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**INDUCTION OF MALARIAL ANAEMIA BY HAEMOZOIN MEDIATED
SUPPRESSION OF ERYTHROPOIESIS THROUGH DYSREGULATION OF
IL-10 AND IL-12 CYTOKINES**

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**A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF
SCIENCE (APPLIED PARASITOLOGY) IN THE SCHOOL OF PURE AND
APPLIED SCIENCES OF KENYATTA UNIVERSITY**

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*Induction of
malarial anaemia by*



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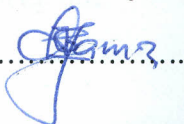
DECLARATIONS

This thesis is my original work, and has not been presented for a degree in any other University or any other award.

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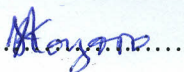
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DEDICATION

This thesis is dedicated to my wife Hellen Ng'uono, father Jacob Yamo, my children Vivian Atieno, Brian Yamo, Vallery Achieng' and Esther Akinyi for whom I have much love and respect. Without their support and encouragement, I would not have had the determination to go over this steep hill.

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TABLE OF CONTENTS

DECLARATIONS	ii
DEDICATION	iii
ACKNOWLEDGEMENT.....	iv
LIST OF APPENDICES	ix
LIST OF TABLES	x
LIST OF FIGURES.....	xi
ACRONYMS AND ABBREVIATIONS	xii
ABSTRACT	xv
CHAPTER ONE.....	1
1.0 GENERAL INTRODUCTION	1
1.1 Background	1
1.2 Justification of the Study	2
1.3 Research Questions	3
1.4 Null Hypotheses	3
1.5 Objectives	3
1.5.1 General Objective.....	3
1.5.2 Specific Objectives.....	4
CHAPTER 2.....	5
2.0 LITERATURE REVIEW	5
2.1 Malaria Transmission	5
2.2 Epidemiology of Malaria.....	5
2.2.1 World Distribution of Malaria.....	5
2.2.2 Malaria Situation in Kenya.....	5

2.2.3 Economic Importance of Malaria.....	6
2.2.4 The Life Cycle of Human Malaria Parasites.....	6
2.3 Clinical Manifestations of Malaria.....	8
2.4 Control of Malaria.....	8
2.4.1 Treatment and Prophylaxis.....	8
2.4.2 Vector Control.....	9
2.5 Immunity to Malaria.....	10
2.5.1 Sickle Cell Trait and Malaria.....	10
2.5.2 Neutrophils and Macrophages.....	11
2.5.3. Haemozoin and Phagocytic Cells.....	12
2.5.4 Cytokines in Malaria.....	13
2.5.4.1 Interleukin-12 (IL-12).....	13
2.5.4.2 Tumour Necrosis Factor- α (TNF- α).....	14
2.5.4.3 Interleukin-10 (IL-10).....	14
2.5.4.4 Interferon-gamma (IFN- γ).....	15
2.6 Inflammatory Response.....	15
2.7 Adaptive Immunity.....	16
2.8 Pathology of Malaria.....	17
2.8.1 Immunopathogenic processes.....	17
2.8.2 Increased Destruction of RBCs.....	17
2.8.3 Decreased Production of RBCs.....	19
3.0 MATERIALS AND METHODS.....	22
3.1 Study Area.....	22
3.2 Study Population.....	23
3.2.1 Sample Size Determination.....	23

3.3 Inclusion Criteria.....	23
3.4 Definition of Anaemia.....	24
3.5 Determination of Parasitaemia.....	24
3.6 Haemozoin Counts from Monocytes and Neutrophils.....	25
3.7 Hookworm Investigation.....	25
3.8 Reticulocyte Count.....	25
3.9 Full Haemogram Measurement.....	26
3.10 Multiplex Assay.....	26
3.10.1 Principle of the Assay.....	26
3.11 Ethical Considerations.....	27
3.12 Statistical Analysis.....	27
CHAPTER 4.....	28
4.0 RESULTS.....	28
4.1 The Effect of Monocyte Acquired Hz on the Production of Interleukin IL-10, IL-12, Tumour Necrosis Factor- α (TNF- α) and Interferon - γ (IFN- γ) in Children with MA.....	28
4.1.1 Clinical and Demographic Characteristics of Study Participants.....	28
4.2 Parasitological Parameters of Study Subjects Grouped By Clinical Status.....	30
4.3 Determination of the Relationship between in Vivo Innate Inflammatory Mediator Production and Monocyte Acquisition of PfHz.....	32
4.4 Determination of the Relationship between HCM and Cytokine Production.....	35
4.5 Effects of Naturally Acquired PfHz in Production of Cytokine.....	37
4.6 Determination of the Relationship between IL-10, IL-12, TNF- α and IFN- γ Production and Malarial Anaemia.....	40
4.7 Erythrocytic Characteristics of Study Participants at Enrollment.....	42
4.8 Leucocytic Characteristics of Study Participants at Enrollment.....	44

4.9 The Effects of Naturally-Acquired Hz on Haematological Parameters	46
4.10 The Effects of Naturally-Acquired Hz on leucocytic parameters	49
CHAPTER FIVE:	55
5.0 DISCUSSION	55
5.1 Effect of Hz on Inflammatory Mediator Production in Infants and Children with Malarial Anaemia	55
5.2 Effect of Hz on Haematological Outcomes in Infants and Children with Malarial Anaemia	58
CHAPTER SIX	62
6.0 CONCLUSIONS AND RECOMMENDATIONS	62
6.1 Conclusions	62
6.2 Recommendations	62
REFERENCES	63
Appendix 1: Global distribution of malaria	76
Appendix 2: Distribution of endemic malaria in Kenya	77
Appendix 3: Life cycle of human <i>Plasmodium</i>	78
Appendix 4: Structure of heme and haemozoin	79
Appendix 5: UNM/ KEMRI study area	80
Appendix 6: Consent form	81
Appendix 7: Child questionnaire	83
Appendix 8: Child laboratory tests	86

LIST OF APPENDICES

Appendix 1: Global distribution of malaria	76
Appendix 2: Distribution of endemic malaria in Kenya	77
Appendix 3: Life cycle of human <i>Plasmodium</i>	78
Appendix 4: Structure of heme and haemozoin	79
Appendix 5: UNM/KEMRI study area	80
Appendix 6: Consent form	81
Appendix 7: Child questionnaire.....	83
Appendix 8: Child laboratory tests.....	86

LIST OF TABLES

Table 1: Demographic and Clinical Characteristics of Study Participants at enrollment .	29
Table 2: Parasitological Characteristics of Participants at Enrollment	31
Table 3: Cytokine Levels and Cytokine Ratios among Children with Malarial Anaemia	34
Table 4: Relationship between HCM and Cytokine Production	36
Table 5: Association between Cytokine Production and Disease Severity	41
Table 6: Erythrocytic Counts and Indices of Study Participants at Enrollement.....	43
Table 7: Levels of Leucocytes among Study Participants.....	45
Table 8: The Relationship between HCM and Erythrocytic Parameters	48
Table 9: The relationship between HCM and Leucocytic and Platelets Parametres.....	50
Table 10: Relationship between HCM, Parasitaemia and Haematological Parameters...	51

ACRONYMS AND ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ART	Anti-Retroviral Therapy
BFU-E	Burst Forming Units-Erythroids
CD	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention
CFU-E	Colony Forming Units-Erythroids
CFU-GEMM	Colony Forming Units-Granulocyte, Erythroid, Macrophage and Megakaryocytes
CM	Cerebral Malaria
CR-1	Complement Receptor type -1
DVBD	Division of Vector Borne Diseases
ELISA	Enzyme Linked Immunosorbent Assay
G6PD	Glucose-6-Phosphate Dehydrogenase
GDP	Gross Domestic Product
Hb	Haemoglobin
HC	Healthy Control
HCM	Haemozoin Containing Monocytes
HCN	Haemozoin Containing Neutrophils
Hct	Haematocrit
HDP	High Density Parasitaemia
HIS	Health Information System
HIV	Human Immunodeficiency Virus
Hz	Haemozoin
IFN- γ	Interferon gamma

IgM	Immunoglobulin mu
IL-10	Interleukin-10
IL-12	Interleukin-12
ITN	Insecticide Treated Net
KEMRI	Kenya Medical Research Institute
MA	Malarial Anaemia
MCH	Maternal Child Health
MCHC	Mean Cell Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MdMA	Moderate Malarial Anaemia
MHC	Major Histocompatibility Complex
M/MA	Mild Malarial Anaemia
MOH	Ministry of Health
NERB	National Ethical Review Board
NIH	National Institute of Health
NK cells	Natural Killer Cells
NO	Nitric Oxide
NOS - 2	Nitric Oxide Synthase - 2
OPD	Out-Patient Department
PBMC	Peripheral Blood Mononuclear Cells
<i>Pf</i> H ₂	<i>Plasmodium falciparum</i> Haemozoin
<i>p</i> RBC	Parasitized Red Blood Cells
RBC	Red Blood Cell
RPI	Reticulocyte Production Index
SDH	Siaya District Hospital

SMA	Severe Malarial Anaemia
SPSS	Statistical Package for Social Sciences
TGF	Transforming Growth Factor
T _H	T _{Helper} Cells
TNF	Tumour Necrosis Factor
UM	Uncomplicated Malaria
USA	United States of America
WBC	White Blood Cell
WHO	World Health Organization

ABSTRACT

Severe malarial anaemia (SMA) is the leading cause of morbidity and mortality in young children less than five years residing in a *Plasmodium falciparum* holoendemic transmission area of western Kenya. Although the immunopathogenesis of SMA is largely uncharacterized, recent studies have shown that circulating interleukin-12 (IL-12) production is suppressed during malarial anaemia. Since interleukin-10 (IL-10) and tumour necrosis factor alpha (TNF- α) suppress IL-12 production in a number of diseases, the profiles of these regulatory cytokines were investigated to determine the mechanism for IL-12 suppression in children with malarial anaemia. Moreover, monocytes and neutrophils acquire haemozoin (Hz) naturally during a malarial infection. In this study children aged three to thirty three months with Hz and healthy controls visiting the hospital were studied in a longitudinal prospective hospital-based design. Sample size was determined using sample size software. Malaria screening was performed using Giemsa-stained thick and thin blood films. Parasites were counted against 300 leucocytes and parasite density estimated using each individual's white blood cell (WBC) count. Hz was counted from 30 monocytes and 100 neutrophils and the level of haemozoination compared with haemoglobin levels, IL-10 and IL-12 production. Full haemogram was estimated using a Coulter counter. Multiplex assay was used to determine IL-10, IFN- γ , TNF- α and IL-12p70 in plasma levels, which were then compared with Hz and haemoglobin (Hb) levels. Differences between median cytokine concentrations in various groups were tested using Kruskal Wallis test and pair wise comparisons using Mann-Whitney U test. The ratio of IL-10:IL-12 was calculated for each individual and the difference among median ratio between various groups was compared using Mann-Whitney U test. Chi-square test was used to compare proportions. Results revealed that ingestion of naturally acquired malarial pigment (Hz) by monocytes increased the production of IL-10 and TNF- α , relative to IL-12, which correlated with increased severity of malarial anaemia. Further results revealed that elevated levels of Hz containing monocytes (HCM) (>10 %) was associated with significantly higher numbers of WBC ($P < 0.0001$), lymphocytes ($P < 0.0001$), and monocytes ($P < 0.0001$). In contrast, high HCM (> 10 %) were inversely associated with red blood cells count ($P < 0.0001$), Hb ($P < 0.0001$), haematocrit ($P < 0.0001$), reticulocyte counts ($P < 0.05$), and platelet counts ($P < 0.0001$). It was concluded that elevated levels of Hz-deposition in monocytes are associated with increased severity of anaemia, leukocytic parameters and thrombocytopenia.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background

Malaria is one of the most prevalent infectious diseases of parasitic origin occurring in the tropical world. Of the four species of malaria parasites which infect man, *Plasmodium falciparum* presents the most serious and life threatening clinical episodes (Bruce-Chwatt, 1985) resulting in 300-500 million clinical cases and 1.5-2.7 million deaths annually with children less than five years and pregnant women being the most affected (Bremman *et al.*, 2004; WHO, 2005). The population at risk of malaria continue to rise due to the rapid spread of drug-resistant parasites which has been compounded by the emergence of insecticide-resistant *Anopheles* mosquito vectors (Hume *et al.*, 2003). In addition, migration into malarious areas, changing agricultural practices such as irrigation projects, increased demands on public health systems due to the spread of HIV in impoverished countries, and long-term climatic changes such as global warming, contribute to the growing incidence of malaria (Lindblade *et al.*, 2000; Sachs and Malaney, 2002). It has been estimated that if no effective intervention strategies are undertaken, the number of malaria cases will double over the next 20 years (Bremen, 2001).

Severe malarial anaemia (SMA) and cerebral malaria (CM) are two of the most important causes of malaria-related morbidity and mortality (Bloland *et al.*, 1999; Molyneux, 2000). However, compared to extensive investigation of the immune response, vaccine development, and genomics, malarial anaemia has received relatively little attention (Chang and Stevenson, 2004). Most of the research conducted in areas of malarial anaemia (MA) have concentrated on epidemiologic studies, and relatively little effort has

been made to investigate the mechanisms underlying the development and progression of malarial anaemia (Chang and Stevenson, 2004). This complacency could have been due to the fact that malaria can be treated with commonly available drugs and through blood transfusion. However, with the emergence of multi-drug resistant strains of *P. falciparum* and high prevalence of HIV/AIDS infection in malaria endemic regions suggests that these strategies pose potential risks and complications. Hence, there is an urgent need to unravel the mechanisms and processes of pathophysiology of malaria, as this information would be important in the development of novel therapies against the disease.

1.2 Justification of the Study

Malaria is holoendemic in western Kenya and Coast province. However the incidence in western Kenya is higher than it is in Coast province and the transmission is stable throughout the year. In this area the transmission of malaria among children aged between 1-4 years is 83% with prevalence of anaemia being 90%. In this area severe anaemia is the most important clinical manifestation of malaria infection. Since *in vitro* studies have shown that Hz enhances the production of IL-10, IL-1 β , and TNF- α but suppresses the production of IL-12 (Deshpande and Shastry, 2004) a pro-inflammatory cytokine which is associated with increased erythropoiesis in malaria models (Mohan and Stevenson, 1998a), this study investigated the effect of naturally acquired *Pf*Hz on cytokine production *in vivo* and its relationship with malarial anaemia. In addition, since profound haematological alterations have been associated with malaria in semi-immune population (Erhart *et al.*, 2004), this study further investigated the impact of naturally acquired *Pf*Hz on haematological outcomes in children with malaria residing in a malaria holoendemic area of western Kenya. The findings of this study will contribute to the understanding of the pathophysiology of malarial anaemia.

1.3 Research Questions

- i. What is the role of monocyte- and neutrophil-acquired Hz in modulating the production of IL-10, IL-12, TNF- α and IFN- γ in children with malaria?
- ii. What is the relationship between IL-10, IL-12, TNF- α and IFN- γ cytokine production and anaemia?
- iii. What impact does Hz deposition on monocytes and neutrophils have on haematological indices in children with malaria?

1.4 Null Hypotheses

- i. Ingestion of Hz by monocytes and neutrophils is not associated with the circulating levels of IL-10, IL-12, TNF- α and IFN- γ in children with malaria.
- ii. Production of IL-10, IL-12, TNF- α and IFN- γ is not associated with malarial anaemia.
- iii. Ingestion of Hz by monocytes and neutrophils is not associated with haematological outcomes in children with malaria.

1.5 Objectives

1.5.1 General Objective

To determine the effect of Hz on the production of IL-10, IL-12, TNF- α and IFN- γ and the relationship between Hz and haematological outcomes in young children with malaria.

1.5.2 Specific Objectives

- i. To determine the effect of monocyte- and neutrophil-acquired Hz on the production of IL-10, IL-12, TNF- α and IFN- γ in children with malaria.
- ii. To determine the relationship between IL-10, IL-12, TNF- α and IFN- γ cytokine production and MA in children with malaria.
- iii. To determine the effect of Hz deposited in monocytes and neutrophils on haematological outcomes in children with MA.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Malaria Transmission

Malaria is caused by parasites of the genus *Plasmodium* that are spread from person to person through the bites of an infected mosquito. There are four species of human malaria parasites which include *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. *P. falciparum* is the most common and is responsible for the most serious type of malaria infection (WHO, 2005). Malaria transmission differs in intensity and regularity depending on local factors such as rainfall patterns, proximity of mosquito breeding sites and mosquito species. The species of mosquitoes responsible for malaria transmission in sub-Saharan Africa include *Anopheles gambiae*, *An. funestus*, and *An. arabiensis* (Beach *et al.*, 1993).

2.2 Epidemiology of Malaria

2.2.1 World Distribution of Malaria

By the end of 2006, more than 109 countries and territories were endemic for malaria and about 3.3 billion people lived in areas at risk of malaria transmission, while 247 million clinical cases were reported (WHO, 2008). Malaria is found mainly in the tropics and sub-tropics between 64°N and 34°S (Bruce-Chwatt, 1980). Malaria is endemic in some regions while in others it occurs in epidemics (Appendix 1).

2.2.2 Malaria Situation in Kenya

In Kenya, *P. falciparum* is the leading cause of morbidity and mortality, particularly among pregnant women and children under the age of 5 years (Division of Malaria Control, 2001). Studies in Kenya indicate that malaria accounts for about 30% of

outpatient attendance and 19% of admissions and 20-25% of deaths (Division of Malaria Control, 2001). Each year, approximately 1.5 million women become pregnant the majority of whom live in areas with moderate-to-intense transmission, and thus are at risk of being infected with malaria (Guyatt and Snow, 2001). Kenya is facing serious problems of drug resistance with chloroquine and sulfadoxine-pyrimethamine being abandoned due to efficacy failure (Obonyo *et al.*, 2003). There are five malaria transmission zones in Kenya (Appendix 2)

2.2.3 Economic Importance of Malaria

Malaria has been associated with major negative economic effects in countries where it is endemic. In countries where malaria is common, average per capita GDP has risen (between 1965 and 1990) only 0.4% per year, compared to 2.4% per year in other countries (Sachs and Melaney, 2002). The economic impact includes cost of health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism (Greenwood and Mutabingwa, 2002). In malaria endemic countries the disease may account for as much as 40% of public health expenditure, 30-50% of inpatient admissions, and up to 50% of outpatient visits (WHO, 2005). In Kenya, an estimated 170 million working days are lost each year due to malaria, suggesting that it is the most important cause of poverty in Kenya (Division of Malaria Control, 2001).

2.2.4 The Life Cycle of Human Malaria Parasites

The life cycle of *P. falciparum* is complex, having both intra- and extracellular stages (Appendix 3) as well as other stages in the vector. During a bloodmeal, the female Anopheles mosquito injects sporozoites into the host's bloodstream. These sporozoites

migrate to the liver and invade hepatocytes. After days of replication by asexual division, the sporozoite-infected hepatocytes produce exoerythrocytic schizonts that rupture and release thousands of merozoites into the bloodstream (Struik and Riley, 2004). Merozoites invade erythrocytes and develop into erythrocytic schizonts via asexual mitotic replication. The rupture of erythrocytic schizonts is characterized by episodes of fever, rigours, nausea, and headaches that result from the systemic pro-inflammatory cytokine cascade initiated by cells of the innate immune system (Miller *et al.*, 2002). This cyclical pattern of blood-stage infection continues for 48-72 hours (Struik and Riley, 2004). In some instances, merozoites do not invade erythrocytes, but instead differentiate into male and female gametocytes. Following ingestion by a mosquito, these gametocytes develop into extracellular gametes. Male and female gametes then fuse during sexual replication in the mosquito midgut to produce motile zygotes that penetrate the midgut wall and form oocysts. Following meiosis, oocysts develop into haploid sporozoites that migrate to the mosquito salivary glands and can be transmitted to the next host (Appendix 3).

2.2.4.1 Development of Haemozoin

Haemozoin (Hz, malarial haemozoin) is formed in the digestive vacuoles of late trophozoites and schizonts during intra-erythrocytic development (Day *et al.*, 1996). During this process, malaria parasites digest haemoglobin using protease enzyme leading to the formation of amino acids which they use for their nutrition (Goldberg *et al.*, 1991). Heme which is released as a by-product, is oxidized by tissue oxygen to toxic haematin [aquaferriprotoporphyrin IX, H₂O-Fe (III) PPIX], which is detoxified by conversion to Hz through a process of polymerization catalyzed by heme polymerase (Slater and Cerami, 1992). This enzyme links heme groups via iron carboxylate bonds (Slater and

Cerami, 1992). The core Fe (III) PPIX structure of H₂ is surrounded by proteins, carbohydrates, lipids, and nucleic acids (Goldie *et al.*, 1990; Francis *et al.*, 1997) (Appendix 4).

2.3 Clinical Manifestations of Malaria

Clinical features of malaria are very diverse and may range from mild discomfort to severe disease. Different species of malaria parasites produce fevers of different frequencies depending on the length of the schizogonic cycle. Initial attacks are characterized by fever that may relapse depending on species (Kochar *et al.*, 2007). In areas where malaria is holoendemic, clinical manifestations of malaria are variable (Bruce-Chwatt, 1985) with children less than 5 years and pregnant women and HIV-1 positive patients suffering severe forms of the disease (Snow *et al.*, 1999; Andrews and Lanzer, 2002). In areas of unstable transmission, malaria affects people of all ages (Miller and Warrell, 1994; Snow *et al.*, 1997) due to their poorly developed immunity. In such populations, malaria is characterized by cerebral complications, hyperparasitaemia, hypoglycaemia, respiratory distress, SMA, renal failure and pulmonary oedema (WHO, 2000). Symptoms associated with cerebral malaria (CM) are rarely reported from adults residing in areas with stable transmission (Marsh *et al.*, 1996; Newton *et al.*, 1998). SMA is more common in children in holoendemic areas (Snow *et al.*, 1997; Bloland *et al.*, 1999).

2.4 Control of Malaria

2.4.1 Treatment and Prophylaxis

Treatment of malaria involves supportive measures as well as specific antimalarial drugs. When properly treated, a complete recovery can be expected. Chloroquine was the

treatment of choice for uncomplicated malaria but was replaced by sulphadoxine pyrimethamine which in turn has also been replaced by Artemether-Lumefantrine on the recommendation of WHO (WHO, 2005). On the other hand complicated malaria is treated with intravenous quinine (Pasvol, 2005). There are several other substances that are used for treatment and, partially for prophylaxis. Many drugs may be used for both purposes; larger doses are used to treat cases of malaria while lower doses are used for prophylaxis. Prophylaxis is recommended for visitors to malaria endemic areas.

2.4.2 Vector Control

The objective of malaria control is to prevent mortality and reduce morbidity as well as economic losses due to disease (Trigg and Kondrachine, 1998). Control of malaria vectors revolves around destruction of mosquito breeding sites through drainage and use of insecticides. Use of insecticide treated nets (ITNs) is being promoted through public and private sector partnership to help in breaking human-vector contact. It has been demonstrated that consistent use of ITNs reduces malaria in children less than 5 years residing in malaria holoendemic transmission areas by 25% (Phillips-Howard *et al.*, 2003; ter Kuile *et al.*, 2003). Previous studies indicate that children sleeping under nets impregnated with insecticides have lower levels of parasitaemia and marked improvement in anaemia compared to those not using them (Phillips-Howard *et al.*, 2003). Malaria control efforts have been complicated with the development of mosquito resistance to insecticides (Matambo *et al.*, 2007). Other methods for vector control include use of predators and source filling (Service, 1986).

2.5 Immunity to Malaria

Several factors contribute towards protection against *P. falciparum* malaria in humans. These include innate non-antigen-specific immunity and immunity acquired after several attacks of malaria (Biswas *et al.*, 2008). Several genetic factors influence protection against malaria. They may prevent a malaria infection from occurring or confer protection against the severe forms of malaria. These factors include erythrocyte genetic defects such as sickle cell trait, α - and β - thalasseмииs, deficiency of glucose-6-phosphate dehydrogenase (G6PD), Duffy blood group antigen and foetal haemoglobin (HbF) (Shear *et al.*, 1998).

2.5.1 Sickle Cell Trait and Malaria

The sickle cell gene defect is due to a mutation of a single nucleotide (A to T) which results in glutamic acid being substituted by valine at position 6 within the β -globin gene (Ashley-Koch *et al.*, 2000). Haemoglobin S with this mutation is referred to as HbS, as opposed to the normal adult HbAA. HbS can be inherited as HbAS. This is normally a benign mutation, causing no apparent effects on the secondary, tertiary, or quaternary structure of Hb (Ashley-Koch *et al.*, 2000). Under conditions of low oxygen concentration, there is polymerization of the HbS (Ashley-Koch *et al.*, 2000). In people heterozygous for HbS (HbAS), polymerization problems are minor, while in those homozygous for HbS (HbSS), the presence of long chain polymers of HbS distort the shape of the RBC, from a smooth biconcave shape to ragged and full of spikes, making it fragile and susceptible to breaking within capillaries (Ashley-Koch *et al.*, 2000). People heterozygous for HbS (HbAS) only have symptoms if they are deprived of oxygen (for example, while climbing a mountain). Those afflicted with the disease experience very painful vaso-occlusive crises (Ashley-Koch *et al.*, 2000). This occurs when HbS

becomes deoxygenated and undergoes an abnormal change in shape and consistency, which leads to a change in shape and plasticity in the cell wall of the RBC, an occurrence called sickling. It is believed that carriers (sickle cell trait) are relatively resistant to malaria (Hebbel, 2003; Ayi *et al.*, 2004). Since the gene is incompletely recessive, carriers have a few sickled RBCs at all times, not enough to cause symptoms, but enough to give resistance to malaria (Nagel and Roth, 1989). Because of this, heterozygotes have a higher fitness than either of the homozygotes. This is known as heterozygote advantage. In a carrier, the presence of malaria parasites causes RBC to rupture, making *Plasmodium* unable to complete its life cycle. Furthermore, polymerization of Hb affects the ability of the parasite to digest Hb. Therefore, in areas where malaria is holoendemic, people's chances of survival actually increase if they carry sickle cell trait (Greenwood *et al.*, 1988; Hill, 1992). Other innate immune responses include α and β thalassemia, Duffy antigens and HbF.

2.5.2 Neutrophils and Macrophages

The major effector cells for parasite clearance during blood-stage infection appear to be mononuclear and polymorphonuclear leucocytes. Phagocytes mediate protection through phagocytosis of merozoites, schizonts and gametocytes (Roberts and Weidanz, 1978). Polymorphonuclear neutrophils have been reported to inhibit the asexual multiplication of *P. falciparum* *in vitro* (Brown and Greenwood, 1985). Human neutrophil mediates the killing of *P. falciparum* through increased fatty acids (Kumaratilake and Ferrante, 1994), increased respiratory burst and degranulation through the priming of polyenoic acid (Hardy *et al.*, 1991). Neutrophils which have ingested Hz have increased production of TNF- α , IFN- γ and TNF- β , especially in the presence of malarial antibody (Kumaratilake and Ferrante, 1994). Monocyte-derived macrophages and polymorphonuclear cells are

able to kill late stages of intra-erythrocytic parasites in the absence of antibodies through phagocytosis (Nnalue and Friedman, 1988).

2.5.3. Haemozoin and Phagocytic Cells

At the end of schizogonic cycle, *P. falciparum* releases merozoites and Hz, which are ingested by phagocytic cells as part of the first line of immune responses. The interaction between Hz and immune cells causes dysfunction of accessory cells such as impairment of phagocytosis (Schwarzer *et al.*, 1992), expression of MHC class II antigens, CD54, CD11c (Schwarzer *et al.*, 1998) and cytokine production, which has been implicated in cytokine-mediated manifestation of malarial anaemia (Arese and Schwarzer, 1997). Monocytes which have ingested Hz secrete large amount of TNF- α , IL-10 and IL-1 β (Pichyangkul *et al.*, 1994; Mordmuller *et al.*, 1998; Deshpande and Shastry, 2004). Although IL-10, IFN- γ , TNF- α and IL-6 are often elevated in acute malaria (McDevitt *et al.*, 2004) it is only IL-10 which suppresses the release of IL-12 from macrophages (Keller *et al.*, 2006) leading to reduced numbers of BFU-E and CFU-E in the bone marrow and spleen (Mohan and Stevenson, 1998b). It has been reported that sustained production of IL-12 during malarial anaemia provides protection against severe forms of malaria in murine models (Sam and Stevenson, 1999). It has further been observed that the level of IL-12 correlate with the extent of anaemia and that B6 mice, which are refractory to *P. chabaudi* AS infection, had a high IL-12 level during infection compared to susceptible AJ mice (Mohan and Stevenson, 1998a).

The balance between Th1 and Th2 immune effector molecules are of central importance for the host response to diseases. It has been demonstrated that low IL-10/TNF- α ratio is associated with malarial anaemia in children residing in a malaria holoendemic area

(Othoro *et al.*, 1999), suggesting that insufficient IL-10 response to high TNF- α levels may have a central role in the pathophysiology of malaria. Further studies have shown that the balance between regulatory cytokines such as IL-10, transforming growth factor (TGF)- β and inflammatory cytokines such as IL-12, IFN- γ and TNF- α is critical in malaria pathogenesis (Li *et al.*, 2003; Omer *et al.*, 2003). It is therefore clear that IL-12 is one of the critical factors for controlling malarial infection, thus its suppression may lead to malarial anaemia. Although immunological mechanisms that govern the pathogenesis of SMA are still largely undefined, the overall cytokine production in various stages of malaria infection and the relative balance of pro- and anti-inflammatory cytokines determine whether a pathogenic or a protective outcome develops (Romagnani *et al.*, 1997; Torre *et al.*, 2002).

2.5.4 Cytokines in Malaria

Cytokines are immunoregulatory proteins originating from macrophages, T cells and natural killer (NK) cells that are produced in response to different infections. CD4⁺ T cells produce a profile of cytokines that regulate effector functions and influence T cell maturation (Romagnani *et al.*, 1997). T-helper type 1 (T_H1) cells express cytokines such as IL-12, IFN- γ , TNF- α among others, while T-helper type 2 (T_H2) cells produce anti-inflammatory cytokines such as IL-10 and IL-13.

2.5.4.1 Interleukin-12 (IL-12)

Interleukin-12 is a heterodimeric molecule composed of IL-12p35 and IL-12p40 subunits produced mainly by monocytes (Kobayashi *et al.*, 1989). Studies involving murine models of malaria suggest that production of IL-12 provides protection through increased erythropoiesis (Sam and Stevenson, 1999). This is supported by the fact that recombinant

IL-12 (rIL-12) injected into susceptible mice gives them 100% protection against *P. yoelii* challenge (Sedegah *et al.*, 1994; Hoffman *et al.*, 1997). Conversely, the inability of IL-12 deficient mice to control parasitaemia is restored after administration of rIL-12 (Su and Stevenson, 2002). Although the mechanisms responsible for IL-12 suppression in human malaria are still unknown, *in vitro* studies suggest that IL-12p70 is negatively regulated by IL-10 (Aste-Amezaga *et al.*, 1998; Ho *et al.*, 1998; Deshpande and Shastri, 2004).

2.5.4.2 Tumour Necrosis Factor- α (TNF- α)

TNF- α is produced mainly by activated macrophages and less by other cell types. It is encoded on genes which are located within the MHC complex on chromosome 6; hence it is referred to as class III MHC protein. It has been suggested that an active membrane bound form of TNF- α may exist that can mediate tumour killing through direct contact. TNF- α plays an important early role in the inflammatory response during malaria (Jacobs *et al.*, 1996). Although available evidence suggests that TNF- α may act directly on BFU-E, its effect on CFU-E is indirect and mediated by the local release of other cytokines such as IFN- γ from accessory cells (Felli *et al.*, 2005).

2.5.4.3 Interleukin-10 (IL-10)

Interleukin-10 is a protein produced by T_H2 cells, CD8 T cells, monocytes, keratinocytes and activated B cells. It was originally called cytokine synthesis inhibitory factor because of its ability to inhibit cytokine production by activated T lymphocytes (Abbas and Lichtman, 1994). In particular, IL-10 inhibits the production of IL-12 and IFN- γ by T_H1 cells and therefore tips the regulatory balance in favour of humoral immune responses (Delves and Roitt, 1998). IL-10 also inhibits cytokine production by natural killer cells (NK-cells) and macrophages (Delves and Roitt, 1998). IL-10 also has a direct

stimulatory effect on B cells and promotes antibody production (Mujuzi *et al.*, 2006). Several studies have shown that IL-10 levels are elevated during malarial infections (Peyron *et al.*, 1994; Wenisch *et al.*, 1995; Keller *et al.*, 2006).

2.5.4.4 Interferon–gamma (IFN- γ)

Interferon–gamma is a dimerized soluble cytokine that is the only member of the type II class of interferon's (Gray and Goeddel, 1983). This interferon was originally called macrophage-activating factor. It is secreted by all CD8 T cells, some CD4 T cells and to a lesser degree NK cells (Thale and Kiderlen, 2005). IFN- γ increases the expression of class II MHC proteins on class II bearing cells, thus enhancing antigen presentation to CD4+ T lymphocytes (Kemp and Bruunsgaard, 2001). IFN- γ is also effective as a host defence because it is able to kill intracellular parasites (Jackson *et al.*, 2001).

2.6 Inflammatory Response

Plasmodium evades the immune response by altering its surface antigens throughout various stages of its life cycle (Bull, 1998; Zambrano-Villa *et al.*, 2002). Each phase of the life cycle is characterized by the transient expression of species-specific proteins that are highly polymorphic and antigenically variable. The erythrocytic stage of *P. falciparum* infection plays an important role in the pathology of malaria and is characterized by dysregulatory processes that prevent the induction of robust immune responses (Luke and Hoffman, 2003). During schizogony, parasitized red blood cells (pRBC) burst, creating RBC ghosts and releasing daughter merozoites and Hz into the blood stream (Nguyen *et al.*, 1995). Phagocytic cells ingest free Hz and mature pRBCs and other RBC remnants (Nguyen *et al.*, 1995), as well as unparasitized RBCs coated with malaria antibodies (Day *et al.*, 1996). Ingestion of opsonized Hz by monocytes impairs their expression of MHC class II

molecules, CD54, and CD11c, as well as generation of the oxidative burst, protein kinase C activity, and their ability to repeat phagocytosis (Arese and Schwarzer, 1997; Schwarzer *et al.*, 1992, 1993, 1998). Hz released into the circulation upon schizont rupture are concentrated in resident macrophages of the liver, bone marrow and spleen (Schwarzer *et al.*, 1998). Impaired antigen presentation to T cells results from the suppression of dendritic cell maturation by pRBCs (Urban *et al.*, 1999). Parasitized RBCs can also cause apoptosis of malaria-specific B cells and T cells (Good, 2005). *P. falciparum* infection is characterized by anaemia, and impaired circulation resulting from peripheral hypotension and the adherence of infected erythrocytes to the vascular endothelium (Stevenson and Riley, 2004).

2.7 Adaptive Immunity

Active immune factors are acquired as people interact with the environment. People resident in malaria-endemic regions acquire this immunity from prolonged natural exposure to malaria parasites (Maire *et al.*, 2006). The immunity, which is protective against parasites and clinical disease, results only after continued exposure from multiple infections with malaria parasites over time. Clinical immunity provides protection against severe effects of malaria, but fails to provide protection against infection with malaria parasites. After several years of continued exposure, people develop immunity that limits high-density parasitaemia (Maire *et al.*, 2006). However, it does not lead to sterile protection. The transmission intensity influences the course of development for both clinical and parasitic immunity. Where malaria transmission is intense, young children bear the brunt of the disease, but as they grow older, they build up immunity and are relatively protected against the disease (CDC, 2004). In areas of low malaria endemicity, both children and adults suffer severe

disease and high parasitaemia since exposure is less. Immune individuals lose their immunity if they relocate to malaria free area for several years.

2.8 Pathology of Malaria

2.8.1 Immunopathogenic processes

These are now recognized as having a central role in severe malaria, with pro-inflammatory cytokine cascades leading to complex downstream metabolic changes. Cytokine-induced failure of oxygen utilization is likely to play an important role. Pro-inflammatory cytokines and anti-inflammatory cytokines, such as interleukin-10 (IL-10), have been proposed to have a protective or counter-regulatory role. Tumour necrosis factor-alpha (TNF- α) is raised in those with severe malaria and has been implicated in the pathogenesis of murine cerebral malaria. TNF- α is also raised in placental malaria and is associated with low birth weight (Mackintosh, *et al.*, 2004)

2.8.2 Increased Destruction of RBCs

Parasite multiplication results in decreased haematocrit level due to rupture of pRBC when schizonts mature and merozoites are released. However, the severity of malarial anaemia, in which the haematocrit level may approach 15% or less [where anaemia is defined as a haematocrit of <33%] does not correlate with the degree of parasitaemia (Ong'echa *et al.*, 2006). It has been demonstrated that uninfected RBCs in malaria patients have a shortened life span compared to that of healthy individuals (Looareesuwan *et al.*, 1991; Jakeman *et al.*, 1999). Studies show that for every RBC destroyed from the direct effect of the parasite on average 8.5 uninfected cells are destroyed (Jakeman *et al.*, 1999). Based on these data, it was concluded that the destruction of uninfected RBC may be the leading cause of the SMA (Jakeman *et al.*,

1999). The mechanism underlying the shortened life span of uninfected RBCs, however, is not clear. It has been postulated that reactive oxygen species produced during malaria infection modify RBC membrane proteins, which become targets for auto-antibodies (Jakobsen *et al.*, 1998). Host immunoglobulins may also recognise parasite antigens expressed on RBCs leading to antibody deposition on the red cell surface and subsequent phagocytosis by macrophages (McGregor *et al.*, 1968; Adam *et al.*, 1981; Brojer *et al.*, 1989; Waitumbi *et al.*, 2000). During acute *P. falciparum* infection there is proliferation and hyperactivity of macrophages in the reticulo-endothelial system cells (RES) leading to increased clearance of both pRBCs and unparasitized RBCs (Mendez *et al.*, 2000). Antibody-independent phagocytosis of RBCs also occurs via exposure of phosphatidyl serine and/or galactose residues due to cell membrane injury triggered by reactive oxygen species produced by the immune response to malaria parasites (Bratosin *et al.*, 1998; Serghides *et al.*, 2003). Furthermore, studies by Smith *et al.* (2003) demonstrated that *P. falciparum* infected RBCs are recognised and phagocytosed by human monocytes and macrophages in an opsonin-independent manner via the macrophage class B scavenger receptor (CD36). In addition, complement-mediated phagocytosis and/or haemolysis may be involved (Woodruff *et al.*, 1979; Ritter *et al.*, 1993). These studies additionally demonstrated that immunoglobulin M (IgM) auto-antibodies specific for the glycolytic enzyme triosephosphate isomerase are detected in the serum of malaria patients suffering prolonged haemolysis (Woodruff *et al.*, 1979; Ritter *et al.*, 1993). These auto-antibodies may activate the complement system and induce RBC lysis, which may lead to prolonged haemolysis. Loss of the complement regulatory proteins, complement receptor type 1 (CR1) and CD55, from the RBC surface has also been shown to correlate with SMA (Waitumbi *et al.*, 2000; Stoute *et al.*, 2003) as these proteins are important in protecting cells from complement-mediated damage by controlling the complement activation

cascade. The loss of CR1 and CD55 from the RBC surface may result in increased lysis and/or phagocytosis of uninfected RBCs.

2.8.3 Decreased Production of RBCs

Without treatment, malaria patients often present with a sub-optimal absolute number of reticulocytes in the peripheral blood for the degree of anaemia (Abdalla *et al.*, 1980). Inadequate reticulocyte production suggests insufficient erythropoiesis, which may be a result of hypo-proliferative erythropoiesis or of hyper-proliferative, yet ineffective erythropoiesis (Wickramasinghe and Abdalla, 2000). Increased or decreased erythropoiesis is generally determined by the cellularity of the erythroid cells in haematopoietic tissues. Ineffective erythropoiesis is often associated with intramedullary destruction of erythroid precursors by mechanisms such as erythrophagocytosis, or dysplastic features of these precursors, which can be recognised by light microscopy, such as grossly deformed nuclei, nuclear budding, bi- and multi-nuclearity, megaloblastic changes, internuclear chromatin bridges, and karyorrhexis (Wickramasinghe and Abdalla, 2000). In addition, electron microscopy also reveals ultrastructural abnormalities of erythroblasts, including abnormally long intranuclear clefts, marked irregularities of nuclear shape, multi-nuclearity, myelinisation or loss of parts of the nuclear membrane, intracytoplasmic circular double membranes, abnormally large autophagic vacuoles, and varying degrees of iron-loading of mitochondria (Wickramasinghe *et al.*, 1987; Wickramasinghe *et al.*, 1989; Abdalla, 1990). Acute malaria infection is generally associated with reduced total erythropoietic activity. Bone marrow cellularity is often normal or reduced in combination with erythroid hypoplasia suggesting hypo-proliferative erythropoiesis (Abdalla *et al.*, 1980). Whether or not the residual erythropoietic activity is ineffective is unclear. Other studies demonstrated a marked

loss of polychromatic erythroblasts during acute *P. falciparum* infection (Dormer *et al.*, 1983), while others reported dysplastic features of erythroid precursors in the bone marrow of non-immune Europeans with acute-phase malaria infection (Knuttggen, 1987). In addition, ferrokinetic studies in acute malaria show that RBC iron utilisation, a measure of effective erythropoiesis, is reduced (Srichaikul *et al.*, 1967; Wickramasinghe and Abdalla, 2000). However, no evidence of dyserythropoiesis was observed among children in India with acute *P. falciparum* malaria (Das *et al.*, 1999; Jakeman *et al.*, 1999). Based on these observations, it has been proposed that suppressed erythropoiesis plays only a minor role in the pathogenesis of malarial anaemia during acute infection in populations with low malaria transmission (Das *et al.*, 1999; Jakeman *et al.*, 1999). In contrast, chronic malaria infection is often associated with erythroid hyperplasia accompanied by inappropriately low levels of reticulocytosis (Wickramasinghe and Abdalla, 2000) suggesting that ineffective erythropoiesis may play a more significant role in chronic than acute malaria infection. Previously it was observed that there was severe dyserythropoiesis in patients with chronic *P. falciparum* (Weatherall *et al.*, 1983; Abdalla, 1990). Erythrophagocytosis is often present in these patients (Wickramasinghe *et al.*, 1989). Furthermore, a study investigating the cell cycle distribution of erythroblasts from Gambian children with either acute or chronic *P. falciparum* malaria suggested a perturbation of erythroblast kinetics (Wickramasinghe *et al.*, 1982). These abnormalities were more marked in children with chronic than acute malaria and they predominantly affected the early polychromatic erythroblast.

Although SMA is often associated with peripheral parasite density, recent studies found no significant relationship between parasitaemia and disease severity (McElroy *et al.*, 1994; Ong'echa *et al.*, 2006) suggesting that other mechanisms could be involved. Studies have shown that detection of HCM in peripheral circulation is associated with disease

severity in areas of holoendemic transmission and increased mortality in areas of low transmission (Nguyen *et al.*, 1995; Lyke *et al.*, 2003; Mujuzi *et al.*, 2006). However, the mechanism involved is yet to be elucidated.

CHAPTER 3

3.0 MATERIALS AND METHODS**3.1 Study Area**

The study was conducted at Siaya District Hospital (SDH), which is about 30 Kms from Lake Victoria in Nyanza province which is about 30 Kms from Lake Victoria (Appendix 5). Malaria transmission is holoendemic with residents receiving 100-300 infectious bites per person per year (Beier *et al.*, 1994). The mosquito vectors in this area are *An. gambiae s.s.*, *An. arabiensis* and *An. funestus* (Beach *et al.*, 1993). Although malaria transmission is holoendemic in the area, its peaks are experienced just after the long and short rains which start in March-August and November to December, respectively (Bloland *et al.*, 1999). SMA is the primary cause of morbidity and mortality with cerebral manifestations of malaria being only rarely reported (McElroy *et al.*, 1994; Lackritz *et al.*, 1997; Ong'echa *et al.*, 2006).

Annual rainfall ranges between 800mm-2000mm, with an average temperature of 15-30°C. The residents of the district are mainly of the Luo ethnic group (> 96%), the population being homogeneous (Bloland *et al.*, 1999). The poverty level is estimated at 58.02% (Republic of Kenya, 2001). The population of the district is estimated at about 558,989 with 15% of the population being below 5 years of age. The district has an area of about 1,523 Km² (Government of Kenya, 1999). There are 81,400 children below the age of 5 years. The number of women aged between 15-49 years is 134,157. The population growth rate stands at 0.9%. The infant and under 5 years of age mortality rate are 176/1000 and 257/1000 live births, respectively (Siaya District Health System Annual Bulletin, 2003).

3.2 Study Population

Study participants were children below three years of age whose guardians accepted to participate and voluntarily signed informed consent form (Appendix 6).

3.2.1 Sample Size Determination

Sample size was calculated using sample size calculation software (Dupont and Plummer, 1990). To compare haematological and immunological alteration between PCM positive and PCM negative for a confidence interval of 95%, and power of 95%. Assuming an alpha of 0.05, HCM rate among healthy controls of 1%, rate of HCM among cases of 16%. A total of 285 children were enrolled in each arm of the study. Since there are two arms in the study a total of 570 children was enrolled. 30% of participants were randomly selected from each PCM category for cytokine assay.

3.3 Inclusion Criteria

The study participants were included if they had no signs of cerebral malaria, history of transfusion, HIV-1 infection, G6PD deficient or hookworm infestation. Eight hundred and twenty five children were screened, 87 were excluded because they were aparasitaemic with Hb < 11; HIV-1 infection, 38; HbS, 28; G6PD deficient, 19; refusal to give consent by the parent, 37.

Six hundred and sixteen participants who met the inclusion criteria were categorized into five groups: (i) healthy controls (HC; Hb \geq 11.0 g/dL, malaria-negative smear for malaria parasites presenting at the hospital for routine immunization and free from the symptoms of any acute or chronic disease; $n = 59$) (ii) Uncomplicated malaria (UM; Hb \geq 11.0 g/dL; with a malaria-positive smear for *P. falciparum* (of any density) and free from the

symptoms of severe malaria such as hypoglycaemia; $n = 35$), (iii) mild malarial anaemia (MMA; $8.0 \leq \text{Hb} < 11.0$ g/dL; with a malaria-positive smear for *P. falciparum* (of any density) and free from the symptoms of severe malaria such as hypoglycaemia; $n = 141$), (iv) moderate malarial anaemia (MdMA; $6.0 \leq \text{Hb} < 8.0$ g/dL; with a malaria-positive smear for *P. falciparum* (of any density) and free from the symptoms of severe malaria such as hypoglycaemia; $n = 159$), (v) severe malarial anaemia (SMA; $\text{Hb} < 6.0$ g/dL; with a malaria-positive smear for *P. falciparum* (of any density); $n = 222$).

3.4 Definition of Anaemia

Anaemia was defined as $\text{Hb} < 11.0$ g/dl in children less than 5 years residing in developing countries (Akhwale *et al.*, 2004). Although WHO defines SMA as an Hb of < 5 g/dL and parasitaemia of any density, in this study a cut off of 6.0 g/dl was used based on previous Hb distribution within an age- and geographically- matched reference population in western Kenya (McElroy *et al.*, 1999).

3.5 Determination of Parasitaemia

Thick and thin blood films were made from blood samples collected from participants and stained in 3% Giemsa's stain in buffer (pH 7.2) for 45 minutes and examined under oil immersion (WHO, 1991). At least 100 oil immersion fields were examined before a slide was deemed to be negative (WHO, 1991). Parasites were counted against 300 white blood cells (WBC) and parasite density estimated using each participant's WBC count obtained from a Coulter® AcT diff2™ counter (Beckman Coulter Corp., Miami, USA).

3.6 Haemozoin Counts from Monocytes and Neutrophils

On each slide, 100 neutrophils and 30 monocytes were examined for Hz (Nguyen *et al.*, 1995; Lyke *et al.*, 2003). The percentage haemozoination of monocyte was calculated as follows:

- Haemozoin containing monocytes (HCM)/ μl = (number of monocytes with haemozoin, HCM/30) \times (absolute WBC \times percent of monocytes).
- Haemozoin Containing Neutrophils (HCN) per microlitre = (number of haemozoinated neutrophils, HCN/100) \times (absolute WBC \times percent of neutrophils).

The level of Hz was scored as 1 = 0% PC; 2 = < 10% HCM (Low); and 3 = \geq 10% HCM (High) (Were *et al.*, 2006). Neutrophils were scored in the same manner. To investigate the relationship between HCM and HCN with MA in these children, the level of haemozoination was compared with haematological and immunological parameters.

3.7 Hookworm Investigation

To rule out anaemia due to hookworm infection, Ritchie's formal ether concentration method was performed (Zimmerman and Needham, 1995). Briefly, a pea-size stool sample was emulsified thoroughly in 7 ml of 4% formalin and strained through four layers of wet gauze into a centrifuge tube and 3 ml of ether added to dissolve fats and reduce the specific gravity of the debris. The emulsion was centrifuged at 400-500g for 3 minutes. The supernatant was discarded and the deposits stained with iodine solution and examined for intestinal helminths (Zimmerman and Needham, 1995).

3.8 Reticulocyte Count

Reticulocyte count was carried out as previously described (Rowan *et al.*, 1996). Briefly, equal volume of fresh blood and new methylene blue were mixed and incubated

at 37°C for 30 minutes after which smears were made. Reticulocyte count was determined by counting the number of reticulocytes per 1000 mature RBCs and expressing the reticulocyte count as a percentage of the total (reticulocytes and mature RBCs) (Rowan *et al.*, 1996).

3.9 Full Haemogram Measurement

Full haemogram was determined using a Coulter® A^oT diff2™ (Beckman Coulter Corp., Miami, USA). It works on the principle that if a tube with a small aperture on the wall is immersed into a suspension of low electrolyte concentration, the particles pass through the aperture causing the displacement of the volume of electrolyte equivalent to the volume of the particles from the sensing zone. This causes a short impedance across the aperture. This change can be measured as a voltage pulse. The pulse height is proportional to the volume of the sensed particles.

3.10 Multiplex Assay

Invitrogen's multiplex bead immunoassay kits are developed to maximize flexibility in experimental design, permitting the measurement of one or multiple proteins in panels.

3.10.1 Principle of the Assay

Beads of defined spectral properties are conjugated to protein-specific capture antibodies and added along with samples (including standards of known protein concentration, control samples, and test samples), into the wells of a filter-bottom microtitre plate where proteins bind to the capture antibodies during a 2 hour incubation. After washing the beads, protein-specific biotinylated detector antibodies are added and incubated with the beads for 1 hour. During this incubation, the protein-specific biotinylated detector

antibodies bind to the appropriate immobilized proteins. After removal of excess biotinylated detector antibodies, streptavidin conjugated to the fluorescent protein, R-Phycoerythrin (Streptavidin-RPE), is added and allowed to incubate for 30 minutes. The Streptavidin-RPE binds to the biotinylated detector antibodies associated with the immune complexes on the beads, forming a four-member solid phase sandwich. After washing, the beads were analyzed with the Luminex detection system.

3.11 Ethical Considerations

The National Ethical Review Board (NERB) of KEMRI, the National Institute of Health (NIH), Ethical Review Committees of the University of Pittsburgh and University of New Mexico granted permission for this study. Any information that was collected about the patient was treated with confidentiality. Written consent was obtained from participants before they were entered into the study. Participation in the study was voluntary.

3.12 Statistical Analysis

Data was entered into SPSS program (SPSS 12.0 for windows, SPSS Inc., Chicago, IL). The difference between the proportion of children with HCM and those without were compared using chi-square test while the difference between the clinical groups was compared using Kruskal Wallis test and where significance was noted, pair-wise comparison was done using Mann-Whitney U test for medians. Spearman's correlation analysis was used to determine the relationship between HCM, parasitaemia and anaemia and also between cytokines, haemoglobin and parasitaemia. All tests were two-tailed with P values < 0.05 being considered significant.

CHAPTER 4

4.0 RESULTS

4.1 The Effect of Monocyte Acquired Hz on the Production of Interleukin IL-10, IL-12, Tumour Necrosis Factor- α (TNF- α) and Interferon - γ (IFN- γ) in Children with MA

4.1.1 Clinical and Demographic Characteristics of Study Participants

The clinical and demographic characteristics of study participants ($n = 616$) are summarized in Table 1. Proportions of males versus females were comparable among the clinical groups; healthy controls (HC), uncomplicated malaria (UM), mild malarial anaemia (M/MA), moderate malarial anaemia (MdMA), and severe malaria anaemia (SMA) ($P = 0.102$). However, age ($P < 0.0001$), axillary temperature ($P < 0.0001$) and glucose levels ($P = 0.010$) differed significantly between the clinical groups. In addition, no significant differences in the age and temperature were noted between the parasitaemic groups (UM, M/MA, MdMA, and SMA) ($P > 0.050$). SMA was more prevalent among the youngest children [median age of 8.0 (5.9-13.0) months]. Relative to the HC group, glucose levels were significantly elevated among children with UM ($P = 0.017$), M/MA ($P = 0.004$), and SMA ($P = 0.001$), but not MdMA ($P = 0.078$). However, glucose levels were comparable among the parasitaemic groups.

Table 1: Demographic and Clinical Characteristics of Study Participants at enrollment

Characteristics	HC (n=59)	UM (n=35)	M/MA (n=141)	MdMA (n=159)	SMA (n=222)	<i>p</i>
Age (months)	6.0 (4.0-11.0)	9.5 (6.3-21.8)*	9.5 (6.0-17.0)**	12.0 (7-18.0)**	8.0 (5.9-13.0)*	<0.0001 ^a
Gender (M/F)	23/36	16/19	83/58	77/82	110/112	0.102 ^b
Temperature (°C)	36.6 (36.0-37.1)	37.8 (36.6-39.3)**	37.5 (36.9-38.6)**	37.6 (36.5-39.0)**	37.5 (36.9-38.5)**	<0.0001 ^a
Glucose (mmol/L)	4.8 (4.2-5.2)	5.2 (4.7-6.1)*	5.3 (4.6-6.2)*	5.0 (4.4-5.8)	5.1 (4.6-6.1)**	0.010 ^a

HC= Healthy Controls; UM=Uncomplicated Malaria; M/MA= Mild Malarial Anaemia; MdMA= Moderate Malarial Anaemia; SMA=Severe Malarial Anaemia. Data are presented as median (Q1-Q3) unless otherwise stated. *denote $P < 0.05$; ** denote $P < 0.001$

4.2 Parasitological Parameters of Study Subjects Grouped By Clinical Status

The parasite density and prevalence of high density parasitaemia (HDP; defined as parasitaemia $> 10,000/\mu\text{L}$) were comparable among the different categories (UM, M/MA, MdMA, and SMA) ($P = 0.251$ and $P = 0.526$, respectively; Table 2). The overall prevalence of HCM and HCN was 48.7% and 9.3%, respectively. The density of HCM differed significantly among the parasitaemic groups with the SMA group having the highest count ($P < 0.0001$), with prevalence of HCM increasing with MA severity ($P < 0.0001$). The density of HCN between parasitaemic groups were comparable ($P < 0.065$). Although the prevalence of HCN differed significantly among the parasitaemic groups ($P = 0.048$), it comprised only 9.3% ($n=57$) of the study population and all the individuals with HCN also had HCM. Deposition of H₂ on monocytes was therefore used as the index of haemozoination.

Table 2: Parasitological Characteristics of Participants at Enrollment

Characteristics	UM (n=35)	M/MA (n=141)	MdMA (n=159)	SMA (n=222)	<i>P</i>
Parasitaemia (μL)	47,480 (22,200-91,464)	27,947 (7,912-76,632)	26,669 (8,168-63,020)	28,468 (8,790-69,343)	0.251 ^a
HDP, <i>n</i> (%)	29 (82.9)	103 (72.5)	112 (70.4)	163 (72.4)	0.526 ^b
HCM /mm ³	0 (0-0)	0 (0-0)**	0 (0-309)**	1,224 (0-3,532)**	<0.0001 ^a
HCM, <i>n</i> (%)	3 (8.6)*	42 (29.8)**	70 (44.0)**	156 (70.3)**	<0.0001 ^b
HCN/mm ³	0 (0-0)	0 (0-0)*	0 (0-0)	0 (0-0)**	0.065 ^a
HCN, <i>n</i> (%)	2 (5.7)	12 (8.5)	8 (5.0)	30 (13.1)*	0.048 ^b

UM = Uncompleted Malaria; M/MA= Mild Malarial Anaemia; MdMA= Moderate Malarial Anaemia; SMA= Severe Malarial Anaemia; HDP = High Density Parasitaemia; HCM = Haemozoin Containing Monocyte; HCN = Haemozoin Containing Neutrophils. Data are presented as median (Q1-Q3) unless otherwise stated. * denotes *P*-value < 0.05 while ** denotes *P*-value < 0.001.

4.3 Determination of the Relationship between *in Vivo* Innate Inflammatory Mediator Production and Monocyte Acquisition of *Pf*Hz

To examine *in vivo* relationships between IL-12p70, IL-10, IFN- γ , TNF- α and their ratios, children were categorised into groups with different severities of malarial anaemia (Table 3). Circulating IL-10 levels were significantly elevated across the clinical categories (HC, UM, M/MA, MdMA, and SMA) ($P < 0.0001$). Pair-wise comparison revealed that relative to the HC, the IL-10 levels increased significantly in the UM ($P = 0.003$), M/MA ($P < 0.0001$), MdMA ($P < 0.012$), and SMA ($P < 0.0001$) groups. The highest levels were noted among M/MA group, from which point, levels decreased as the disease progressed in severity. A significant decrease was noted among the MdMA and SMA compared to M/MA ($P = 0.016$ and $P = 0.020$, respectively). Circulating IL-12p70 levels were significantly decreased across clinical groups ($P < 0.001$), with MdMA group having the lowest levels (Table 3). In addition, pair-wise comparison revealed significant reduction of circulating IL-12p70 in the UM ($P = 0.015$), MdMA ($P = 0.041$), and SMA ($P = 0.001$) groups relative to the HC group. Plasma IFN- γ levels were significantly different across the groups ($P = 0.044$). Pair-wise comparison illustrated that IFN- γ levels were significantly reduced among HC vs. SMA ($P = 0.028$). The circulating TNF- α levels were comparable between the groups ($P = 0.809$). The production of IL-10 relative to that of IL-12p70 increased significantly across clinical categories ($P < 0.0001$); with M/MA group having the highest IL-10/IL-12p70 ratio [0.8 (0.4-2.4)]. Pair-wise comparison illustrated that IL-10/IL-12p70 ratio was significantly higher in all the parasitaemic groups relative to the HC group ($P < 0.0001$ for all the comparisons). Similarly, the ratio of IL-10/IFN- γ was significantly increased across the clinical groups ($P < 0.0001$). Further comparison revealed that M/MA, MdMA and SMA groups had significantly increased IL-10/IFN- γ ratios relative to the HC group ($P < 0.0001$, $P = 0.004$, and $P < 0.0001$, respectively), but not the UM group ($P = 0.132$). IL-10/TNF- α ratio varied

significantly across the clinical categories ($P < 0.0001$). The highest levels were noted among the M/MA group [15.0 (5.3-51.0)]. Significant increase in the IL-10/TNF- α ratio was noted among the UM and M/MA compared to HC, ($P = 0.015$, and $P < 0.0001$, respectively). IL-12p70/IFN- γ and IL-12p70/TNF- α ratios were not significantly different across the clinical groups ($P = 0.077$) and ($P = 0.057$).

Table 1: Cytokine Levels and Cytokine Ratios among Children with Malarial Anaemia

Cytokine/ratio	HC (n=22)	UM (n=26)	M/MA (n=54)	MdMA (n=24)	SMA (n=109)	<i>P</i>
IL-10	69.3 (31-158)	300 (84-944)*	570 (176-925)**	160 (54-687)*	259 (118-615)**	<0.0001
IL-12p70	661 (355-984)	389 (233-598)*	443 (321-713)	261 (242-385)*	358 (272-504)**	<0.001
IFN- γ	25 (0.2-72.1)	21 (2.1-94.8)	11.4 (2.5-41.4)	11.4 (1.9-23.9)	4.6 (1.8-21.6)*	0.044
TNF- α	29 (5.7-107)	31 (15-49)	31 (10-65)	22 (14-48)	32 (17-76)	0.809
IL-10/IL-12p70	0.1 (0.07-0.2)	0.6 (0.2-2.0)**	0.8 (0.4-2.4)**	0.4 (0.2-1.3)**	0.6 (0.3-1.4)**	<0.0001
IL-10/IFN- γ	2.6 (1.1-23.4)	20.5 (1.3-137)	62.5 (7.7-395)**	24.9 (7-151)*	54.7 (10.9-197)**	<0.0001
IL-10/TNF- α	2.2 (0.6-5.4)	11.7 (2.7-30)*	15 (5.3-51)**	6.8 (3.3-22)**	9.3 (4.0-17)**	<0.0001
IL-12p70/IFN- γ	23 (6.4-237)	19 (3.7-84)	35 (12.6-361)	30 (19-391)	65 (18.7-226)	0.077
IL-12p70/TNF- α	24.3 (10-53.4)	13.3 (8.7-16.6)	18.6 (7.5-41)	19.7 (11.1-27.5)	11.9 (6.5-25.3)	0.057

Interleukin (IL-10); Interleukin (IL-12p70); IFN- γ = Interferon gamma; TNF- α = Tumour Necrosis Factor- alpha. HC= Healthy Control; UM = Uncompleted Malaria; M/MA= Mild Malarial Anaemia; MdMA= Moderate Malarial Anaemia; SMA= Severe Malarial Anaemia. Data are presented as median (Q1-Q3) unless otherwise stated. * denotes $P < 0.05$ while ** denotes $P < 0.001$

4.4 Determination of the Relationship between HCM and Cytokine Production

Since Hz is acquired naturally during malaria, this study investigated whether *in vivo* acquisition of Hz was associated with alterations of cytokine profiles among malaria infected children. The present study demonstrate that IL-10 levels increased significantly with the increasing deposition of Hz in monocytes across the HCM categories ($P < 0.001$) whereas IL-12p70 levels were comparable across the HCM categories ($P = 0.138$) (Table 4). Circulating IFN- γ levels were significantly decreased across the HCM categories ($P = 0.016$). Relative to IL-10, the ratio of IL-10 to TNF- α levels increased significantly with increasing Hz deposition in monocytes ($P < 0.002$) with the highest ratio being in the < 10% HCM ($P < 0.001$) relative to the HC group. Further analysis revealed that relative to the HC group, circulating IFN- γ levels were significantly decreased in the <10% HCM ($P=0.008$) but not $\geq 10\%$ HCM ($P = 0.111$). The relative production of IL-12 and IL-10 increased significantly across the HCM categories ($P < 0.001$). The relative production of IL-10 and IFN- γ were significantly increased across the HCM categories ($P < 0.001$), with the <10% HCM group having the highest ratio relative to the HC group ($P < 0.001$). In addition, the ratio of IL-12p70 and IFN- γ significantly differed across the HCM categories ($P = 0.043$). There was a significant increase in the IL-12p70/IFN- γ ratio in the < 10% HCM ($P = 0.015$) but not in $\geq 10\%$ HCM ($P = 0.301$) relative to the HC group.

Table 4 : Relationship between HCM and Cytokine Production

Cytokine /ratio	HC (n=21)	0%HCM (n=108)	<10%HCM (n=24)	≥10%HCM (n=82)	<i>P</i>
IL-10	69.3(31-158)	232 (86-712)**	290 (175-757)**	239 (117-710)**	<0.001
IL-12p70	661(355-984)	408 (298-661)	473 (304-781)	368 (266-484)	0.138
IFN- γ	25 (0.2-72.1)	11.4 (2.0-41.8)	2.1 (0.9-24.0)*	6.3 (1.8-24.2)	0.016
TNF- α	29 (2.6- 1.1-23.4)	28 (9.7-58)	35 (2.2-76)	32 (1.5-173)	0.285
IL-10/IL-12p70	0.1 (0.07-0.2)	0.5 (0.2-1.5)**	0.6 (0.3-1.3)**	0.6 (0.3-1.6)**	0.001
IL-10/TNF- α	2.6(1.1-23.4)	8.5 (3.5-29)**	9.6 (3.8-14.4)*	9.0 (3.6-18.7)**	0.002
IL-10/IFN- γ	2.2 (0.6-5.4)	27 (2.7-136)*	122 (25.4-495)**	41 (10.4-193)**	0.001
IL-12/TNF- α	23(6.4-237)	16 (8.4-35)	13 (6.0-27)	12 (6.6-28)	0.473
IL-12/IFN- γ	24.3(10-53.4)	34 (10.2-188)	265 (29-565)*	52 (17-185)	0.043

Interleukin (IL-10); Interleukin (IL-12p70); IFN- γ = Interferon Gamma; TNF- α = Tumour Necrosis Factor-alpha. Data are presented as median (Q1-Q3) unless otherwise stated. * denotes *P*-value < 0.05 relative to the 0% HCM group.

4.5 Effects of Naturally Acquired *Pf*H_z in Production of Cytokine

To determine if suppression of circulating IL-12p70 and over-expression of IL-10 and/or TNF- α in children with SMA were related to mononuclear cell ingestion of *Pf*H_z, children were categorised into groups according to the presence and absence of HCM in circulation. IL-12p70 levels were reduced in HCM (+) group ($P < 0.001$) (Figure 1), while IL-10 levels were significantly elevated in HCM (+) group ($P = 0.028$) (Figure 1B). In contrast, circulating levels of TNF- α and IFN- γ were comparable between the HCM groups ($P = 0.153$, and $P = 0.552$, respectively; Figure 1C and 1D). Additional analyses revealed that IL-10/IL-12p70, IL-10/TNF- α , IL-10/IFN- γ , and IL-12p70/IFN- γ were also comparable between the HCM groups ($P = 0.133$, $P = 0.516$, $P = 0.805$, and $P = 0.295$, respectively; Figure 1E, 1F, 1G, 1D, and 1H, respectively). However, IL-12p70/TNF- α ratio was significantly lower in the HCM (+) group relative to the HCM (-) group ($P = 0.002$) (Figure 1I).

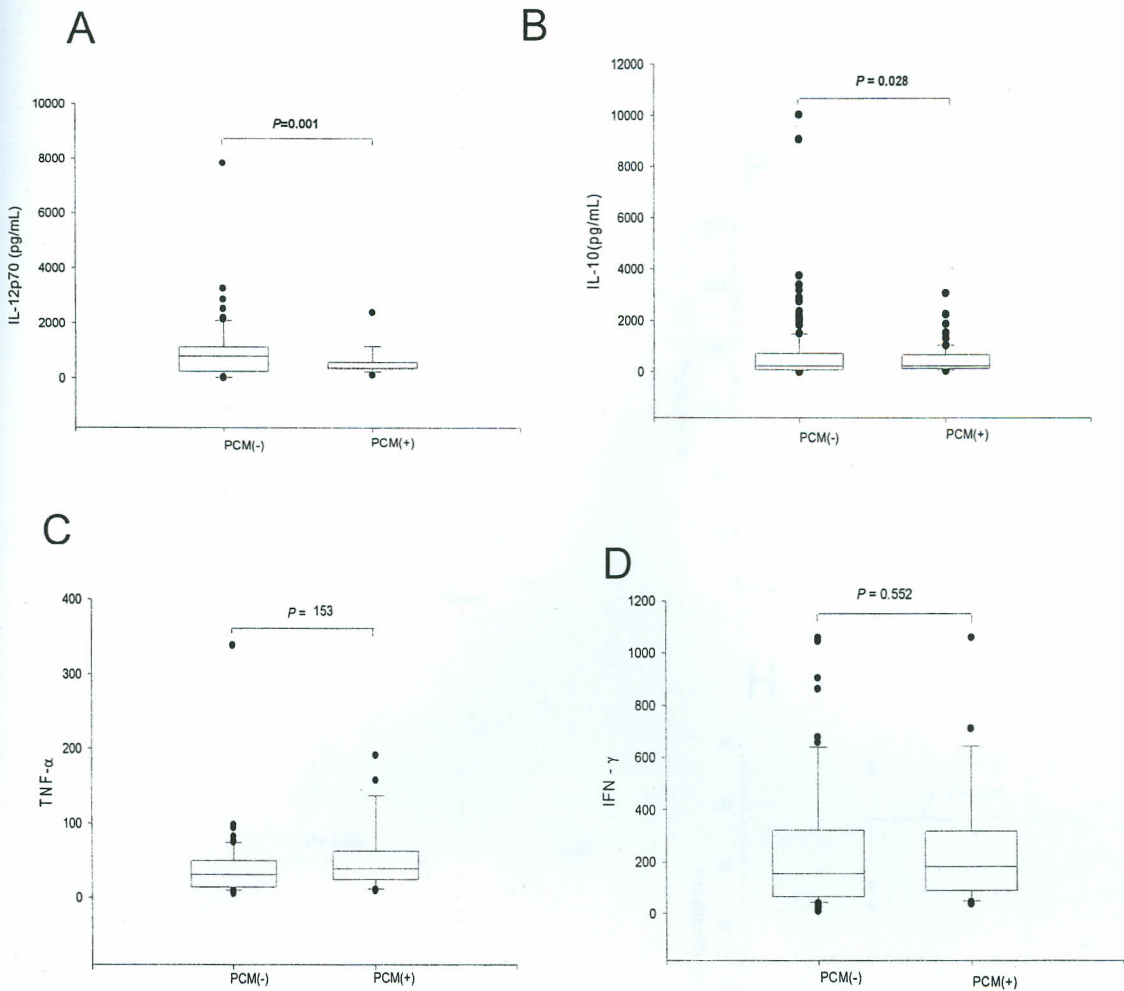


Figure 1: Effects of Naturally Acquired *PfHz* on Circulating Cytokine Concentration.

Children with acute *P. falciparum* (n = 235) were grouped according to the presence or absence of HCM as follows: HCM (-) (n = 159); and HCM (+) (n = 76). Plasma levels of (A), IL-12p70; (B), IL-10; (C) - TNF- α and (D), IFN- γ were determined by a Multiplex bead assay. Each box represents the interquartile range while the line through the box represents the median. The whiskers show the 10th and the 90th percentiles and the closed circles are outliers. Statistical difference was determined by Mann-Whitney U test for medians.

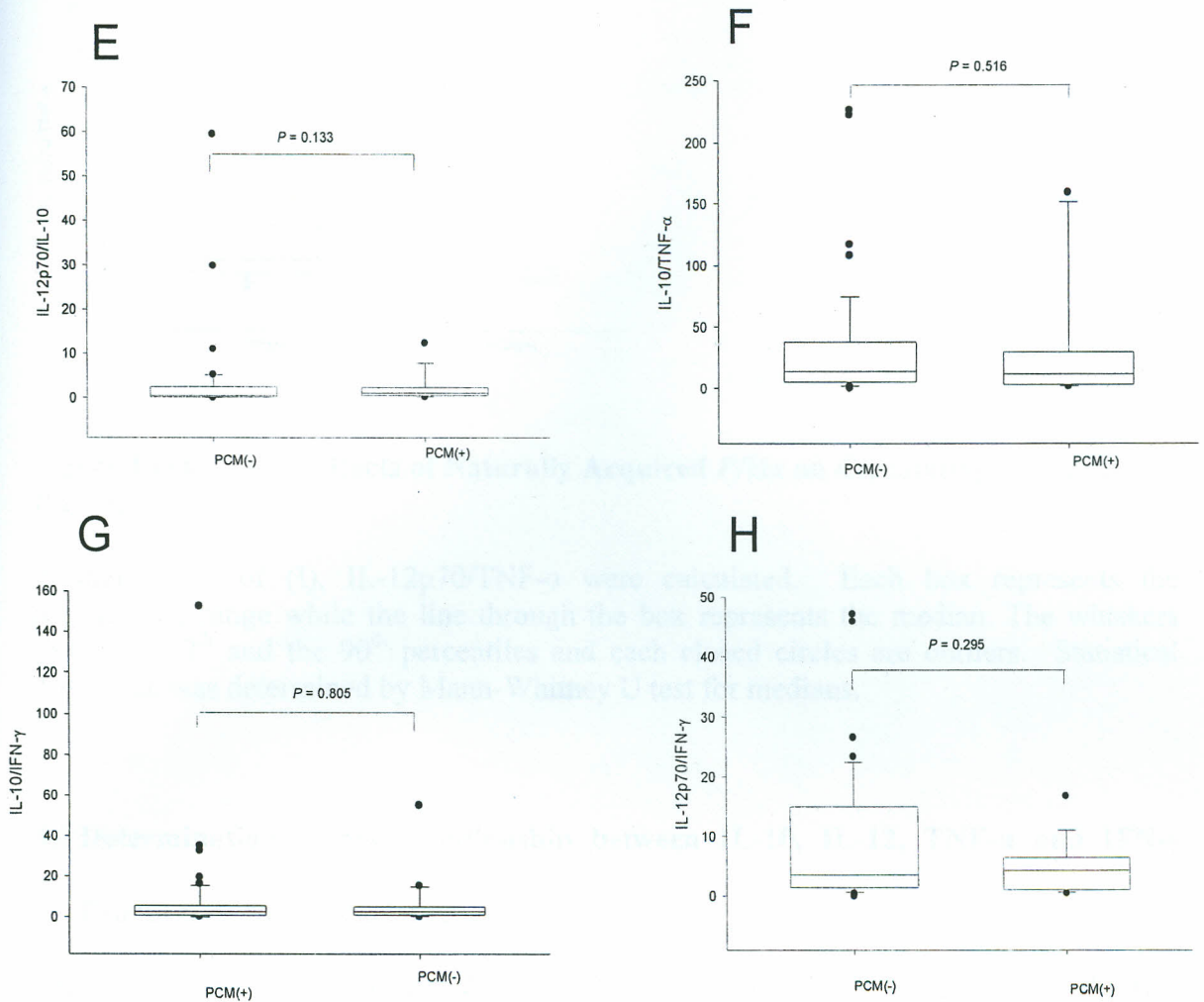


Figure 1 continued: Effects of Naturally Acquired *PfHz* on Circulating Cytokine Ratios.

Interleukin (IL-10); Interleukin (IL-12p70); IFN- γ = Interferon gamma; TNF- α = Tumour necrosis factor-alpha. Relative plasma levels of: (E), IL-10/IL-12p70 (F), IL-10/TNF- α ; (G), IL-10/IFN- γ and IL-12p70/IFN- γ (H) and IL-12p70/TNF- α (I) were calculated. Each box represents the interquartile range while the line through the box represents the median. The whiskers show the 10th and the 90th percentiles and each closed circles are outliers.

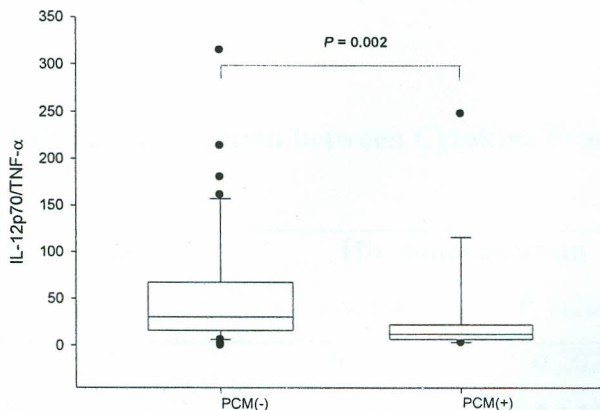


Figure 1 continued: Effects of Naturally Acquired *Pf*Hz on Circulating Cytokine Ratios.

Plasma levels of (I), IL-12p70/TNF- α were calculated. Each box represents the interquartile range while the line through the box represents the median. The whiskers show the 10th and the 90th percentiles and each closed circles are outliers. Statistical difference was determined by Mann-Whitney U test for medians.

4.6 Determination of the Relationship between IL-10, IL-12, TNF- α and IFN- γ

Production and Malarial Anaemia

The relationships between circulating cytokine concentrations (and ratios) and the two primary end points of disease severity in areas with holoendemic transmission (i.e., the Hb concentration and parasitaemia level) were examined by Spearman's correlation analysis. Results showed that plasma levels of IFN- γ ($r = 0.184$, $P < 0.008$) and IL-12p70/IFN- γ ratio ($r = -0.164$, $P < 0.017$), but not IL-10, IL-12p70, or TNF- α were significantly correlated with the Hb concentrations (Table 5). Examination of absolute cytokine levels and cytokine ratios revealed that IL-12p70, IL-10/IFN- γ and IL-12p70/TNF- α ratios were inversely correlated with parasitemia ($r = -0.159$, $P = 0.017$; $r = -0.190$, $P = 0.006$; $r = -0.247$, $P < 0.0001$, respectively), while IL-10, TNF- α , and IL-

IL-12p70/IL-10 were positively associated with parasitaemia ($r = 0.244$, $P < 0.0001$; $r = 0.144$, $P = 0.037$; $r = 0.337$, $P < 0.0001$, respectively; (Table 5).

Table 5: Association between Cytokine Production and Disease Severity

Cytokine	Hb concentration		Parasitaemia/ μ l	
	r-value	P-value.	r-value	P-value
IL-12p70	0.088	0.202	-0.159	0.017
IL-10	-0.007	0.159	0.244	<0.0001
TNF- α	-0.035	0.611	0.144	0.037
IFN- γ	0.184	0.008	-0.066	0.342
IL-12p70/IL-10	0.042	0.555	0.337	<0.0001
IL-10/TNF- α	-0.097	0.159	0.118	0.086
IL-10/IFN- γ	-0.103	0.137	-0.190	0.006
IL-12p70/TNF- α	0.074	0.285	-0.247	<0.0001
IL-12p70/IFN- γ	-0.164	0.017	0.010	0.883

Relationship between haemoglobin levels, parasitaemia/ μ L and cytokine parameters were determined for children with malaria ($n = 213$) using the Spearman's correlation analysis. Significance level was set at $P < 0.05$. The figures in bold represent relationships those were significantly associated.

4.7 Erythrocytic Characteristics of Study Participants at Enrollment

A significant decrease in the Hb levels, RBC counts and indices with increasing malarial anaemia was observed ($P < 0.0001$ in all cases) (Table 6). Pair-wise comparison demonstrated a significant decrease in the Hb levels, RBC counts and RBC indices (Hct, MCV and MCH) between the MA groups relative to the HC group ($P < 0.0001$ in all cases). Although the UM group had significantly reduced RBC counts and Hb levels relative to the HC group, the RBC indices (Hct, MCV, MCH and MCHC) were comparable between the two groups ($P > 0.050$). Reticulocyte production index (RPI) was stratified as $RPI < 2$ and $RPI \geq 3$ (Koepke and Koepke, 1986). RPI was significantly different across clinical categories ($P = 0.027$). Pair-wise comparison did not reveal significant differences between HC vs. UM, *M/MA*, *MdMA* and *SMA* ($P = 0.984$), ($P = 0.489$), ($P = 0.562$), ($P = 0.121$), respectively. Significant differences were however noted between UM vs. *M/MA*, *MdMA*, *SMA* ($P = 0.006$), ($P = 0.015$), ($P = 0.006$), respectively. On the other hand $RPI > 3$ was similar across clinical categories ($P = 0.259$).

Table 6: Erythrocytic Counts and Indices of Study Participants at enrollment

Characteristics	HC (n=59)	UM (n=36)	M/MA (n=141)	MdMA (n=159)	SMA (n=221)	<i>p</i>
RBC ($\times 10^6/\mu\text{L}$)	4.9 (4.7-5.5)	4.8 (4.4-5.2)**	4.3 (4.0-4.6)**	3.2 (2.9-3.9)**	2.1 (1.7-2.6)**	<0.0001^a
Haemoglobin (g/dL)	11.6 (11.3-12.0)	11.4 (11.1-12.0)*	9.6 (8.8-10.3)**	6.6 (6.2-7.2)**	4.8 (4.1-5.5)**	<0.0001^a
Haematocrit (%)	35.6 (34.6-36.9)	35.4 (33.6-36.9)	29.5 (27.4-31.7)**	21.2 (19.8-23.1)**	15.3 (12.6-17.5)**	<0.0001^a
MCV (fl)	72 (69-74.5)	76.0 (68.4-79.3)	69.2 (63.1-73.4)**	67.8 (61.8-74)*	71.7 (64.5-77.2)	<0.0001^a
MCH (pg)	23.6 (22.3-24.8)	25.1 (23.0-26)	22.6 (20.4-24.1)**	21.3 (19.2-23.7)**	22.6 (20.3-25.0)*	<0.0001^a
MCHC (g/dL)	33 (32-33.5)	33.4 (32.4-33.7)	32.4 (31.4-33.1)	31.7 (30-33)**	31.7 (30-33.1)**	<0.0001^a
RPI (%)	0.6 (0.4-1.0)	0.6 (0.4-1.1)	0.7 (0.4-1.2)	0.7 (0.3-1.3)	0.5 (0.3-1.1)	0.027
RPI<2 <i>n</i> , (%)	53 (89.8)	37 (88.6)	135 (95.0)	140 (88.1)	212 (95.5)	0.022^b
RPI>3 <i>n</i> , (%)	2 (3.4)	2 (5.7)	2 (1.4)	2 (1.3)	4 (1.8)	0.259 ^b

UM = Uncompleted malaria; M/MA = Mild Malarial Anaemia; MdMA = Moderate Malarial Anaemia; SMA = Severe Malarial Anaemia. MCV = Mean Corpuscular Volume; MCH = Mean Cell Haemoglobin; MCHC = Mean Cell Haemoglobin Concentration; RPI= Reticulocyte Production Index. Data presented as Median (Q1-Q3). *denotes *P*-value < 0.05 while **denotes *P*-value <0.001.

4.8 Leucocytic Characteristics of Study Participants at Enrollment

WBC count differed significantly across the clinical categories ($P < 0.0001$) (Table 7). Relative to the HC group, the WBC counts were significantly increased in the SMA group ($P < 0.050$), but comparable with the UM, M/MA and MdMA groups ($P > 0.050$). The lymphocyte counts also differed significantly across the clinical groups ($P < 0.0001$), with the UM ($P < 0.010$), M/MA ($P < 0.050$), and MdMA ($P < 0.050$) groups having significantly reduced lymphocyte counts relative to the HC group. However, the SMA and HC groups had comparable levels of lymphocytes. The monocyte counts also differed significantly across the clinical groups ($P < 0.0001$), with the MdMA ($P < 0.050$) and SMA ($P < 0.010$) groups having significantly elevated monocyte counts relative to the HC group. The granulocyte counts differed significantly across the clinical groups ($P < 0.0001$); being significantly elevated in all the parasitaemic groups relative to the HC group ($P < 0.010$ for all the comparisons). The platelet counts were also significantly elevated across the clinical categories ($P < 0.0001$), being highest in the HC group and progressively decreasing with MA severity (HC>UM>M/MA>MdMA>SMA). Relative to the HC group, all the parasitaemic groups had significantly reduced platelet counts ($P < 0.010$ for all the comparisons).

Table 7: Levels of Leucocytes among Study Participants

Characteristics	HC (n=59)	UM (n=36)	M/MA (n=141)	MdMA (n=159)	SMA (n=221)	<i>p</i>
WBC ($\times 10^3/\mu\text{L}$)	10.1 (8.1-14.3)	11.9 (8.7-16)	11.0 (8.8-14.1)	11.3 (9.0-15.7)	13.5 (9.8-15.7)*	<0.0001
Lymphocytes ($\times 10^9/\mu\text{L}$)	6.5 (4.5-8.1)	3.9 (2.9-5.9)**	5.2 (3.7-7.8)*	5.8 (4.2-8.5)*	6.6 (4.4-9.8)	<0.0001
Monocytes ($\times 10^9/\mu\text{L}$)	0.8 (0.6-1.1)	0.8 (0.5-1.1)	0.9 (0.6-1.2)	1.1 (0.7-1.5)*	1.4 (0.8-2.1)**	<0.0001
Granulocytes ($\times 10^9/\mu\text{L}$)	3.0 (1.9-4.2)	6.6 (3.8-9.8)**	4.5 (3.1-6.3)**	4.1 (2.4-7.0)**	4.8 (3.1-7.4)**	<0.0001
Platelets ($\times 10^3/\mu\text{L}$)	412 (308-521)	257 (208-350)**	199 (106-279)**	157 (103-229)**	148 (108 -204)**	<0.0001

UM = Uncompleted malaria; M/MA = Mild Malarial Anaemia; MdMA= Moderate Malarial Anaemia; SMA = Severe Malarial Anaemia; WBC = White blood cells. Data presented as Median (Q1-Q3). * denotes *P*-value < 0.05 while ** denotes *P*-value < 0.01.

4.9 The Effects of Naturally-Acquired Hz on Haematological Parameters

To investigate the role of HCM on haematological alterations, 557 participants with malaria (Table 8) were categorised according to various levels of HCM as follows: 0% HCM, ($n=283$), < 10% HCM ($n=76$), and $\geq 10\%$ HCM ($n=198$). Results revealed that parasitaemia differed significantly across the HCM categories ($P < 0.01$) with an increasing parasitaemia being associated with increasing HCM density (Table 8). Further analyses showed that parasite density was significantly higher in the $\geq 10\%$ HCM ($P < 0.003$) group relative to the 0% HCM group. The prevalence of HDP did not differ significantly across the HCM groups ($P < 0.066$), but ironically, the 0% HCM group had significantly more individuals with HDP relative to the < 10% HCM ($P < 0.049$) but not $\geq 10\%$ HCM ($P < 0.080$). However, there was no significant difference in the parasite densities or prevalence of HDP between the < 10% HCM and $\geq 10\%$ HCM groups ($P = 0.682$). The prevalence of SMA also differed significantly across the HCM categories with the < 10% HCM ($P < 0.01$) and $\geq 10\%$ HCM ($P < 0.01$) groups having significantly more individuals relative to the 0% HCM group.

The RBC counts, Hb levels, and haematocrit were significantly reduced among the three HCM categories ($P < 0.0001$). Children with < 10% HCM, as well as those with $\geq 10\%$ HCM, had significantly lower RBC counts, Hb levels, and Hct relative to children with *P. falciparum* parasitaemia without HCM ($P < 0.01$ for all cases). The RBC indices MCV and MCH were comparable among the HCM categories ($P = 0.150$ and $P = 0.559$, respectively). MCHC, on the other hand, differed significantly among the HCM groups ($P = 0.024$) with the < 10% HCM group having lower MCHC relative to children with no HCM ($P < 0.05$). RPI, a measure of the erythropoietic response, was comparable across the HCM categories ($P = 0.219$). Similarly, RPI < 2 (suppression of erythropoiesis) and

RPI ≥ 3 (appropriate erythropoietic response for the degree of anaemia) were comparable among the different HCM categories ($P = 0.761$ and $P = 0.070$, respectively).

Table 8: The Relationship between HCM and Erythrocytic Parameters

Characteristics	0% HCM (<i>n</i> = 285)	< 10% HCM (<i>n</i> = 111)	≥ 10% HCM (<i>n</i> = 160)	<i>P</i>
Parasitological parameters				
Parasitaemia / μ L	26,025 (7,006-63,249)	26,880 (10,437-68,677)**	42,086 (10,482-101,745)**	<0.0001 ^a
HDP, <i>n</i> (%)	195 (68.4)	87 (78.4)**	122 (76.3)**	<0.066 ^b
SMA, <i>n</i> (%)	65 (22.8)	58 (52.3)**	98 (61.3)**	<0.0001 ^b
RBC indices				
RBC ($\times 10^6/\mu$ L)	3.9 (2.9-4.7)	2.8 (2.2-3.7)**	2.6 (1.9-3.2)**	<0.0001 ^a
Hb (g/dL)	8.3 (6.3-11.0)	5.9 (4.9-7.7)**	5.6 (4.6-6.7)**	<0.0001 ^a
Hct (%)	25.7 (20.2-32.9)	19.0 (15.9-24.1) **	17.5 (14.0-21.5)**	<0.0001 ^a
MCV (fl)	70.0 (64.1-74.7)	71.7 (64.0-78.6)	69.5 (64.0-75.8)	0.150 ^a
MCH (pg)	22.6 (20.4-24.4)	23.0 (20.3-23.1)	22.3 (20.5-24.5)	0.559 ^a
MCHC (g/dL)	32.4 (31.3-33.3)	31.9 (30.7-33)*	32.0 (30.5-33.4)	0.024 ^a
RPI	0.6 (0.4-1.1)	0.6 (0.3-1.3)	0.5 (0.3-1.2)	0.219 ^a
RPI < 2 <i>n</i> (%)	266 (92.4)	67 (60.3)	150 (93.9)	0.583 ^b
RPI ≥ 3 <i>n</i> (%)	5 (1.7)	3 (4.1)	2 (1.0)	0.070 ^b

HCM = Haemozoin Containing Monocytes; HDP = High Density Parasitaemia; SMA = Severe Malarial Anaemia; RBC = Red Blood Cells; Hb = Haemoglobin; MCV = Mean Corpuscular Volume; MCH = Mean Cell Haemoglobin; MCHC = Mean Cell Haemoglobin Concentration; RPI = Reticulocyte Production Index

4.10 The Effects of Naturally-Acquired Hz on leucocytic parameters

The WBC, lymphocyte, monocyte, and platelet counts differed significantly across the HCM groups ($P < 0.0001$ for all cases; Table 9). However, the granulocyte counts were comparable among the three HCM categories ($P = 0.340$). The MPV significantly increased while Pct significantly decreased with increasing HCM ($P < 0.0001$ for both). In addition, pair-wise comparisons revealed that relative to the 0% HCM group, both $< 10\%$ HCM and $\geq 10\%$ HCM groups had significantly increased MPV ($P < 0.05$ and $P < 0.01$, respectively) and decreased Pct ($P < 0.01$ for both).

Table 9: The Relationship between HCM and Leucocytic and Platelets Parameters

Characteristics	0% HCM (n = 285)	< 10% HCM (n = 111)	≥ 10% HCM (n = 160)	P
WBC indices				
WBC ($\times 10^3/\mu\text{L}$)	10.8 (8.6-14.6)	11.1 (8.3-16.0)	12.7 (10.2-17.2)**	<0.0001
Lymphocytes ($\times 10^3/\mu\text{L}$)	5.2 (3.7-7.3)	5.6 (4.0-8.2)	6.3 (5.2-8.9)**	<0.0001
Monocytes ($\times 10^3/\mu\text{L}$)	0.9 (0.6-1.3)	1.1 (0.7-1.5)*	1.3 (0.9-2.0)**	<0.0001
Granulocytes ($\times 10^3/\mu\text{L}$)	4.4 (3.0-6.4)	4.3 (2.6-6.6)	4.7 (2.9-7.4)	0.340
Platelet indices				
Plt ($\times 10^3/\mu\text{L}$)	199.5 (129-314)	155 (100-213)**	141 (98-195)**	<0.0001
MPV (fl)	8.0 (7.3-8.8)	8.4 (7.6-9.4)*	8.8 (8.0-9.6)**	<0.0001
Pct (%)	0.16 (0.07-0.2)	0.13 (0.07-0.2)**	0.09 (0.06-0.12)**	<0.0001

WBC = White Blood Cells; Plt = Platelet; MPV = Mean Platelet Volume; Pct = Plateletcrit; HCM = Haemozoin Containing Monocytes

4.11 Relationship between HCM, Parasitaemia and Haematological Parameters

Association analyses between HCM, parasitaemia, and haematological parameters showed that HCM was inversely correlated with Hb levels ($r = -0.431$, $P < 0.0001$), RBC counts ($r = -0.421$, $P < 0.0001$), and platelet counts ($r = -0.133$, $P = 0.001$; Table 10). However, HCM was further positively correlated with WBC counts ($r = 0.243$, $P < 0.0001$), lymphocyte counts ($r = 0.302$, $P < 0.0001$), and monocyte counts ($r = 0.362$, $P < 0.0001$), but not with granulocyte counts ($r = 0.041$, $P < 0.333$). Parasitaemia, on the other hand, was positively correlated with WBC counts ($r = 0.184$, $P < 0.0001$) and granulocyte counts ($r = 0.393$, $P < 0.0001$), but inversely correlated with platelet counts ($r = -0.124$, $P = 0.003$; Table 10).

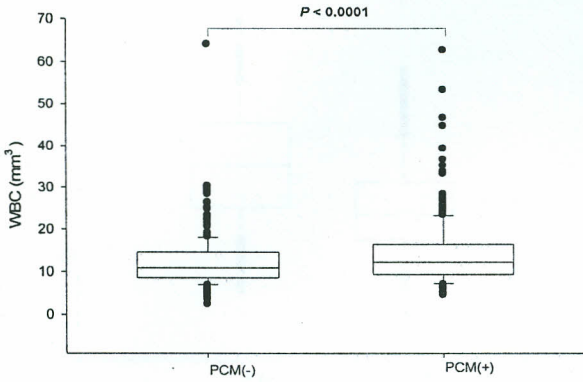
Table 10: Relationship between HCM, Parasitaemia and Haematological Parameters

Haematological characteristic	HCM		Parasitaemia	
	r-value	P-value	r-value	P-value
Hb (g/dL)	- 0.431	<0.0001	- 0.048	0.300
RBC ($\times 10^6/\mu\text{L}$)	- 0.421	<0.0001	- 0.036	0.390
WBC ($\times 10^3/\mu\text{L}$)	0.243	<0.0001	0.184	<0.0001
Lymphocytes ($\times 10^3/\mu\text{L}$)	0.302	<0.0001	0.084	0.046
Monocytes ($\times 10^3/\mu\text{L}$)	0.362	<0.0001	0.066	0.119
Granulocytes ($\times 10^3/\mu\text{L}$)	0.025	0.559	0.393	<0.0001
Platelets ($\times 10^3/\mu\text{L}$)	- 0.133	0.001	- 0.123	0.003

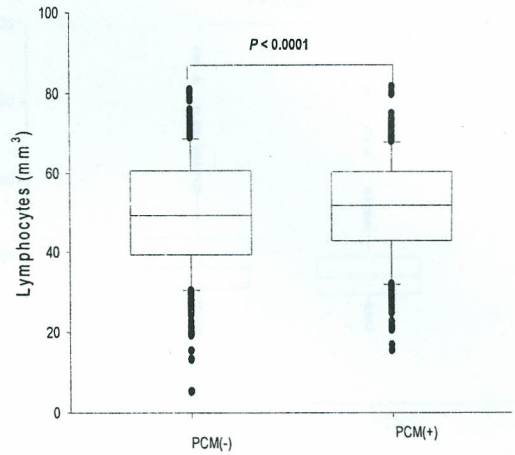
Relationship between total HCM counts ($/\mu\text{L}$), parasitaemia ($/\mu\text{L}$) and haematological parameters were determined for children with acute *P.falciparum* malaria ($n = 557$). The relationship was determined by Spearman's correlation test.

To further establish the predictor(s) of SMA in children with malaria, multiple step-wise linear regression analyses were used to examine the impact of age, parasitaemia, HCM, WBC, lymphocytes, monocytes and granulocytes on clinical variables that were associated with haemoglobin at the $P < 0.01$ level in the bivariate analyses. Each variable at the $P < 0.01$ level of significance was included in the model and excluded step-wise if they failed to give a significant ($P < 0.05$) overall effect on the model. Haemoglobin was entered first into the model as the dependent variable, followed by the block of predictor (covariates) variables: age, parasitaemia, HCM, WBC, lymphocytes, monocytes and granulocytes. HCM (standardized linear regression coefficient, $\beta = -0.239$, $P < 0.001$) and lymphocytes ($\beta = -0.232$, $P < 0.001$) were independently associated with Hb. To further explore relationship between HCM and haematological alterations and determine whether HCM had any role in haematological alterations during *P.falciparum* infection, participants ($n = 556$) were categorized as HCM (-) ($n = 285$) and HCM (+) ($n = 271$). WBC, lymphocytes and monocytes were significantly increased among the HCM (+) group ($P < 0.0001$) (Figure 2A, 2B and 2C) respectively while granulocytes were not different among HCM (+) and the HCM (-) groups ($P = 0.537$; Figure 2D). Hb levels and platelet counts were significantly reduced among HCM (+) group ($P < 0.0001$; Figure 2E and 2F, respectively).

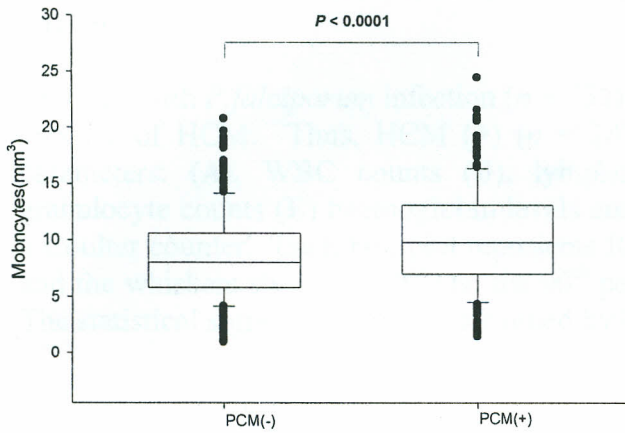
A



B



C



D

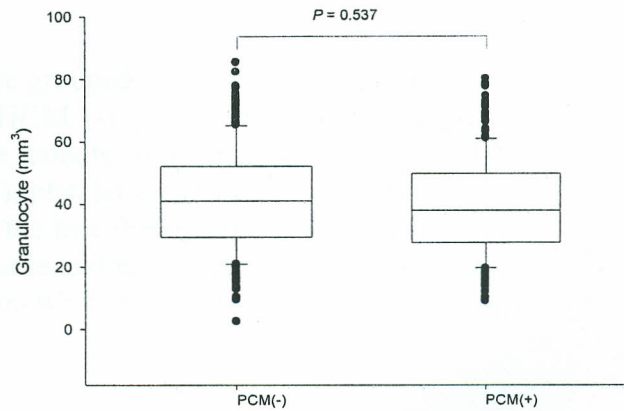
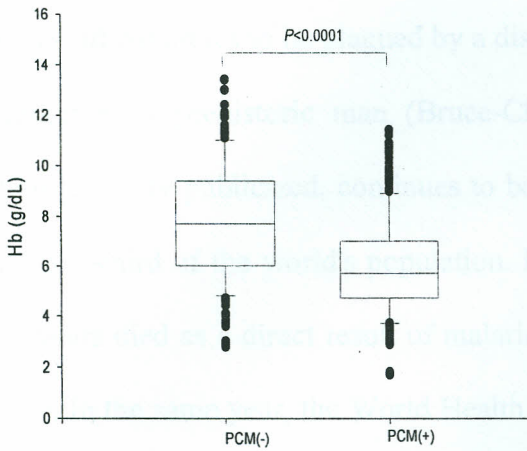


Figure 2: Effects of Naturally Acquired *Pf*H_z on Haematological Parameters

Children with *P. falciparum* infection ($n = 556$) were grouped according to the presence or absence of HCM. Thus HCM (+) ($n = 283$); HCM (-) ($n = 273$). Haematological parameters (A), WBC counts (B), lymphocyte counts (C), monocyte counts (D) granulocyte counts (E) haemoglobin levels and (F) platelet counts were measured using coulter counter.

E



F

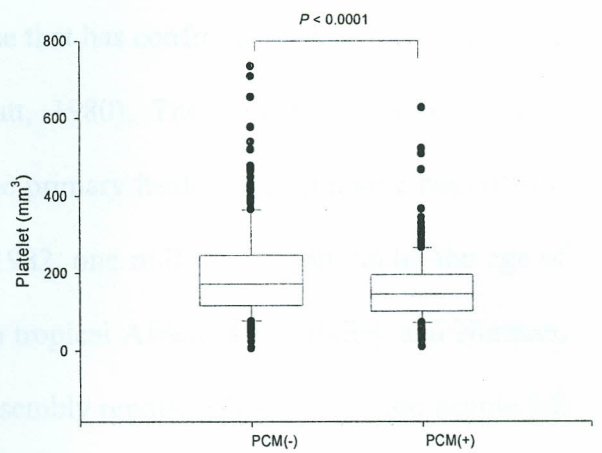


Figure 2 continued: Effects of Naturally Acquired *PfHz* on Haematological Parameters.

Children with *P. falciparum* infection ($n = 556$) were grouped according to the presence or absence of HCM. Thus, HCM (+) ($n = 283$); HCM (-) ($n = 273$). Haematological parameters: (A), WBC counts (B), lymphocyte counts (C), monocyte counts (D) granulocyte counts (E) haemoglobin levels and (F), platelet counts were measured using a Coulter counter. Each box plot represents IQR, the line through the box is the median and the whiskers show the 10th and the 90th percentile and closed circles are the outliers. The statistical significance was determined by Mann-Whitney U test for medians.

CHAPTER FIVE:

5.0 DISCUSSION

The world continues to be plagued by a disease that has confronted her arguably since the emergence of prehistoric man (Bruce-Chwatt, 1980). The global spread of malaria, although rarely publicized, continues to be the primary health and economic concern for over one-third of the world's population. In 1982, one million children under the age of five years died as a direct result of malaria in tropical Africa alone (Bailey and Norman, 1982). In the same year, the World Health Assembly reported that 150 million people fell victim to a malaria parasite, resulting in 2 million deaths (Bailey and Norman, 1982). Now, two decades later, those numbers are even higher. Three hundred million cases of malaria are reported and, unfortunately, more people die from malaria today than 30 years ago. Estimates indicate that 1.5 to 2.7 million people die from malaria yearly, 70-90% of which are children under the age of five years (Phillips, 2001; Bloland, 2001). The victims of malaria, however, need not be counted only among those who die. For those who survived the infection, the debilitating nature of this disease presents a bleak outlook for their survival in future sicknesses and reduces their life expectancy (Krier and Baker, 1980). Malaria accounts for 25-50% of all hospital admissions in Africa (Krishna, 1997).

5.1 Effect of Hz on Inflammatory Mediator Production in Infants and Children with Malarial Anaemia

This study evaluated the role of innate inflammatory mediator production in the development of malarial anaemia among young children and determined whether there was a relationship with Hz containing monocytes (HCM). Differences between plasma cytokine levels of children with uncomplicated malaria (UM) and children with severe malarial anaemia (SMA) were examined to elucidate the potential involvement of these

cytokines in the development of uncomplicated versus SMA. Results revealed significant variation in plasma levels of IL-10, and IL-12p40/p70, IL-10/TNF- α and IL-10/IFN- γ among clinical categories suggesting a complex relationship between cytokines and malarial anaemia (MA). IL-12p70 is a cytokine which plays an important role in enhancing erythropoiesis through increased CFU-E and BFU-E in the bone marrow in murine models (Mohan and Stevenson, 1998b). The present study observed that IL-12p70 was suppressed across clinical and HCM categories. In addition, the observation that levels of IFN- γ were significantly different between those with HCM and those without HCM, is important since high levels of IFN- γ has been shown to induce nitric oxide synthase-2 (NOS-2) and nitric oxide (NO) production, which are important for the killing of intracellular parasites or limiting their growth (Brunet, 2001). As such, ability to mount a rapid IFN- γ response has been linked to a more favourable clinical outcome in most animal models of malaria and in humans (Deloron *et al.*, 1991; Luty *et al.*, 1999).

Suppression of IL-12 is associated with risk of severe malaria (Sedegah *et al.*, 1994; Jacobs *et al.*, 1996; Luty *et al.*, 2000; Dodoo *et al.*, 2002; Malaguarnera *et al.*, 2002; Mohan and Stevenson, 1998b; Perkins *et al.*, 2000). Although these pro-inflammatory mediators play an important protective role, excess inflammatory responses could lead to severe pathology, hence the importance of anti-inflammatory cytokines to regulate the excessive pro-inflammatory response (Dodoo *et al.*, 2002). The finding in this study that the relative production of pro-inflammatory and anti-inflammatory cytokines differs among children suffering from varying degrees of MA is consistent with this fact. Due to its function in fever induction, TNF- α plays a central role in the early pro-inflammatory response that determines protection against malaria, and therefore, may be expected to differ among various levels of disease categories.

Previous studies show that IL-4 is suppressed in PBMC cultures stimulated with Hz (Deshpande and Shastry, 2004). Since IL-4 enhances IL-12 production by amplifying transcription of the genes encoding the IL-12p35 and p40 subunits (D'Andrea *et al.*, 1992), it is likely that the low levels of IL-12 in children with SMA/HCM could be due to low transcription of IL-12 subunits. However, recent transcriptional kinetic studies observed that the levels of IL-12p35 had sustained induction over 72 hr following stimulation with *Pf*Hz while IL-12p40 transcripts peaked after 24hrs and rapidly declined thereafter (Ong'echa *et al.*, 2008). These observations suggest that the observed suppression of IL-12 during SMA could partly be due to suppressed production of IL-12p40 transcripts by Hz uptake. Since IL-12 levels in this study were found to be significantly suppressed between HC and other groups with differing levels of MA, this suggests a shift in the balance between Th1 and Th2 cells that has been shown to exist among malaria-exposed individuals in *P. falciparum*-endemic areas.

Pair-wise comparisons of plasma TNF- α , IFN- γ , IL-10, IL-12p40/p70 and their relative levels in the HC versus other levels of MA showed that IL-10 levels were significantly different between HC and other categories, while IL-12 was only significantly different between HC and SMA groups. However, the relative production of IL-10 over IL-12p70, TNF- α , and IFN- γ was significantly different between HC and other clinical categories, suggesting that the anti-inflammatory response in the later stages of the infection played a critical role in the development of MA.

5.2 Effect of H_z on Haematological Outcomes in Infants and Children with Malarial Anaemia

Severe malarial anaemia (SMA) is a major cause of morbidity and mortality in areas where *P. falciparum* malaria transmission is holoendemic. Although the disease has multifactorial aetiology, the mechanisms that govern its pathogenesis are poorly understood. However, increased destruction of both infected and uninfected RBCs and suppression of erythropoiesis are largely involved (Mendez *et al.*, 2000; Ekvall *et al.*, 2001; Chang and Stevenson, 2004). SMA not only occurs in individuals with chronic infection and low parasitaemia, but also in individuals with acute falciparum malaria with high parasitaemia (Wickramasinghe and Abdalla, 2000), thereby suggesting a complex relationship between the host and parasite, or its products. Previous studies have shown that in uncomplicated acute *P. falciparum* malaria, haematocrit levels are usually normal within the first 24 hours following the onset of fever, followed by a decrease (Wickramasinghe and Abdalla, 2000). Although treatment results in clearance of parasitaemia, haematocrit levels often continue to fall (Phillips and Warrell, 1986) leading to about 17% mortality among non blood transfused children and 15% of blood transfused children discharged from hospital after their parasitaemia cleared (Lackritz *et al.*, 1997). This observation is consistent with previous findings that found no relationship between peripheral parasitaemia and anaemia (McElroy *et al.*, 1994; Ong'echa *et al.*, 2006), further suggesting that factors other than parasitaemia are responsible for development and sustenance of malarial anaemia after parasites have been cleared. Results presented here show that Hb, RBC parameters and their indices were all significantly reduced ($P < 0.0001$) across the clinical spectrum. However, parasite density was not significantly different across the clinical categories ($P < 0.251$), supporting these earlier observations. Studies done to determine the effect of *P. falciparum* on leucocytic

cells has been controversial. While some studies have found leucocytopenia among *P. falciparum*-infected individuals (Perrin *et al.*, 1982; Rojanasthien *et al.*, 1992; Erhart *et al.*, 2004), others have found leucocytosis (Modiano *et al.*, 2001; Ladhani *et al.*, 2002). This study showed an increase of all leucocytic parameters across clinical groups which progressed with worsening clinical state of the patients. Further analysis revealed that lymphocyte, monocyte and granulocyte counts were significantly higher among young children with SMA compared with the UM group.

This study further observed that HCM induces high IL-10 levels among children with MA, which is consistent with previous findings (Deshpande and Shastry, 2004; Keller *et al.*, 2006). In addition, this study has shown that children with SMA not only had the highest HCM counts, but also the lowest platelet counts. Furthermore, increased IL-10 levels have been associated with thrombocytopaenia (Casals-Pascual *et al.*, 2006). These findings support the hypothesis that pathological changes in haematological parameters were partially associated with HCM through an IL-10 dependent mechanism.

Although the results in this study revealed that there was no relationship between parasitaemia and clinical category, or between HDP and clinical categories, both median parasitaemia and prevalence of HDP were significantly associated with % HCM categories. Children with HCM $\geq 10\%$ had the highest median parasitaemia compared to 0% HCM ($P < 0.0001$), whereas HDP was most prevalent in the 0% HCM group compared to $\geq 10\%$ HCM ($P < 0.0001$). Comparison between $< 10\%$ HCM and $\geq 10\%$ HCM did not reveal any significant difference ($P < 0.887$), suggesting that peripheral parasitaemia and HCM are not significantly related. All leukocyte indices, except granulocyte counts, were significantly different between Hz categories. Consistent with the hypothesis that HCM leads to MA, median RBC counts, haematocrit, and Hb levels

were lowest in the $\geq 10\%$ HCM category, the group in which subjects with SMA were the most common. These results suggest that the ingestion of *Pf*Hz by peripheral blood monocytes mediates haematological alterations in children with malaria.

The acquisition of *Pf*Hz has also been associated with SMA among children living in malaria holoendemic areas (Amodu *et al.*, 1998; Casals-Pascual *et al.*, 2006; Keller *et al.*, 2006; Mujuzi *et al.*, 2006) and increased mortality among adults residing in low malaria transmission areas (Nguyen *et al.*, 1995). Findings of this study show a significant inverse relationship between HCM and disease status. Furthermore, multiple step-wise linear regression analyses revealed that HCM and lymphocytes were the most constant predictors of MA, suggesting that peripheral parasitaemia could be playing only a marginal role in the development of MA. Although the mechanism(s) through which HCM contributes to MA is not clear, studies involving murine models and humans indicate that suppression of IL-12 was related to the development of SMA (Mohan and Stevenson, 1998a; Luty *et al.*, 2000; Perkins *et al.*, 2000; Malaguarnera *et al.*, 2002). *In vitro* PBMC cultures stimulated with *Pf*Hz showed over-expression of TNF- α and IL-10 (Deshpande and Shastry, 2004), while only IL-10 was reported to be responsible for the suppression of IL-12p70 (Keller *et al.*, 2006). These observations suggest that *Pf*Hz induces anaemia through an IL-10 dependent mechanism.

Previous studies have reported thrombocytopenia in children with SMA (Skudowitz *et al.*, 1973; Gerardin *et al.*, 2002; Erhart *et al.*, 2004). The present observations show that platelet count and plateletcrit were significantly reduced across the Hz categories, except for platelet distribution width. Although several mechanisms for thrombocytopenia have been suggested, including platelet pooling, decreased platelet life span and increased

phagocytic clearance (Skudowitz *et al.*, 1973), recent findings show that natural acquisition of *PfHz* by monocytes among children with *P. falciparum* suppresses RANTES production (Were *et al.*, 2006). Although severe thrombocytopaenia is frequently reported among patients with *P. falciparum* infection, it is unclear why disseminated intravascular coagulopathy is uncommon among malaria patients (Geradin *et al.*, 2002).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. There is a significant increase of IL-10 and a concomitant decrease of IL-12p70 and IFN- γ with worsening MA.
2. Haemozoin is associated with suppressed IL-12p70 and high IFN- γ levels in malaria patients with MA.
3. There is a significant increase in lymphocytes and monocytes with a concomitant significant decrease with increasing HCM and MA.
4. All erythrocytic count and indices decreased significantly with increasing HCM.
5. This study found that HCM increased with worsening disease state in a dose-dependent manner suggesting that the quantity and distribution of engulfed Hz is a better measure of disease severity than peripheral parasite density in malaria holoendemic areas.

6.2 Recommendations

- 1 Children with signs and symptoms of malaria with negative blood slides but have altered haematological profile should be thoroughly re-evaluated for malaria.
- 2 Assessment of HCM should be included during malaria microscopy as an additional indicator in malaria diagnosis.
- 3 A detailed study should be undertaken to determine if endogenous IL-12 could be given to malaria patients to enhance recovery.
- 4 A detailed study should be undertaken to determine if endogenous IL-12 could be given as a Th-1 type response promoting adjuvant for anti-malaria vaccines.

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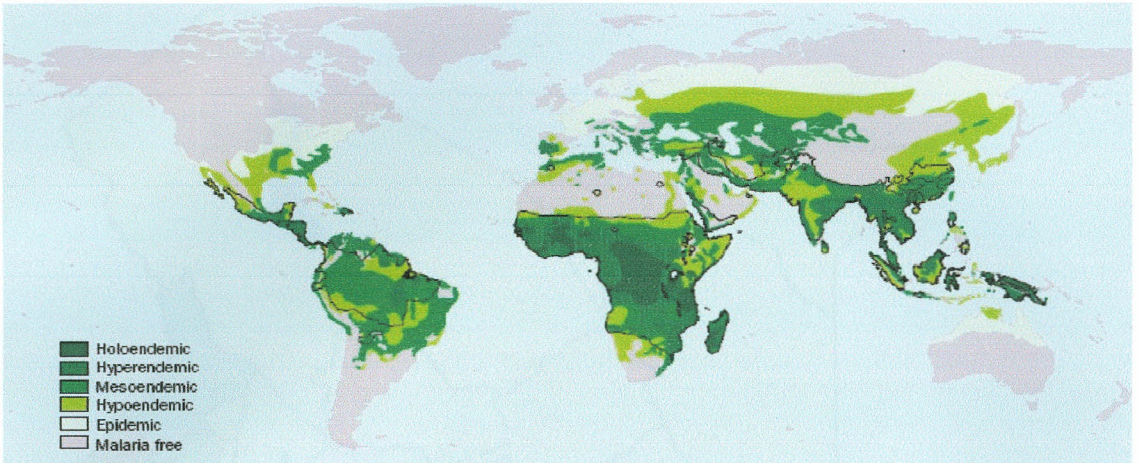
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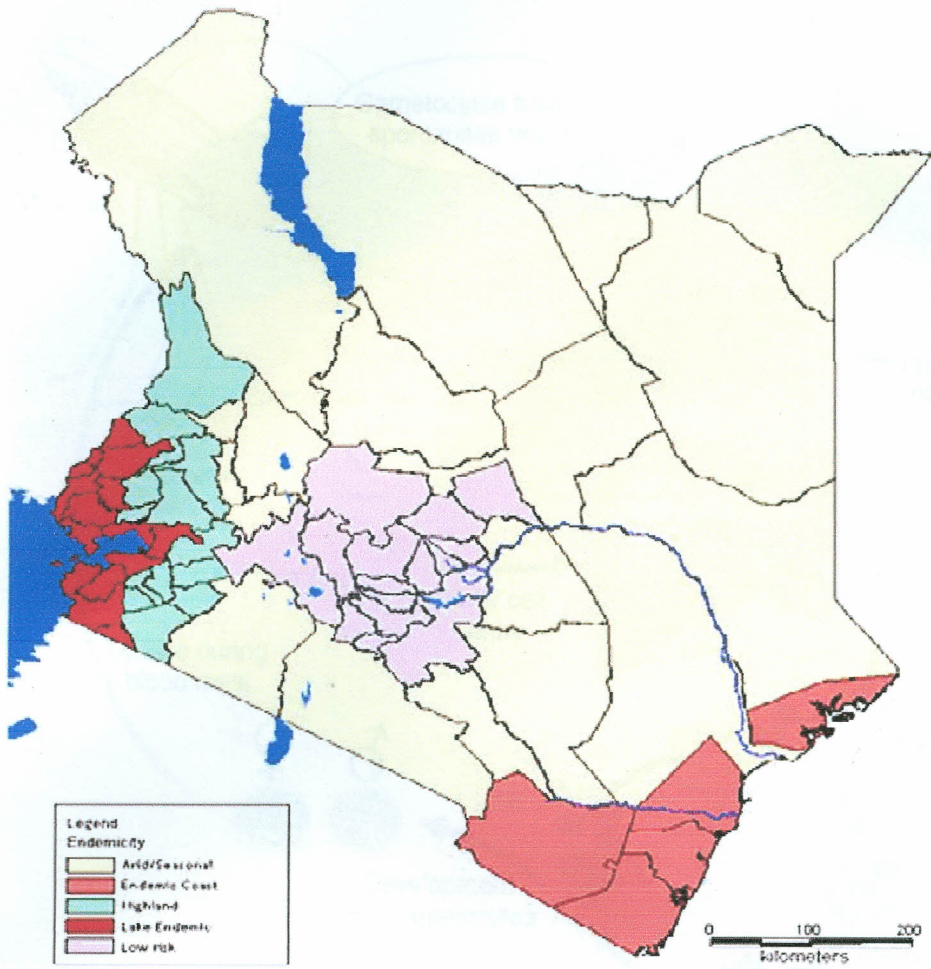
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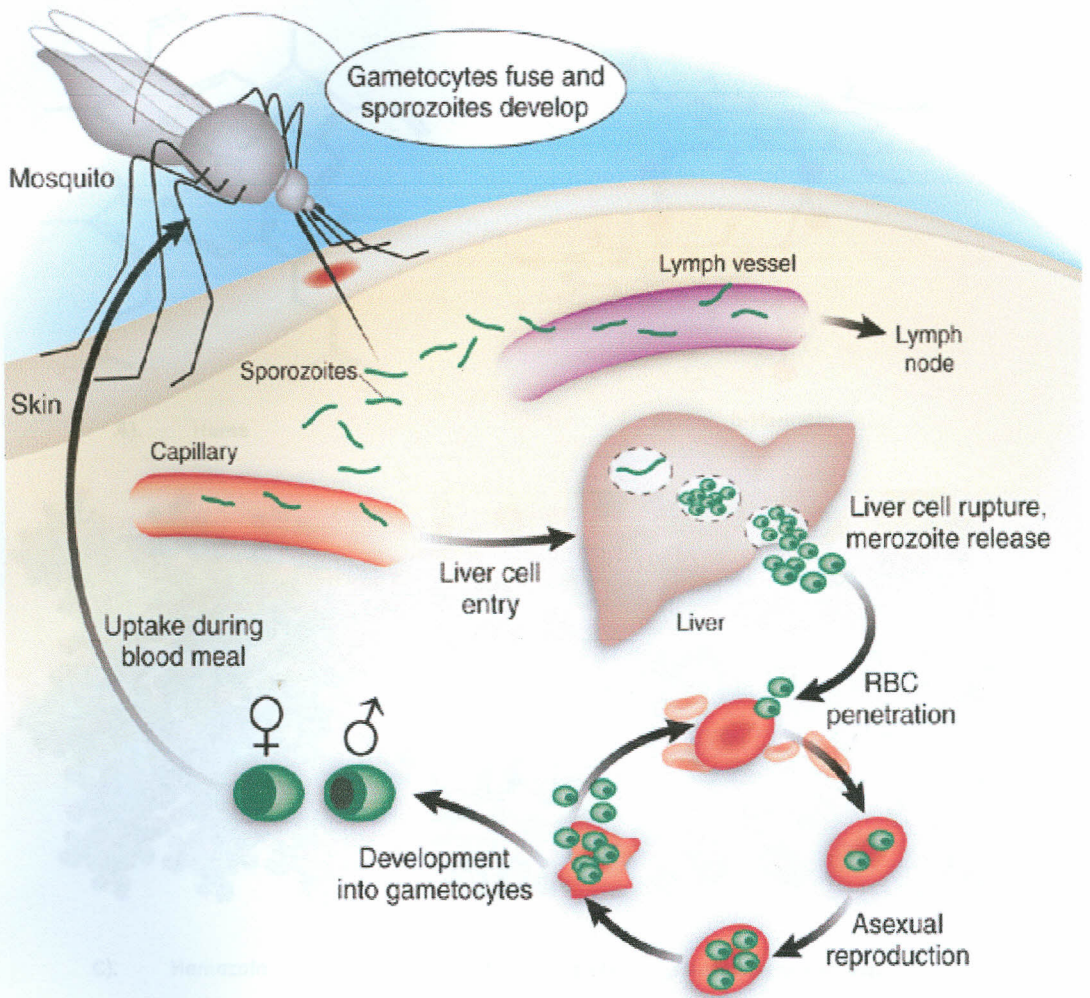
Appendix 1: Global distribution of malaria

Simon *et al.*, 2004

Appendix 2: Distribution of endemic malaria in Kenya

Source: [http://www.nmcp.or.ke/images/kenya.gif\(1.2.3\)](http://www.nmcp.or.ke/images/kenya.gif(1.2.3))

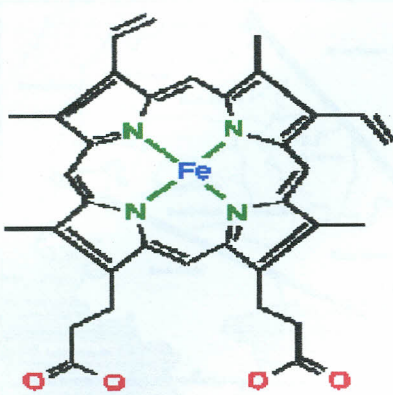
Appendix 3: Life cycle of human *Plasmodium*



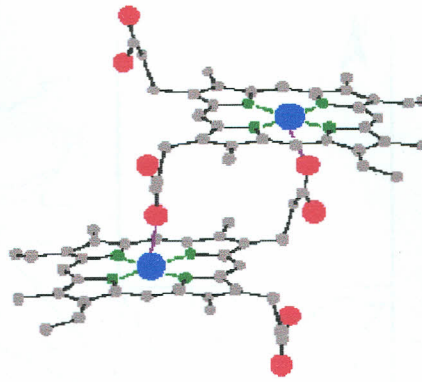
(Source: Jones and Good, 2006;

<http://www.nature.com/nm/journal/v12/n2/full/nm0206-170.htm>

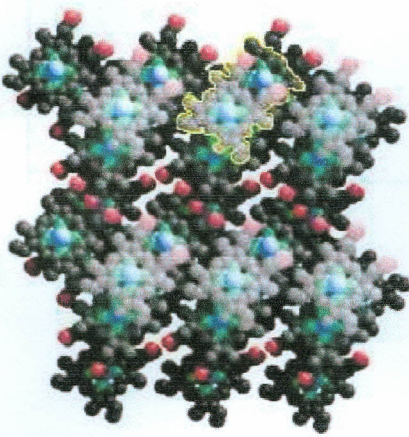
Appendix 4: Structure of heme and haemozoin



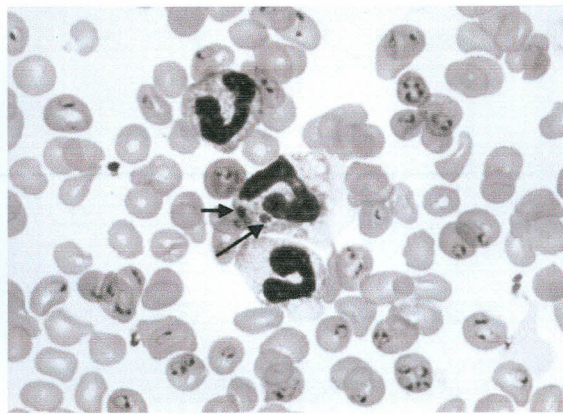
A). Heme



B). β -Hematin



C). Hemozoin

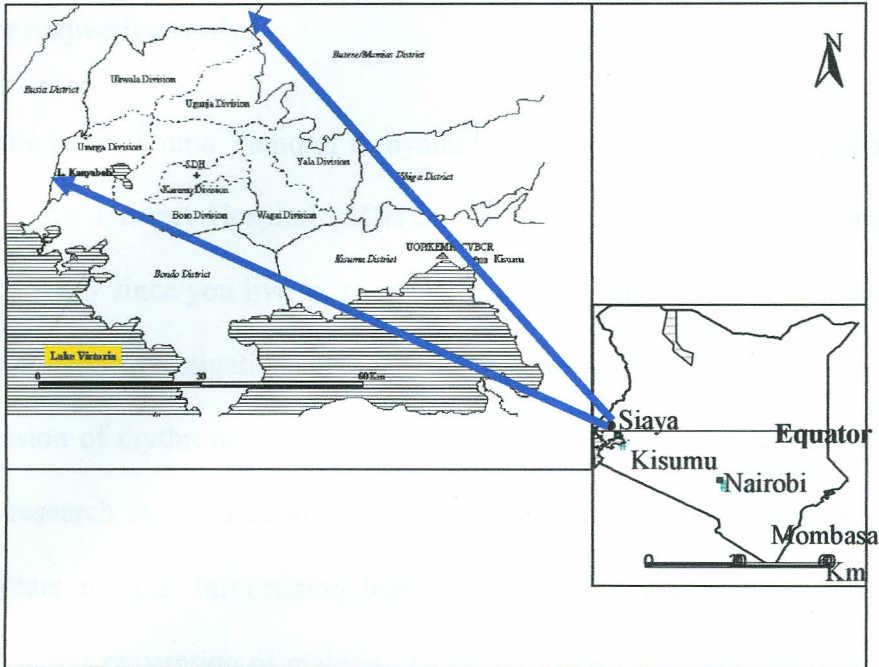


D). Hemozoin (pointed by the arrows)

Source: (Pagola *et al.*, 2000)

A). Chemical structure of heme; B). Ball and stick model of β -hematin. Reciprocal bonds between oxygen and iron shown in purple; C). Space filling model of proposed haemozoin structure (modified from Pagola, 2000). A single hematin unit is outlined in yellow; D). Amber intracytoplasmic haemozoin (arrows) visualized by light microscopy within a polymorphonuclear

Appendix 5: UNM/ KEMRI study area



Appendix 6: Consent form

Consent for parent/guardian of child participation in malarial anaemia hospital-based prospective study.

My name is Mr. Ouma Yamo of Kenyatta University. I am carrying out a research study at the Siaya District Hospital (SDH). I invite the participation of your child in this research study since you live in an area where nearly all children get malaria. The title of the study is “Investigation of malarial anaemia induction by haemozoin mediated suppression of erythropoiesis through dysregulation of IL-10/IL-12 cytokine”. The aim of the research study is to understand how malarial anaemia develops during the first three years of life. Information learned from this research study may help improve treatment and prevention of malaria. Since I want to see how malarial anaemia develops in young children, this research study will look at the first and second episodes (times) your child gets malaria. Therefore, I will need to see your child at hospital several times- the first and second times he or she gets the disease. I will then provide you with a schedule and ask that you bring your child back to SDH. We are also taking samples from healthy children that are attending the clinic for their routine vaccinations. This will allow us to compare healthy children with children who have malaria. The study will enroll a total of 200 children, which will be distributed among the different health risk groups.

During the first and second visits, I will ask you about your child’s health and examine him/her see if your child has malaria and signs of low blood (anaemia), we will collect blood (several drops) by sticking your child’s heel or finger with a small needle (lancet). We may draw a small amount of blood from a vein in your child’s arm (about 1 teaspoonful) if necessary to test how your child’s body fights malaria. If your child is

healthy, we will not need to see you again at hospital. However, we ask that you bring your child back to hospital at the first sign of a fever. Stool samples will be collected from your child to test if the child has intestinal worms which may cause anaemia.

Parent /guardian's name: _____ Child's name: _____
 (Please Print) (Please Print)

Date: _____ Study #: _____

Parent/ guardian's statement:

The above research study has been explained to me. The consent form has been read to me, and my questions have been answered to my satisfaction. I have received a copy of this form. I understand that taking part in this research study is voluntary and my child cannot participate without my consent. I agree for my child to take part in this research study. By signing this form, I give my consent for my child's participation in this research study.

Signatures:

Parent/ guardian's signature: _____ Date: _____

Witness Signature: _____ Date: _____

Appendix 7: Child questionnaire

1 Interviewer _____

2 Date of visit. ___/___/___

3 Child's study Number. -----

4 Child's date of birth..... ___/___/___

5 Age of child..... Weeks

(DETAILS)

Province: _____

Town: _____

Location: _____

Sub-location: _____

Village: _____ Compound: _____ Hse: _____

Name of husband----- Name of homestead-----

Landmarks near to homestead-----

10. Has the child been hospitalized? _____

1=Yes =====> GO TO 17**2= No.**

11. If YES, how many times? ____

12. Reason for last hospitalization 1: ____

13. Reason for first hospitalization 2 ____

14. Since the last visit, did the child receive a transfusion?

1=Yes =====> GO TO 15 2=NO

15. If YES, how many transfusions? _____

24. In the last month, did the child have diarrhea (>3 loose stools/day)?

1 = Yes =====> GO TO 25 2 = No

25 If YES, beginning with the most recent episode please record the starting and ending date of each episode. If the diarrhea is still continuing, please leave the spaces for Date Ended" blank, and indicate the diarrhea is continuing.

<u>Date Started</u>	<u>Date ended</u>	<u>Diarrhea continues</u>
-----	-----	-----

26 in the last month, did the child take any malaria medication?

1 = Yes 2 = No 9 = don't know

Name of Medicine	Date Started	Date ended	# of days
------------------	--------------	------------	-----------

27. If YES, please provide the following Information (please record what the mother report giving the child). If the child is still taking the medication, please use today's date for the "Date ended"

Doses / day			
-----	-----	-----	-----
-----	-----	-----	-----

Does the child have any other illness? (1= Yes 2= No 9= don't know 7 =NA

If YES, what is the illness?

01= Fever 02 = Dif. Breathing 03 = Cough 04 = Abdominal pain 05=
 Vomiting 06 = Headache 07 = Loss of appetite 8 = other, specify

Treatment given:

(TR00)00 = None

(TR01)01 = *Hematinics*

(TR02) 02 =Antimalarial

(TR03)03 =crystalline /Gentamycin inj.

(TR04)04 =Ointment/ creams

(TR05)05 = Antifungal (G.V etc)

(TR06)06 = Multivites

(TR07)07 =Antiboitics

(TR08)08 = Septrin

(TR09) 09 = others specify _____

OTHER NOTES

DIAGNOSIS SUMMARY/ OTHER NOTES

Appendix 8: Child laboratory tests

Study number: __. ____. Birth date: __/__/__. Visit date: __/__/__.

Enrolment _____ Follow-up _____

Body temp: _____⁰C. Fansidar treatment: Yes __, No __. Iron supplement: Yes __, No __

Other infectious diseases: Yes __, No __; if yes specify _____.

Please check off as Samples are collected

Haemoglobin _____

Malaria smear _____

Blood for full haemogram and immunology studies _____

Laboratory Results of Child's Samples

Haemoglobin __. 9 = Not Done

IL-10----- pg/mL IL-12-----pg/mL

TNF- α -----pg/mL IFN- γ -----pg/mL

Blood smear results_

1= positive 2= negative 9= Not Done

(If positive, parasite-count _____ / 300 Species _____

Haemozoin-----/30monocytes ----- /100 neutrophils

Abstract # 997 presented at the 55th ASTMH meeting (Atlanta, GA, USA, 2006)

THE IMPACT OF *PLASMODIUM FALCIPARUM* HAEMOZOIN ON
HAEMATOLOGICAL INDICES OF INFANTS AND YOUNG CHILDREN RESIDING
IN A MALARIA HOLOENDEMIC AREA OF WESTERN KENYA

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ABSTRACT

Acute malaria in children in holoendemic *Plasmodium falciparum* transmission areas is frequently characterized by severe haematological complications. Our previous investigations in children with malarial anaemia (MA) show that although concomitant peripheral parasite density and anaemia severity are not significantly related, deposition of naturally acquired haemozoin (Hz) in neutrophils and monocytes is significantly associated with malaria disease severity. Since the role of Hz in promoting haematological abnormalities remains largely undefined, haematological complications associated with Hz-deposition in leukocytes was investigated in children (n=558) presenting with acute malaria (aged 2-33 mos) at Siaya District Hospital, western Kenya, a holoendemic *P. falciparum* transmission area. Haematological indices were determined on a Beckman-Coulter CounterTM, while haemozoin-containing monocytes (HCM) and haemozoin-containing neutrophils (HCN) were determined on Giemsa-stained peripheral

blood smears. There was a lower prevalence of HCN (9.3%) relative to HCM (48.7%) in children with MA. Based on the short- vs. prolonged-clearance kinetics of HCN and HCM, respectively, this finding suggests that MA in this population is characterized by chronic falciparum infections. Examination of haematological parameters revealed that elevated HCM (>10%) were associated with higher numbers of white blood cells ($P<0.001$), lymphocytes ($P<0.001$), and monocytes ($P<0.001$). In contrast, high HCM (>10%) were inversely associated with red blood cells ($P<0.001$), haemoglobin ($P<0.001$), hematocrit ($P<0.001$), reticulocyte counts ($P<0.05$), and platelet counts ($P<0.001$). Taken together, these results demonstrate that elevated levels of Hz-deposition in monocytes are associated with elevated leukocytic parameters, increased severity of anaemia, and thrombocytopenia in children with acute malaria.

Abstract # 549 presented at the 56th ASTMH meeting (Philadelphia, PA, USA, 2007)

DECREASED IL-10 PRODUCTION IS ASSOCIATED WITH LYMPHOCYTOSIS IN CHILDREN WITH SEVERE MALARIAL ANAEMIA

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Keller², Z. Ng'ang'a¹, JM Ong'echa², and DJ Perkins²

Affiliations: Department of Zoological Sciences, Kenyatta University, Nairobi, Kenya¹, University of Pittsburgh/KEMRI Laboratories of Parasitic and Viral Diseases, Centre for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya², Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, USA³, Centre for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya⁴

Hematological derangements are common phenomena of acute malaria among children in regions with *Plasmodium falciparum* holoendemicity. Our previous studies showed that lymphocyte count and malaria haemozoin-containing monocytes were important predictors of severe malarial anaemia (SMA) in children. Additional studies have shown that lymphocytes are important mediators of protective immunity during blood-stage malaria. Moreover, IL-10 is associated with lymphocytosis in individuals with Epstein Barr virus and Chagas disease. To further explore the role of IL-10 in modulating lymphocytosis during SMA, we investigated the relationship between lymphocytosis and IL-10 in children (n=174, aged 3-31 mos) presenting with acute malaria at Siaya District Hospital, western Kenya. Complete blood counts were performed by an automated hematological analyzer, while parasitemia was determined on Giemsa-stained blood films. Lymphocytosis, monocytosis and granulocytosis were defined by an absolute count above 5500/ μ L, 0.8/ μ L and 8.6/ μ L respectively. Circulating IL-10 levels were measured using enzyme-linked immunosorbent assay. Levels of IL-10 in plasma were inversely

correlated with the lymphocyte count ($r=-0.307$, $P<0.0001$). In addition, lymphocytosis was associated with significantly lower IL-10 levels ($P=0.002$) in children with acute malaria. However IL-10 levels did not differ among children with or without either monocytosis or granulocytosis. Additional analyses revealed that lymphocytosis was most prevalent among children with SMA ($P<0.0001$). Results presented here suggest that reduced levels of IL-10 production may be important for promoting lymphocytosis in children with SMA.