities of the disease.

CHAPTER TWO

2.1 LITERATURE REVIEW

A number of studies have been conducted on TNF-α, IL-10, IL-12 and ICAM-1 levels in malaria, with results showing that they are generally elevated and that they play a role in the pathogenesis, leading to severe malaria and to the clearance of the parasites but the levels of ICAM-1 have not been well elucidated.

In a study conducted in Mali, significantly elevated levels of proinflammatory IL-6, IL-12 and, to a lesser extent, TNF- α in the sera of the severe- falciparum malaria group compared to age-matched healthy children was observed and anti-inflammatory IL-10 was also elevated in severe-malaria group in this study (Lyke *et al* 2006).

In *Plasmodium vivax* infections, the correlation between parasitaemia and serum cytokine levels has been established in a study conducted in Turkey by Zeyrek and group in 2006. Compared to controls, levels of pro-inflammatory cytokines, i.e. IL-1β, IL-6 and IL-12, were significantly higher in patients with parasitaemia. There was a significant positive correlation between serum IL-10 and IL-12 levels and the parasite burden ($r = 0{\cdot}264$, $P = 0{\cdot}024$ and $r = 0{\cdot}264$, $P = 0{\cdot}024$, respectively). Thus, it was concluded that pro-inflammatory response against *P. vivax* gains more importance during periods of increased parasite burden (Zeyrek *et al* 2006). In addition, high fever was correlated with IL-6 and IL-10 levels. Compared to controls, patients with a parasite count greater than 5000/μL had a significantly higher IL-1β and IL-10 levels, while the difference was not significant for patients with a parasite count less than 1000/μL (Zeyrek *et al* 2006). In another study, inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, interferon-gamma (IFN-γ) and IL-6 were highly elevated in acute *Plasmodium falciparum* infections. TNF-α in particular has been associated with cerebral malaria and death in children (Othoro *et al* 1999).

Furthermore, the ratio of IL-6 to IL-10 in plasma also has an effect on the pathogenesis of the disease. Elevated IL-6 to IL-10 ratio in plasma due to relatively IL-10 deficiency predicts fatal outcomes of severe malaria (Perlamann and Trye 2002). IFN-γ, IL-6, IL-10 increased during *Plasmodium falciparum* attack in all children not only with cerebral malaria. In addition, IL-10, a potent anti-inflammatory cytokine increases markedly in severe malaria (White, 2003).Reduced IL-10/IFN-γ ratio has been associated with childhood malaria anemia in areas of high transmissions rates. (Othoro *et al* 1999, White, 2003).

High circulating levels of TNF-α and IFN-γ are more often found in patients with severe malaria than in uncomplicated cases. Extensive deposition of TNF-α, IFN-γ, and IL-1 in organs with massive sequestration (especially in the brain) is more frequently seen in patients who died of cerebral malaria (Chen *et al* 2000).

In many cases, patients with severe malarial anaemia have an increased ratio between the pro-inflammatory cytokine, tumour necrosis factor (TNF)-α, and the anti-inflammatory cytokine, interleukin (IL)-10, and it has been proposed that inflammatory cytokines may be a causative factor for malarial anaemia (Hellebery *et al* 2005)

In addition, patients with severe anemia showed low levels of IL-10, this amount being insufficient to counteract the proinflammatory activity of high concentrations of TNF-α. And Low IL-10/TNF-α ratios are associated with severe anemia, suggesting that IL-10 might play a role in preventing the adverse effects of TNF-α on hematopoiesis. In contrast, higher ratios have been found in children with uncomplicated malaria (Angulo and Fresco 2002, Day *et al* 1999).

On the other hand, Interleukin-12 plays an important role in the adaptive immune response to malaria. Although a correlation with disease severity has been demonstrated, the levels of IL-12 have been found to be paradoxically lower in African children with severe malaria, possibly due to inhibition after phagocytosis

of hemozoin or IL-10 induction. Although the differences were small, IL-12 is reportedly elevated in cases of severe malaria with little significant difference between subsets of severe malaria. The reasons for the lack of IL-1ß elevation and the small TNF- α, and IL-12 elevations may be the result of down regulation by IL-10 (Lyke *et al* 2004).

So far, most of the evidence supporting an immunological involvement in malarial aneamia comes from data related to the role of IL-12 in this pathology. The levels of IL-12, a cytokine that boosts erythropoiesis, are correlated with anemia (Angulo and Fresco 2002). Intercellular adhesion molecule-1 (ICAM-1), binds to the parasite and it is one of the few molecules whose expression is up regulated in malaria. It has been found to be greatest in parasite isolates obtained from patients with cerebral malaria (Gray and Craig 2002).

Recently, a variant of the intracellular adhesion molecule 1 (ICAM-1), $ICAM^{Kilifi}$, has been found more commonly in Kenyan children with severe malaria, although it is not associated with more severe disease in West Africans (Weatherall *et al* 2002). Thus, although these relationships need more work to be well established, it seems likely that genetic variation of both effectors of the immune system and adhesion molecules may play an important role in variable response to *P. falciparum* malaria (Weatherall *et al* 2002).

Under physiological flow conditions, cytoadherence occurs in a stepwise fashion through parasite ligands expressed on the surface of Infected Red Blood Cells(IRBC) and the endothelial receptors CD36, intercellular adhesion molecule-1 (ICAM-1), P-selectin, and vascular adhesion molecule-1. Moreover, rolling on ICAM-1 and P-selectin increases subsequent adhesion to CD36, indicating that receptors can act synergistically. Cytoadherence may activate intracellular signaling pathways in both endothelial cells and IRBC leading to gene expression

of mediators such as cytokines, which could modify the outcome of the infection (May and White, 1999).

Expression of ICAM-1 in the human vascular endothelium can be upregulated by proinflammatory cytokines TNF-α and IL-1α. These cytokines are present at high levels in plasma of malaria infected children and elevated levels are related to disease severity (Matsumotol *et al,* 1998).The marked elevation of ICAM-1 in serum of *P. falciparum* patients may support the concept that dysfunction of the endothelial is vital in pathophysiology of the disease (Maha *et al*, 2001).

In all these cited studies, quantitative analysis of the cytokines and relations with parasitaemia in patients were not emphasized. There is need to examine and evaluate the levels of the cytokines and compare them with parasitaemia of respective patients of various clinical spectra. This will ascertain the importance of cytokine levels in relation to malaria disease pathogenesis and thereby herald novel ways of effectively managing the disease. The variations in human cytokine responses and their link to malaria disease manifestations are the subject of much debate.

2.2 Null Hypothesis

The null hypothesis of this study was: Tumor Necrosis Factor alpha (TNF-α), Interleukin-10 (IL-10), interleukin-12 (IL-12) and intercellular adhesion molecule-1 (ICAM-1) levels are not altered in patients with malaria and with high parasitaemia.

2.3 OBJECTIVES

2.3.1 General objectives

The general objective of this study was to investigate and elucidate whether there is any correlation between cytokine levels, parasitaemia as well as disease severity.

2.4.2 Specific objectives

The specific objectives of the study were:

2.4.2.1 To determine parasite density in patients of varying malaria disease severity.

2.4.2.2 To determine levels of TNF-α, IL10, IL12 and ICAM-1 in patients of varying malaria disease severity

2.4.2.3 To compare the variations across the groups in the levels of TNF-α-, IL-10, IL-12 and ICAM-1 levels and malaria disease parasitaemia and severity.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was a cross- sectional study which was conducted for a period of 1 year from December 2006 to December 2007.

3.2 Study population

The primary study group was children confirmed to have malaria as was determined by attending physicians and confirmed by laboratory evaluation. Samples were collected from 120 children (aged 12 to 144 months)

3.3 Study site

Patients for the study were recruited from the University Teaching Hospital (UTH), Chongwe Clinics in Chongwe District, Mpongwe Mission Hospital in Mpongwe District and Mpulungu Referral Health Centre in Mpulungu District. .

3.4 Inclusion criteria

Children from the ages of 12 months to 144 months were recruited into the study. There were no children aged 134 and 168 months recruited because during the period of data collection children of that age range did not present with fever or did not meet the inclusion criteria. Patients with history of fever proceeding in the past 24 hours or body temperature of 37.5°C or higher with confirmed malaria parasite positive slides as study participants cases and healthy volunteers as study controls. Those without a history of antimalarial treatment in the recent past (at least in the previous two weeks) were also included. Parents/guardians who were willing to have their children undergo voluntary HIV counseling and had their children tested for HIV and those found negative were included in the study. Both verbal and written consent from these patients to participate in the study were sought and only those who consented to the study were recruited into the study.

3.5 Exclusion criteria

Patients excluded from the study were those with any known concurrent chronic illness such as malnutrition, concurrent infection, Tuberculosis or Pneumonia. Patients who were on antimalarial drug treatment in the last two weeks, as well as those not consenting to the study were excluded.

3.6 Sample size

The classical statistical method for determining sample size based on an unknown proportion of markers was used at 95 percent confidence level and 10 percent precision. Since there is no known figure for the prevalence of malaria, a prevalence rate of 50 percent was used to calculate the sample size using the formula below (Mendenhall et al, 1981).

$$n=\frac{Z^2PQ}{d^2}$$

Where n = Sample size

P = Prevalence rate

Z= 1.96 at α= 0.05 (α= desired confidence level)

d= desired width of confidence (precision)

Q= 100- P

Therefore the sample size (n) was determined as:

$$n=\frac{1.96^2 \text{ X } 50 \text{ X } 50}{10^2}$$

$$n= 96.04 \approx 100$$

3.7 Sample collection and processing

Two and half Milliliters (2.5 mls) of venous blood was collected in EDTA containers at the time of recruitment of participants for measurement of cytokines, about 5µl of the blood was used to prepare thin and thick smears. The blood was centrifuged immediately and plasma collected and stored at -40°C until analysis. On the third day blood was collected from the same again, then centrifuged and stored.

3.8 Determination of malaria parasitaemia.

3.8.1 Preparation and staining of thick blood films

About 1µl of blood was collected from the EDTA tubes or from finger pricks and placed on clearly labeled clean slide using the corner of another slide, the blood was spread to make an even thick film. The thick films were allowed to dry protected from flies and dust. The slides were placed in distilled water to dehemoglobinise the prepared smear. A fresh 10 % Giemsa solution in phosphate buffer (pH 7.2) was prepared. The slides were then put into a staining Coplin jar containing the Giemsa solution for 15 minutes. Using running water, the slides were rinsed carefully and gently. The slide were dried in an upright position then examined under oil immersion at 1000 X.

3.8.2 Preparation and staining thin blood films

A single drop of blood was applied onto a clean clearly labeled glass slide. Using a cover slip at an angle of 45°, blood was spread along the slide to make a thin film which film was air dried and fixed in methanol for 30 seconds and dried. A fresh 10 % Giemsa solution in phosphate buffer (pH 7.2) was prepared, and then the slides were stained in a Coplin jar and were left to stain for 30 minutes and rinsed with water. The slides were left in an upright position to dry then examined under oil immersion 1000X.

The thick films were used for parasites density determination and the thin films were used for the identification of malaria parasite species.

3.8.3 Parasites density determination

The method used is based on the number of parasites per µl of blood in a thick film which are counted in relation to a predetermined number of leucocytes. An average of 8000 leucocytes per µl is normally taken as the standard. Two tally counters were used to count parasites and leucocytes separately. In the case where 200 leucocytes were counted and 10 or more parasites would have been identified, results were recorded as parasites per 200 leucocytes. If after 200 leucocytes were counted and 9 or less parasites were counted, counting had to continue until 500 leucocytes were counted and recorded as the number of parasites per 500 leucocytes. In each case, the parasite counts in relation to leukocyte count were expressed as parasites per µl by the following formula:

$$\frac{\text{No. of parasites} \times 8000}{\text{No. of leucocytes}} = \text{No. of parasites per } \mu l$$

(WHO, 1991: Methods in Medical Parasitology)

3.9 Determination of TNF-α IL-10, IL-12 levels, and ICAM-1 expression

TNF-α, IL-10, IL-12 levels and ICAM-1 expression were determined using specific monoclonal antibodies to IL-10, TNF-α, and ICAM-1 using sandwich ELISA at 450 nm wavelength, according to the manufacturer's instructions (R and D systems, catlog numbers; TNF-α-DTA00C ,IL-10-D1000B, IL-12-HS120 and ICAM-1-BBE1B). The details of the ELISA method are as in appendix D.

3.10 Determination of the severity of infection

3.10.1 Full Blood Count (FBC)

FBCs were performed on an ABX Micros analyzer. In order to determine the severity of the disease, Heomoglobin levels were obtained from the Full Blood Count results as follows:

Blood collected in EDTA bottle was used. The required tube holder selection 8 X 40mm was selected for mini vacutainers then the identification mode was selected and patients ID were entered. The sample tube was then installed in the holder and the sample door closed to start the analysis. The analysis cycle lasts 106 seconds

after which the light turned green and the instrument was ready for the next analysis. The results were then printed.

Other manifestations of severe malaria were determined by the WHO classification, by attending physician. Appendix B.

3.11 HIV testing

Two different HIV kits (Abbort Determine ½ and Genie II were used to determine HIV status of children and these tests were run in parallel. Patients were classified as HIV positive if the results of both tests were positive and HIV negative if the results from both tests were negative. The results were classified as indeterminate if they were not being clearly seen. (Genie II HIV-1/HIV-2, Bio-Rad Laboratories). The tests were performed using the manufacturer s instructions.

3.11.1 Determine HIV ½ protocol

Whole blood collected in EDTA bottle was used to perform the test. For each test strip, the protective foil cover was removed. Using a well graduated pipette, 50µl of sample was applied to the sample pad, and then drop of chase buffer was applied within one minute. The results were read after 15 minutes.

The results were interpreted as follows: The test strip has a control window and a patient window. If red bars appeared on both windows then the test was interpreted as positive. If one red bar appeared on the control window then the test was negative and if no bar appeared on the control window, then the test is invalid.

3.12 Ethical consideration and permission

Ethical approval was sought from the University of Zambia-School of medicine Ethics Committee. Individual patients and the legal guardian of each child's consent were sought prior to enrollment and the purpose and benefit of the study were explained. Those who agreed to participate in the study were given consent

forms to sign. Children who were old enough to ascent were allowed to do so only after their parents' approval or consent.

Permission to conduct the study was sought from The University Teaching Hospital Management to conduct the study in the Department of Pediatrics' and Child Health, and from Chongwe, Mpongwe and Mpulungu DHMTs.

3.13 Data analysis

Data was entered in Microsoft excel first then converted and analysed using SPSS 11.0. Differences among the mean concentration of cytokine in various groups were evaluated using Kruskal Wallis and Mann Whikney tests. Correlations between variables were assessed by Spearman's coefficient. Results were expressed as mean (plus/minus) standard deviation.

CHAPTER FOUR

RESULTS

4.1 Study population

There were 102 children recruited in the study. The mean age was 46.41 ± 33.82 months, 55 and 47 were males and females respectively. There were 13 children recruited from UTH, 20 from Chongwe, 54 from Mpongwe and 15 from Mpulungu, as shown in table 1. Children with HIV were excluded and no children were found to have Tuberculosis and Pneumonia.

4.2 Baseline and general findings

The mean age of Children with severe malaria was 44.05± 37.08, and for those with uncomplicated malaria was 45.13± 25.97, while the mean age for the healthy controls was 50.78±44.83, as shown in table 2. The mean weight and height in the severe, uncomplicated malaria, and healthy control groups were 12.75±6.56/88.76±20.99, 13.89±4.8/92.52±17.55 and 15.75±8/96.15±27.16 respectively as shown in table 2. There were no significant differences in the weight and height in the three groups. The mean heomoglobin levels in children with severe malaria were lower (4.33±0.65) as compared to the other groups (9.89±2 uncomplicated malaria and 13.22±1.09 for the healthy controls) and the differences were significant as shown table 2. The mean body temperature was higher in the severe group (38.08±1.21) than in the uncomplicated malaria group (37.68±1.08) and in the healthy control was (36.46±0.34). The difference in temperature between the severe malaria group and uncomplicated malaria groups was not significant.

Table 1: Distribution of study participants by site

Parameter	UTH N=13	Chongwe n=20	Mpongwe n=54	Mpulungu n=15	Overall n=102
Age(months) Range	45.90 (23-132	30.33 (13-126)	71.86 (12-84)	25.44 (19-144)	36.90 (12-144)
Sex ratio(M/F)	9/4	8/11	31/24	7/8	55/47
Severe malaria		0	2	8	20
Uncomplicated malaria	3	10	35	7	55
Healthy controls	0	10	17	0	27

***This data includes normal healthy controls and hence the Temperature range of 36.0-40.2.**

*** Severe malaria was defined as anemia where the Hb was less than or equal to 5g/dl.**

Table 2: Baseline characteristics of the study population

	Severe Malaria n=20	Uncomplicated Malaria n=55	Healthy Control n=27	P-a	P-b	P-c	Overall P-value
Sex ratio(M/F)	9/11	30/25	15/12				54/48
Age(mean/SD) months	44.05± 37.08	45.13± 25.97	50.78±44.83	0.26	0.95	0.34	0.23
Mean weight (Kg)	12.75±6.56	13.89±4.8	15.75±8	0.11	0.13	0.98	0.34
Mean height (Cm)	88.76±20.99	92.52±17.55	96.15±27.16	0.22	0.46	0.54	0.43
Temperature(mean/SD-(Degree celcious))	38.08 ± 1.21	37.68± 1.08	36.46± 0.34	0.34	< 0.001	<0.001	< 0.001
Hb levels (mean/SD-(g/dl))	4.33 ± 0.65	9.89± 2.04	13.22±1.09	< 0.001	< 0.001	<0.001	< 0.001

a- Severe versus Uncomplicated

b- Severe versus Healthy control

c- Uncomplicated versus Healthy control

P- Probability

4.3 Presenting signs and symptoms of the study participants

Table 3 shows the percentages of patients that presented with the listed signs and symptoms. Of the 20 children with severe malaria, eighty percent (80%) of them presented with fever for equal to or less than 7 days duration and twenty percent (20%) had fever for more than 7 days duration. Furthermore, 52.9% and 54.5% of the children with severe and uncomplicated malaria, respectively had headache for less than 7 days duration. Most of the recruited patients were fully conscious except 10 % of the children with severe malaria slightly unconscious as the coma scale was 3, refer to appendix B for the levels of consciousness.

Table 3: Presenting signs and symptoms for the study participants

Clinical Presentation	Severe N=20	Uncomplicated n=55	Control n=27
	No (%)	No (%)	No (%)
Mean Temperature	38.09	37.7	36.46
Fever<7 days	16 (80)	52 (94.5)	0 (0)
Fever>7 days	5 (20)	4 (7.3)	0 (0)
Headache<7 days	9 (52.9)	30 (54.5)	0 (0)
Headache>7 days	2 (11.8)	3 (5.5)	0 (0)
Vomiting<7 days	12 (60)	14 (25.5)	0 (0)
Vomiting>7 days	0 (0)	2 (3.6)	0 (0)
Pallor	18 (90)	15 (27.3)	0 (0)
Pyrexia	20 (100)	32 (58.2)	0 (0)
Respiratory Distress	4 (20)	2 (3.6)	0 (0)
Level Of Consciousness -3	2 (10)	0 (0)	0 (0)
Level Of Consciousness -4	9 (45)	5 (9.1)	0 (0)
Level Of Consciousness -5	9 (45)	50 (90.9)	27(100)

4.4 Parasite density

4.4.1 Parasite density in all study patients

Parasite density was determined in the thick Giemsa stained blood films, and figure 1 shows the number of children with low, moderate and high parasitaemia as described in the legend. The majority of the children (65.8 %) had moderate levels of parasitaemia and those with low and high parasitaemia were 20 % and 14.2 % respectively.

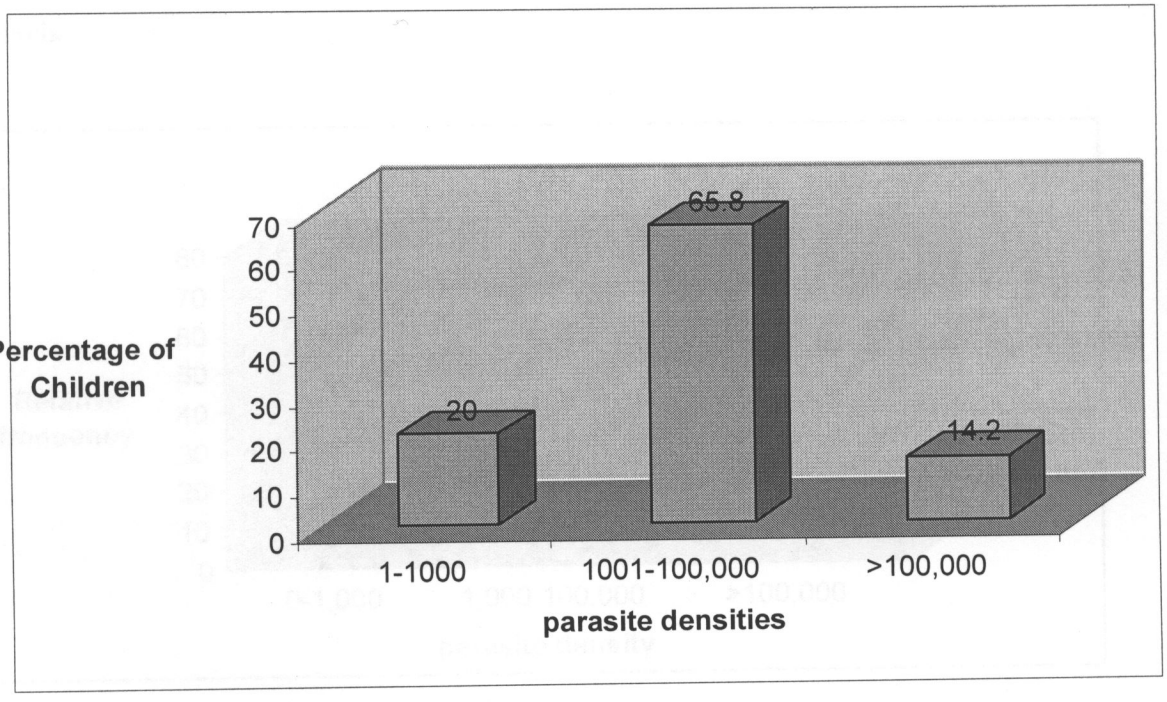
4.4.2 Parasite density in children with severe and uncomplicated malaria

Figure 2 shows that there were more study subjects with moderate parasiteamia with 80% of them being children with severe malaria. Of the 55 children with uncomplicated malaria, 58.2% had moderate parasiteamia. Only 10 % of children with severe malaria and 16.4 % with uncomplicated malaria had high parasitaemia. On the other hand, 10% of those with severe malaria and 25.5% of those with uncomplicated malaria had low parasitaemia.

4.4.3 Heomoglobin levels and parasite density

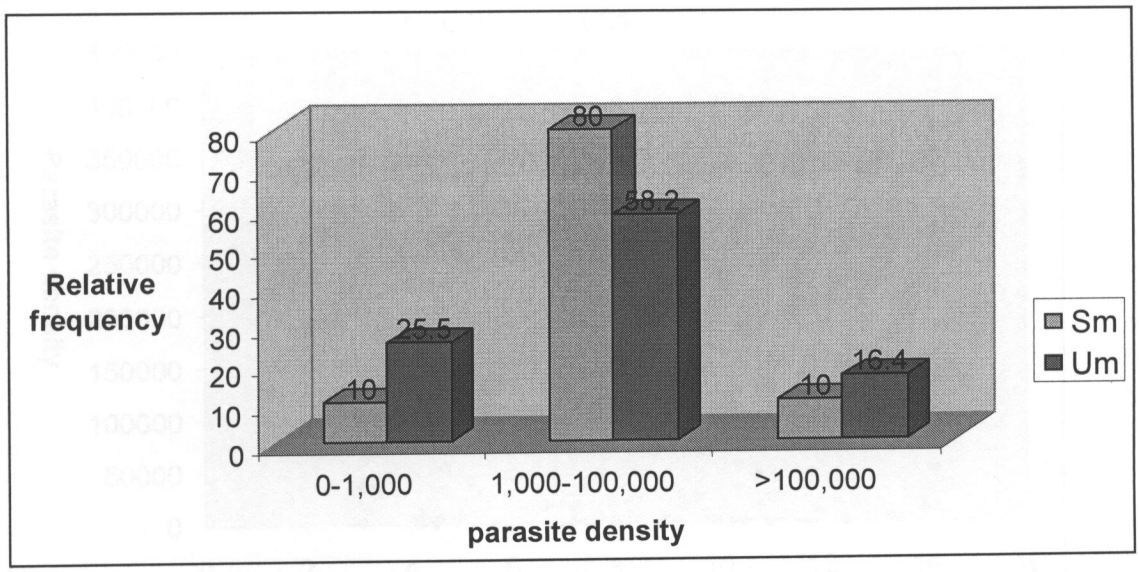
Figure 3 shows the relationship between Haemoglobin levels and parasite densities. There is no relationship between Hb and parasite density as the coefficient of correlation was -0.06 which was far from 1, the correlation was very weak. Most participant had parasite density less than or equal to 5000.

Figure 1: Parasite density in children with malaria in the study population



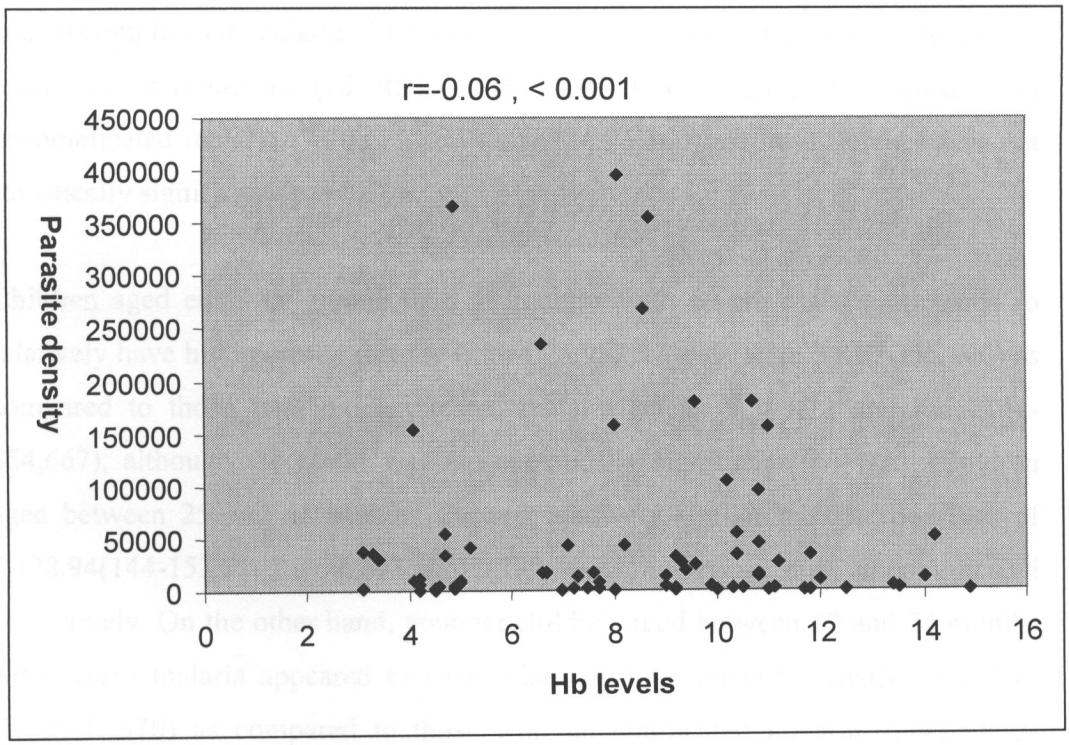
Parasite densities were determined in thick Giemsa stained blood films. The parasite densities were grouped as follows: 0-1,000- low parasiteamia, 1,000-100,000-moderate parasiteamia, >100,000-high -parasiteamia, Sm – Severe malaria, Um- Uncomplicated malaria

Figure 2: Parasite densities in the children with severe and uncomplicated malaria



Parasite densities were determined in thick Giemsa stained blood films. The parasite densities were grouped as follows: 0-1,000- low parasiteamia, 1,000-100,000-moderate parasiteamia, >100,000-high -parasiteamia, Sm – Severe malaria, Um- Uncomplicated malaria

Relation between Hb levels and parasite density



4.4.4 Mean parasite densities in severe and uncomplicated malaria

Table 4 shows the geometric mean parasite densities in the children with severe and uncomplicated malaria. Children with severe malaria had relatively similar levels of parasitaemia (10,545.34 and range 144-356,200) with those with uncomplicated malaria (7,708.79 and range32-395,360) as the difference was not statistically significant (p = 0.68).

Children aged equal or greater than 49 months with severe malaria appeared to relatively have high parasite density (mean 25,957.21 and range 3,890-356,200) as compared to those with uncomplicated malaria (mean 9,595.72 and range144-354,667), although the result was not statistically significant(p =0.6). Children aged between 25 and 48 months showed relatively similar parasite densities of 4,128.94(144-153,710) / 7,393.59(32-395,360) in severe and uncomplicated respectively. On the other hand, younger children aged between 12 and 24 months with severe malaria appeared to have relatively high parasite density 13,359.94 (1,920-41,370) as compared to those with uncomplicated malaria 7,393.59(32-395,360) although the result was not statistically significant.

Table 4: Mean parasiteamia in severe and uncomplicated malaria by age

	Severe malaria	Uncomplicated malaria	P
Parasiteamia (geometric mean and range)	10,545.34(144-356,200)	7,708.79 (32-395,360)	0.68
12-24 years	13,359.94(1,920-41,370)	6,148,66(64-178,070)	0.36
25-48 years	4,128.94(144-153,710)	7,393.59(32-395,360)	0.31
>49 years	25,957.21(3,890-356,200)	9,595.72(144-354,667)	0.6

4.5 Cytokines levels in the study participants

Pre-treatment (day zero) levels of TNF-α (picogram per millitre) were significantly higher in patients than in healthy controls (31.70±34.03/ 17.41±16.45-severe/uncomplicted) and 8.9± 6.67 in healthy controls as shown in table 5. IL-10 levels were slightly high in the uncomplicated malaria group (409.59± 627.39) as compared to the severe malaria group (602.56± 1,032.89), and the observed difference was not statistically significantly. The levels of IL-10 in patients were significantly higher than the levels in the healthy controls (11.21 ± 28.16 and p =<0.001). IL-12 levels were almost similar in the severe and uncomplicated malaria groups 3.82 ± 10.6/3.23 ± 5.89 respectively, p value: 0.44) but the levels in the patients were significantly higher than the levels in the control group (0.83±1.35). On the other hand, the levels of ICAM-1 (in nonagrams per milliliter) were the same in severe malaria and in the uncomplicated malaria groups (447± 380.53, 392.60±167.38 respectively p=0.88), as the difference was not significant. However, ICAM-1 levels were statistically significant higher in patients than healthy controls (447± 380.53 for severe malaria, 392.60±167.38 for uncomplicated and 196.06±125.19 for healthy, p< 0.001).

After treatment (day 3), the levels of TNF-α, IL-10 and IL-12 were reduced in the severe and uncomplicated malaria. ICAM-1 levels in uncomplicated malaria group remained the same as shown in table 5.

Table 5: Baseline and day 3 cytokine results for the study participants

Parameter/Case	Severe Malaria	Uncomplicated Malaria	Healthy controls	P a	P b	P c	Overall P-value
TNF-α (mean/SD)D0	31.70± 34.03	17.41± 16.45	8.9± 6.67	0.88	< 0.001	< 0.001	< 0.001
TNF-α (mean/SD)D3	16.16± 10.65	15.84± 40.70	N/D	0.039	N/D	N/D	< 0.001
IL-10 (mean/SD)D0	409.59± 627.39	602.56± 1,032.89	11.21± 28.16	0.26	< 0.001	< 0.001	< 0.001
IL-10 (mean/SD)D3	118.89± 141.90	77.18± 135.22	N/D	0.79	N/D	N/D	0.79
IL-12 (mean/SD)D0	3.82± 10.6	3.23 ± 5.89	0.83±1.35	0.44	< 0.009	<0.016	<0.001
IL-12 (mean/SD)D3	1.12 ± 2.34	1.73 ± 3.9	N/D	0.54	N/D	N/D	0.54
ICAM-1 (mean/SD)D0	447± 380.53	392.60± 167.38	196.06±125. 19	0.88	<0.001	<0.001	<0.001
ICAM-1 (mean/SD)D3	236.43± 217.32	373.15± 165.42	N/D	0.22	N/D	N/D	0.22

Significant p values determined by Kruskal Wallis test

a- Severe versus Uncomplicated

b- Severe versus Healthy control

c- Uncomplicated versus Healthy control

4.6 Test for associations between study variables

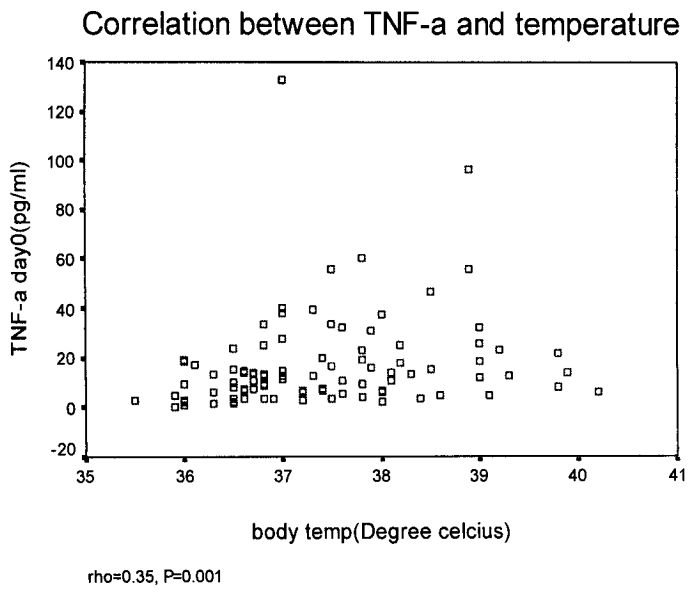
Table 6: Spearman's Correlation coefficient for cytokines with Body temperature, parasite count, Hb levels and Coma scale in study population.

Table 6, is a summary of the correlation coefficients and the P-values of different correlations as determined by the scatter plots as exemplified in figures 4a-e. The correlation coefficients ranged from ± 0.002 to 0.69. Significant association and correlations were observed in Temperature and TNF-α (rho=0.35, < 0.001), and IL-10 (rho=0.47, p< 0,001), showing that temperature increases with increased cytokines. These results also showed a significant association between parasite density and TNF-α (rho=0.23, p< 0.001), IL-10 (rho= 0.59, p< 0.05), IL-12 (rho= 0.21, p <0.05), ICAM-1 and parasite density (rho= 0.45, p=0.001), implies that when the parasite density was high, the mentioned cytokines were high. There was a significant association between TNF-α and Hb (rho= -024, p< 0.05), IL-10 (-0.43, p<0.001), showing that when Hb is low, the named cytokines were high.

	Temperature		Parasite		Hb		Coma	
Cytokine	rho	P	rho	P	rho	P	rho	P
TNF-a	0.35	0.001	0.23	0.029	-0.39	<0.001	-0.04	0.74
IL-10	0.47	<0.001	0.59	<0.001	-0.43	<0.001	0.13	0.21
IL-12	0.028	0.82	0.21	0.038	0.01	0.92	0.11	0.29
ICAM-1	0.19	0.18	0.45	0.001	-0.34	0.012	-0.22	0.12

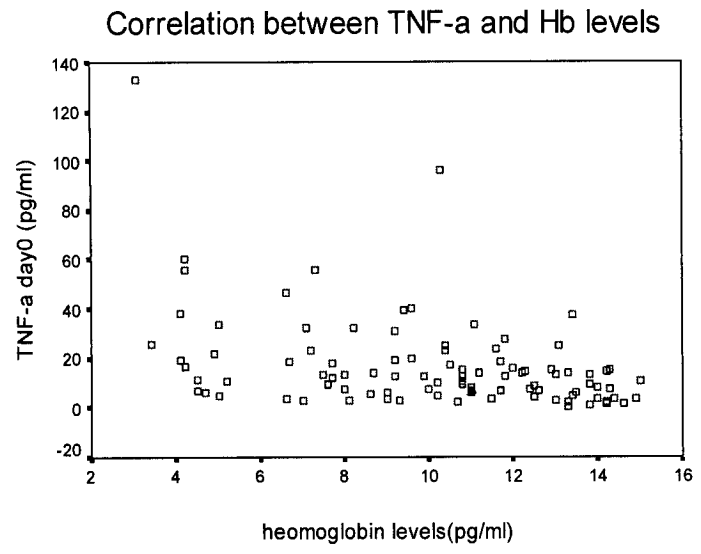
Some scatter graphs showing correlation.

Figure 4a



rho=0.35, P=0.001

Figure 4b



rho=-0.39, P<0.001

Figure 4c

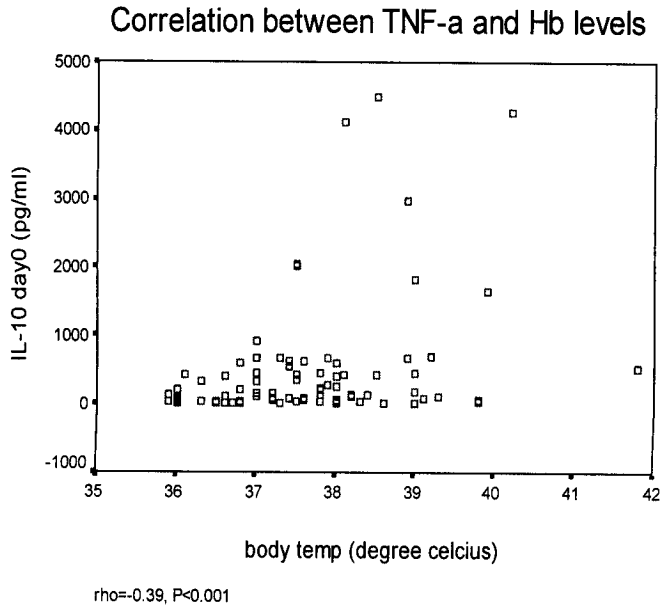


Figure 4d

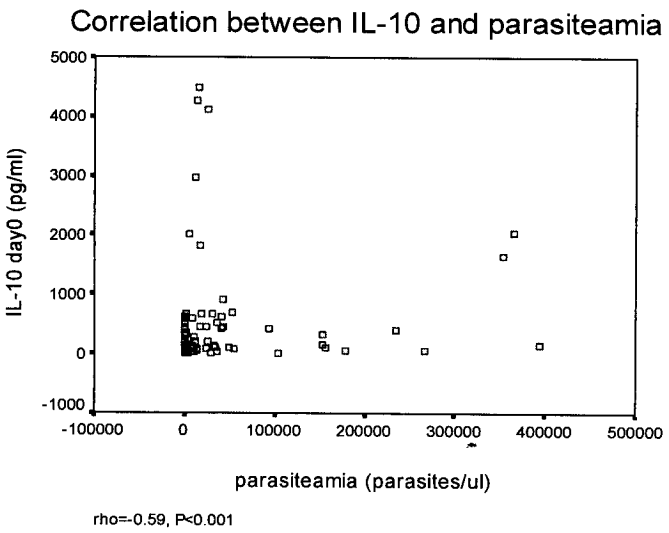
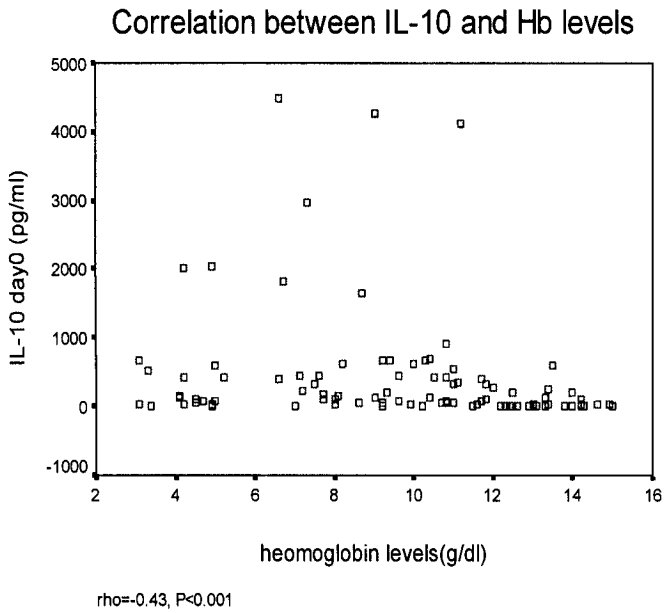


Figure 4e


Correlation between IL-10 and Hb levels
IL-10 day0 (pg/ml)
5000
4000
3000
2000
1000
0
-1000
2
4
6
8
10
12
14
16
heomoglobin levels(g/dl)
rho=-0.43, P<0.001

CHAPTER FIVE

DISCUSSION

As reported elsewhere, cytokines play a key role in the course and outcomes of malaria (Day et al 1999, Zeyrek et al 2006). Despite the fact that there is a lot of literature describing the involvement of cytokines in malaria, the levels and association of certain cytokines and adhesion molecules with disease severity require more studies. In this study we have addressed and investigated some of these.

The levels of parasiteamia were found to be relatively similar in children with severe malaria and those with uncomplicated malaria since the difference was not statistically significant. When grouped into high, moderate and low parasiteamia, more children had moderate levels of parasiteamia and the majority was those with uncomplicated malaria. When the study participants were grouped by age, children aged 49 months and above with severe malaria and those with uncomplicated malaria were found to have relatively similar parasite densities; this was so in younger ones aged between 12 and 24 months, although the differences were not statistically significant. From these finding, it can be concluded that parasite densities do not always correlate to disease severity as found by Issifou and colleagues (Issifou et al, 2003).

The levels of cytokines were generally found to be elevated in malaria infected participants. TNF-α levels were relatively elevated in children with malaria compared to the healthy controls, though not statistically significant, these levels were reduced after three days of treatment. Children aged 25 to 48 months had higher levels of TNF-α as compared to younger children, although the difference was not statistically significant. The study's findings were consistent with Othoro and colleagues' findings (Othoro *et al* 1999). This could be because TNF-α

appears necessary for inhibition of parasiteamia (Clark *et al* 1988) and stimulation of phagocytosis to enhance clearance of parasitised erythrocytes (Newsome F 1984) leading to the clearance of parasites by the third day.

On the other hand, the anti-inflammatory cytokine, IL-10 was slightly elevated in children with uncomplicated malaria than in those with severe malaria although the difference was not statistically significant and after commencement of treatment on the third day the levels had significantly reduced. Older children with uncomplicated malaria had much greater elevated level of IL-10 than children in the other age groups. The younger children appear not to maintain IL-10 levels in response to an inflammatory process hence probably the observed relative lower levels of the cytokine compared to their older counterparts. This in turn may explain the younger children's relative higher susceptibility to anemia as pointed in literature (Nussenblatt V *et al* 2001) as they do not mount enough Th2 response to counteract the suppressive erythropoiesis effects of TNF-α.. The higher levels of IL-10 in older children could be due to their ability to mount a strong T-helper type 2 responses quiet early in infection. Furthermore, IL-10 has an important role in immunoregulation, downregulation of cytokine production (predominantly TNF-α, IL-6, and IL-12), which have Th 1 function as a result Th1 functions are inhibited and natural killer cell activity is promoted (Lyke *et al* 2004).

Furthermore, the pro-inflammatory cytokine, IL-12 which plays an important role in the adaptive immune response to malaria was found to be relatively similar in severe and uncomplicated malaria. Although the difference was very small and not significant, after treatment there was an insignificant reduction. In addition, the levels were high in the younger children aged 24 to 48 months with severe malaria. The study findings are in contrast with findings from two studies by Malaguarnera and colleagues as well as Zeyrek colleagues where the levels of IL-12 were found to be paradoxically lower in African children with severe malaria (Malaguarnera L *et al* 1998 and Zeyrek *et al* 2006) possibly due to

inhibition after phagocytosis of hemozoin or IL-10 induction. In a study by Lyke et al, it was found that IL-12 was elevated in cases of severe malaria with little significant difference between subsets of severe malaria. The possible explanation for the observed low levels of IL-12 in our study and in other studies could have been due to the down regulation by IL-10 which was elevated in all age groups. On the other hand, IL-12 production by monocytes can be inhibited following phagocytosis of small amount of hemozoin, but in our study it was not inhibited implying phagocytosis did not play an important role as reported elsewhere (Luty *et al* 2000).

According to reports in the literature, the levels of ICAM-1 have mainly been associated and extensively studied in cerebral malaria. In our study we did not have any case of cerebral malaria and we investigated the levels in severe anaemia and uncomplicated malaria. The levels of ICAM-1 were found to be relatively similar in children with aneamia compared to those with uncomplicated malaria. Generally the levels in patients were significantly higher than the levels in the control group. Older children with severe malaria had relatively higher levels of ICAM-1 compared to the other groups. This high expression may be attributed to the high parasitaemia density observed in these children and there was correlation between ICAM-1 and parasiteamia. The upregulation of ICAM-1 expression in the acute phase of falciparum malaria can be attributed to an increase in cytokine release, but whether it has a pathological role has not yet been established (Graninger *at el* 1994).After treatment, there was a slight reduction that was not significant.

In this study we assessed the correlation of the studied cytokines with different parameters to establish any associations with the respective disease pathology and clinical presentation so as to investigate their possible usefulness as indicators of disease severity in malaria diagnosis. One of the indicators of disease severity is anemia of equal or less than 5g/dl. There was negative

correlation between Hb and TNF-α (-0.39, p <0.05) supporting our earlier suggestion that TNF-α suppresses erythropoiesis thus contributing to anemia and is consistent with Perkin at el s finding that high TNF-α may promote deleterious effects like severe anemia Perkin *at el* (2000). There also was a negative correlation between Hb and IL-10 (-0.43, p=<0.05) and between ICAM-1 and Hb (rho= -0.34, p<0.05) implying that when the Hb is low, the cytokines are raised and thus can be used for diagnosis.

Parasite density showed correlations with cytokines which were statistically significant. The correlation shown between parasite density and all the cytokines investigated is consistent with the study by Zeyrek and colleagues. In this study conducted by Zeyrek and colleagues in turkey, in *P.vivax* malaria, there was a strong correlation between parasite density and IL-10, IL-12 (Zeyrek at el 2006). Similar findings were observed in another study conducted in Gabon by Issifou and colleague where they found positive strong correlation between parasite density and IL-10 together with TNF-α (Issifou *et al* 2003) This could be explained by the different age group that was studied, the study recruited children aged 6 months to 57 months who have less exposure to malaria or had never been exposed to malaria.

Furthermore, temperature and TNF-α as well as IL-10 showed strong positive correlation. In contrast, there were very weak correlations between these parameters with IL-12 and ICAM-implying that there is no association between them. Studies in cytokines have a number of limitations: Cytokine levels have been demonstrated to vary based on circadian rhythms and with the time course of malaria illness (Lyke *at el* 2004). Secondly, most cytokines are cleared quiet rapidly and have a half life of a few minutes and the circulation levels are influenced by the time of sample is collection. Thirdly, in our study we were unable to rule out or diagnose concomitant bacterial infection, schstosomiasis that could alter the level of cytokine and myocardial, idiopathic lung fibrosis that

could alter ICAM-1 expression. Nevertheless, as also reported elsewhere (Day *at el* 1999) in this study, children were treated with antimalarial alone implying that the sole cause of the symptoms was malaria.

CHAPTER SIX

CONCLUSION

In conclusion, the null hypothesis of the study which states that, Tumor Necrosis Factor alpha (TNF-α), Interleukin-10 (IL-10), interleukin-12 (IL-12) and intercellular adhesion molecule-1 (ICAM-1) levels are not altered in patients with malaria and high parasitaemia, was rejected. This study has demonstrated relatively similar parasite densities in children with severe and uncomplicated malaria. The levels of TNF-α, IL-12, IL-10 and ICAM-1 were observed to be raised in malaria compared to the healthy controls. After commencement of treatment, levels of TNF-α and IL-12 were reduced by day three, though statistically insignificant, while ICAM-1 was unchanged in the uncomplicated malaria. Finally, the observed correlations between cytokines and parasitaemia as well as temperature are suggestive of the pathogenetic role of cytokines in malaria.

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APPENDIX A

Normal range for Cytokines

ICAM-1	147.8 ± 57 ng/mL
IL-10	9.2 ± 1.5pg/mL
IL-12	31.3 pg/mL
TNF-α.	10.1 ± 2.4 pg/mL

Values from Horst Ibelgaufts' cope: Cytokines and Cell online pathfinder Encyclopaedia, range for IL-12 was not given in the literature.

APPENDIX B

World Health Organisation Definitions of Severe malaria

Severe manifestations and complications of *P. falciparum* malaria
In a patient with falciparum malaria in whom other diseases have been excluded, the presence of one or more of the following manifestations is sufficient for a diagnosis of severe falciparum malaria.

Malaria Case Classification	**Definition**
1. Cerebral malaria	Unarousable coma (Blantyre Coma scale-Score Out of 5 Stages 1,2,3, or 4
2. Severe Anaemia	Hemoglobin < 3.1 mmol/l or 5g/dl
3. Metabolic acidosis	Blood pH of <7.35 or plasma bicarbonate concentration of < 22 mmol/L
4. Renal failure	Urine output of < 400 ml in 24 hrs or < 12ml/kg per 24 hrs
5. Pulmonary Oedema	Breathlessness, bilateral crackles
6. Hypoglycaemia	Blood glucose concentration of less than 2.2 mmol/l or < 40 mg/dl)
7. Haemoglobinuria	Black water fever
8. Circulatory Collapse	Systolic BP < 50 mmHg in children 1-5 years or <70 in older ones
9. Hyperpyrexia	Temperature above 40^0C
10. Convulsions	> 2 seizures in 24 hours with regaining of consciousness
11.Disseminated Intravascular Coagulation	Bleeding and clotting disturbances

APPENDIX C

CONSENT FORM FOR PARTICIPANTS IN RESAERCH

STUDY TITLE: DETERMINATION OF TUMOR NECROSIS FACTOR-α, INTERLEUKIN-10, IL-12 AND INTERCELLULAR ADHESION MOLECULE-1 LEVELS IN RELATION TO MALARIA PARASITAEMIA IN CHILDREN.

INTRODUCTION: You are being asked to have your child/ dependant participate in the research study that has been mentioned above. We would like you to be conversant with the benefits, risks and what is expected of you before you decide to take part in the study. The consent form gives information about the study procedure. Once you understand the study and you consent to take part, you will be asked to sign or put your thumb print where indicated in the presence of a witness.

Please note that:

- Your participation in this research is entirely voluntary.
- You may decide not to have your child or dependent participate or to withdraw from the study at any time without losing the child's standard medical care.

PURPOSE OF STUDY

Malaria causes more than 1 million deaths annually worldwide and in Zambia it is the major public heath problem causing death mostly in children below the age of five years and pregnant women. The purpose of this study is to find out if the levels of protein called cytokines and Intercellular adhesion molecules, which tend to change when the body has an infection correlate to the numbers of parasites observed on a blood smear. This will help us in coming up with better ways of managing malaria.

The research will enroll about 330 patients suffering from malaria; children between the age of 1 and 14 years. The participants' participation in the study will last for the duration he/ she will be under treatment at the University Teaching Hospital and at Chipata, Chongwe Clinics and Mpongwe Mission Hospital at the time of recruitment and if not admitted, you will be asked to bring the child on the third day and in this case the study will bear this visit cost for you. If you are interested in participating to this study, you will need to meet the requirements of the enrollment. You will be asked to give your child's blood to be tested for malaria, cytokine and ICAM-1 levels and HIV status.

If the child meets these study requirements and you agree to participate in the study you will be asked to sign or put your thumbprint on the consent form, which will be given to you by study staff in the hospital.

STUDY PROCEDURE

All enrolled Children with fever/ malaria or without fever/ malaria will be recruited into the study. After recruitment, blood sample will be taken from the patients to check for the presence or absence of malaria parasites and for the levels of cytokines and ICAM-1 on the day of admission and on the third day. The procedure for collecting the blood sample involves placing a needle in the vein then slowly drawing blood in to a syringe. The blood to be collected will not be more than a teaspoon (2.5 ml). All results shall be treated with utmost confidentiality and will be communicated to you.

POSSIBLE BENEFITS

There are no direct benefits to your child but you will know your child's condition well and you will be given appropriate medication.

You will not pay for any test or investigation carried out during the study.

RISKS/ DISCOMFORT

Some people may experience little pain or discomfort when blood is drawn and may even feel dizzy. The patient may have a bruise or a swelling at the site of blood drawing.

The emotional and psychological effect of an HIV test result will be dealt with by the standard Voluntary Counseling and Testing or Post Testing Counseling provided routinely in the respective centers.

RESEARCH RELATED INJURY

We do not expect your child to be injured or harmed as a result of participating in the study, but in the case of injury, standard hospital care will be given to your child. The research study will provide the child with the necessary treatment at no cost.

Please note that throughout the study period, care will be taken to respect your child's rights. Signing consent form does not mean loss of rights.

PERSONS TO CONTACT

In case you would like to have more information about this study or you have any questions concerning the study, at any time, please feel free to contact Ms Lungowe Sitali, the principle investigator of the study, at telephone number 097 426711 or 095 426711 or The Secretary, Research Ethics Committee, Telephone 01- 256067.

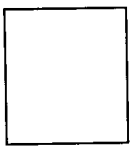
SIGNATURES

If you have read the information ascent or heard it read to you and you have understood the information, voluntarily agree to have your child/dependant participate in the study, please sign your name or put in your thumbprint.

Name of child: _____

Parent/ Guardians name: _____

Parent/ Guardians signature: _____

Or thumbprint 

Date: _____

Witness' Name: _____

Witness' signature: _____

Date: _____

APPENDIX D

Protocols used in the study

IL-10 ELISA protocol

SAMPLE PREPARATION:

The samples were thawed immediately before the assay

REAGENT PREPARATION

All reagents were brought to room temperature before use.

The wash buffer was warmed to room temperature and mixed gently until crystals had completely dissolved then diluted 20 mL of the Wash Buffer Concentrate into 480 mL distilled water to prepare 500 mL of Wash Buffer. Furthermore, Substrate Solution was prepared by mixing Color Reagents A and B together in equal volumes within 15 minutes of use and protect from light.

IL-10 Standard was reconstituted with 1.0 mL of distilled water producing a stock solution of 5000 pg/mL then allowed to sit for 15 minutes with gentle agitation prior to making dilutions.

Eight 1.5 eppedorf tubes were clearly labeled and Pipette 900 µL of Calibrator Diluent RD6P into the first tube which was 500 pg/mL tubes and 500µL of the Calibrator Diluent RD6P was pipetted into the remaining tubes. Using the stock solution serial dilution of (250 pg/mlL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL and 7.8 pg/mL) were produced by transferring 500µL from one tube to the next and mixing each tube thoroughly before the next transfer. The 500 pg/mL standard served as the high standard. The appropriate Calibrator Diluent served as the zero standard (0 pg/mL).

Assay Procedure

Samples were recorded on a plate layout that was provided

Excess microplate strips were removed from the plate frame then they were returned to the foil pouch containing a desiccant pack and resealed. 50µL of Assay Diluent RD1W was added to each well then 200µL of Standard and samples were added per well following the suggested plate layout and covered with the adhesive strip. The plate was incubated for 2 hours at room temperature.

After incubation each well was aspirated and washed, the process was repeated three times for a total of four washes. Washing was performed by filling each well with 400µL Wash Buffer using a squirt bottle. After the last wash, any remaining Wash Buffer was removed by aspirating by inverting the plate and blotting it against clean paper towels.

200µL of IL-10 Conjugate was added to each well then covered with a new adhesive strip and incubated for 2 hours. After the incubation, the plate was washed as before. 200µL of Substrate Solution was added to each well then incubated for 30 minutes at room temperature protected from light by putting plate in the foil pack and sealing. After that, 50µL of Stop Solution was added to each well, and then the optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm.

IL-12 ELISA protocol

Sample collection and storage

The samples were thrawed immediately before the assay

Reagent preparation

All reagents were brought to room temperature before use.

The Wash Buffer was warmed to room temperature and mixed gently until the crystals had completely dissolved 100 mL of the Wash Buffer Concentrate was diluted into 900 mL distilled water to prepare 1000 mL of Wash Buffer. The

Substrate Solution prepared by dissolving the lyophilized Substrate in 6 mL of Substrate Diluent at least 10 minutes before use and mix thoroughly. The rubber stopper was discarded after reconstitution to avoid contamination.

Amplifier Solution was prepared by dissolve the lyophilized Amplifier in 6 mL of Amplifier Diluent at least 10 minutes before use and mixed thoroughly, again here the rubber stopper was discarded after reconstitution.

IL-12 Serum Standard was reconstituted with 4 mL of Calibrator Diluent RD6P. This reconstitution produced a stock solution of 50 pg/mL. The standard was Allowed to sit for 15 minutes with gentle agitation prior to making dilutions.

Eight 1.5 eppedorf tubes were clearly labeled and Pipette 500 µL of Calibrator Diluent RD6P into the first tube which was 25 pg/mL tubes and 500µL of the Calibrator Diluent RD6P was pipetted into the remaining tubes. Using the stock solution serial dilution of (12.5 pg/mL, 6.25 pg/mL, 3.125 pg/mL, 1.562 pg/mL and 0.781 pg/mL) were produced by transferring 500µL from one tube to the next and mixing each tube thoroughly before the next transfer. The 50 pg/mL standard served as the high standard. The appropriate Calibrator Diluent served as the zero standard (0 pg/mL).

Assay procedure

Samples were recorded on a plate layout that was provided

Excess microplate strips were removed from the plate frame then they were returned to the foil pouch containing a desiccant pack and resealed. 50µL of Assay Diluent HD1-5 was added to each well and mixed well since it contained a precipitate. 200µL of Standard and samples were added per well following the suggested plate layout and covered with the adhesive strip. The plate was incubated for 3 hours at room temperature.

After incubation each well was aspirated and washed, the process was repeated three times for a total of four washes. Washing was performed by filling each well with 400μL Wash Buffer using a squirt bottle. After the last wash, any remaining Wash Buffer was removed by aspirating by inverting the plate and blotting it against clean paper towels. The plate was soaked for 30 minutes between each addition of wash buffer.

200μL of IL-12 Conjugate was added to each well then covered with a new adhesive strip and incubated for 2 hours. After the incubation, the plate was washed as before. 50μL of Substrate Solution was added to each well then incubated for 1 hour at room temperature. Later 50μL of amplifier solution was added, then plate covered with a new adhesion strip and incubated for 30 minutes at room temperature. After that, 50μL of Stop Solution was added to each well, and then the optical density of each well was determined within 30 minutes, using a microplate reader set to 490 nm. If wavelength correction is available, set to 650 nm or 690 nm

Sample preparation:

The samples were thrawed immediately before the assay

Reagent preparation

All reagents were brought to room temperature before use.

The wash buffer was warmed to room temperature and mixed gently until crystals had completely dissolved then diluted 20 mL of the Wash Buffer Concentrate into 480 mL distilled water to prepare 500 mL of Wash Buffer. Furthermore, Substrate Solution was prepared by mixing Color Reagents A and B together in equal volumes within 15 minutes of use and protect from light.

TNF-α Standard was reconstituted with 1.0 mL of distilled water producing a stock solution of 10,000 pg/mL then allowed to sit for 15 minutes with gentle agitation prior to making dilutions.

Eight 1.5 eppedorf tubes were clearly labeled and Pipette 900 µL of Calibrator Diluent RD6P and 100µL of the stock solution into the first tube, which was 1000 pg/mL tubes and 500µL of the Calibrator Diluent RD6P was pipetted into the remaining tubes. Using the 1000 µL diluted solution serial dilution of (500pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL and 15.6 pg/mL) were produced by transferring 500µL from one tube to the next and mixing each tube thoroughly before the next transfer. The 1000 pg/mL standard served as the high standard. The appropriate Calibrator Diluent served as the zero standard (0 pg/mL).

Assay procedure

Samples were recorded on a plate layout that was provided

Excess microplate strips were removed from the plate frame then they were returned to the foil pouch containing a desiccant pack and resealed. 50µL of Assay Diluent RD1F was added to each well then 200µL of Standard and samples were added per well following the suggested plate layout and covered with the adhesive strip. The plate was incubated for 2 hours at room temperature.

After incubation each well was aspirated and washed, the process was repeated three times for a total of four washes. Washing was performed by filling each well with 400µL Wash Buffer using a squirt bottle. After the last wash, any remaining Wash Buffer was removed by aspirating by inverting the plate and blotting it against clean paper towels.

200µL of TNF-α Conjugate was added to each well then covered with a new adhesive strip and incubated for 2 hours. After the incubation, the plate was washed as before. 200µL of Substrate Solution was added to each well then incubated for 20 minutes at room temperature protected from light by putting plate in the foil pack and sealing. After that, 50µL of Stop Solution was added to each well, and then the optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm.

ICAM-1

Sample preparation:

The samples were thawed immediately before the assay and diluted time with μL of sample diluent.

Reagent preparation

All reagents were brought to room temperature before use.

The wash buffer was warmed to room temperature and mixed gently until crystals had completely dissolved then diluted 20 mL of the Wash Buffer Concentrate into 480 mL distilled water to prepare 500 mL of Wash Buffer.

ICAM-1 Standard were commercially made and just required reconstitution with 1.0 mL of distilled water then allowed to sit for 15 minutes with gentle agitation prior to making dilutions. Furthermore ICAM-1 control was also reconstituted with 1.0 mL of distilled water.

Assay procedure

Samples were recorded on a plate layout that was provided

Excess microplate strips were removed from the plate frame then they were returned to the foil pouch containing a desiccant pack and resealed. 50μL of Assay Diluent was added to each well then 100μL of Standard and samples were added per well following the suggested plate layout and covered with the adhesive strip. The plate was incubated for 2 hours at room temperature.

After incubation each well was aspirated and washed, the process was repeated three times for a total of four washes. Washing was performed by filling each well with 400μL Wash Buffer using a squirt bottle. After the last wash, any remaining Wash Buffer was removed by aspirating by inverting the plate and blotting it against clean paper towels.

200μL of TNF-α Conjugate was added to each well then covered with a new adhesive strip and incubated for 2 hours. After the incubation, the plate was washed as before. 200μL of Substrate Solution was added to each well then

incubated for 20 minutes at room temperature protected from light by putting plate in the foil pack and sealing. After that, 50μL of Stop Solution was added to each well, and then the optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm.

The mean minimum detectable dose for: TNF-α was 1.6pg/ml,range 0.5-5.5 pg/ml), IL-10 was less than 3.9 pg/ml, IL-12 was less than 0.5 pg/ml, ICAM-1 was less than 0.35 ng/ml for the kits that were used.

MALARIA CYTOKINE, ICAM-1 AND PARASITEAMIA STUDY: PATIENT SCREENING/RECRUITMENT CLINICAL DATA FORM A (To be filled in by Study Investigator)

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

1.1 Health Facility.....

1.2 Type of Case (Complicated or uncomplicated).....

1.3 Date of Admission .....

1.4 Date of Discharge .....

1.5 Date of follow-up.....

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

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?

6.1 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

9.0 Pallor 10.0Pyrexia 11.0 Jaundice 12.0Respiratory distress

13.0 Haemoglobinuria 14.0 Level of Consciousness /Blantyre Coma Scale

15.0 Splenomegaly 16.0 Generalised Lymphadenopathy

17.0 Other Presenting Signs 1 2

MALARIA CYTOKINE, ICAM-1 AND PARASITEAMIA STUDY: PATIENT SCREENING/RECRUITMENT CLINICAL DATA FORM A (To be filled in by Study Investigator)

19.0 Provisional Diagnosis?

20.1 If RVD is part of Provisional Diagnosis, WHO RVD Stage

20.2 If on ART or Other drugs state

20.3 Date of Discharge

FOLLOW UP

PATIENT DETAILS

21.1 Body Weight **21.2 Body Temperature**

PRESENTING SYMPTOMS

22.1 Fever

23.1 Headache

24.1 Vomiting

25.1 Diarrhoea

26.1 Cough

27.1 Single episode of convulsions **27.2 Multiple convulsions**

28.1 Other main complaints? 1 2 3

PRESENTING SIGNS

30.1 Pallor **31.1 Pyrexia** **32.1 Jaundice** **33.1Respiratory distress**

34.1 Haemoglobinuria **35.1 Level of Consciousness /Blantyre Coma Scale**

36.1 Splenomegaly **37.1 Generalised Lymphadenopathy**

38.1 Other Presenting Signs 1 2

MALARIA, CYTOKINE, ICAM-1 AND PARASITEAMIA STUDY: PATIENT SCREENING/RECRUITMENT CLINICAL DATA FORM B (To be filled in by Study Investigator and Laboratory Technologist)

Study ID Number	DD MM YY	Investigator: Technologist:

1.1 Health Facility: .....

1.2 Patient File:.....

1.3 Type of Case (Complicated or Uncomplicated).....

1.4 Date and Time Sample Collected: Date.....Time.....

1.5 Date and Time Sample Received: Date.....Time.....

1.6 Date of Follow-up.....

PARASITOLOGY (Parasitaemia: Giemsa Stain)

*2.1 Parasite count (+, ++, +++) [ ] 2.2 Parasitaemia (Parasites/μL) [ ]

2.3 Infective Plasmodium - 2.3a *P. falciparum*? [ ]

2.3b. *P.Malariae* [ ] 2.4 c. *P. ovale* [ ] 2.5 d. *P. vivax* [ ]

HAEMATOLOGY (FBC AND DIFFERENTIALS)

3.1 WBC [ ] 3.2 HB [ ] 3.3 HCT [ ] 3.4 RBC? [ ] 3.5 PLT? [ ] 3.6 PCT? [ ]

3.7 MCV? [ ] 3.8 MCH? [ ] 3.9 MCHC? [ ]

SEROLOGY

4.0 Retroviral disease Reactive [ ] 4.1 Retroviral disease Non- Reactive [ ]

IMMUNOLOGY: CYTOKINES LEVELS

5.1 TNF-α reading [ ]

5.2 IL-10 Reading [ ]

5.3 IL-12 Reading [ ]

* + to be entered as 1, ++ to be entered as 2, +++ to be entered as 3

MALARIA, CYTOKINE, ICAM-1 AND PARASITEAMIA STUDY: PATIENT SCREENING/RECRUITMENT CLINICAL DATA FORM B (To be filled in by Study Investigator and Laboratory Technologist)

IMMONOLOGY: ICAM-1 Expression

6.1 ICAM-1 Readings [ ]

FOLLOW UP: DAY 3

PARASITOLOGY (Parasitaemia: Giemsa Stain)

***7.1 Parasite count (+, ++, +++)** [ ] **7.2 Parasitaemia (Parasites/μL)** [ ]

IMMUNOLOGY: CYTOKINES LEVELS

8.1 TNF-α reading [ ]

8.2 IL-10 Reading [ ]

8.3 IL-12 Readings [ ]

IMMUNOLOGY: ICAM-1 EXPRESSION

9.1 ICAM-1 Readings [ ]

* + to be entered as 1, ++ to be entered as 2, +++ to be entered as 3

THE UNIVERSITY OF ZAMBIA

RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
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Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

26 September, 2006
Ref.: 003-08-06

Ms Sitali Lungowe
C/O Ms G. Mwangala
ZRA, "Lusaka B"
P.O. Box 35776
LUSAKA

Dear Mr Lungowe ,

RE: RESEARCH PROPOSAL ENTITLED: **"DETERMINATION OF TUMOR NECROSIS FACTOR ALPHA, INTERLEUKIN 10, IL-12 AND INTERCELLULAR ADHESION MOLECULES-1 LEVELS IN RELATION TO MALARIA PARASITEMIA IN CHILDREN"**

The above research proposal was presented to the Research Ethics Committee meeting on 30 August, 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 copy of your final report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.

Yours sincerely,

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

Date of approval: 26 September, 2006 **Date of expiry**: 25 September, 2007

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

7th December 2006

Dr C J Shinondo,
University of Zambia,
School of Medicine,
P.O. Box 50110,
LUSAKA.

Dear Dr Shinondo,

RE: PERMISSION FOR LUNGOWE SITALI AND MABLE MUTENGO, MSc MEDICAL PARASITOLOGY STUDENTS TO CONDUCT RESEARCH IN THE DEPARTMENT OF PAEDIATRICS

We are in receipt of your letter dated 11th September 2006, on the above-mentioned subject.

We are happy to inform you that permission for the two students to conduct the research in the Department of Paediatrics, has been granted. This is on condition that half of the budget for miscellaneous costs be paid to University Teaching Hospital to take care of the utility and disposal of waste bills.

Yours Sincerely,
UNIVERSITY TEACHING HOSPITAL

Dr T Kafula
Deputy Managing Director
For/MANAGING DIRECTOR

Cc Managing Director
Cc Ms Lungowe Sitali - Student
Cc Mrs Mable Mutengo - Student