TITLE PAGE

SERUM LEVELS OF PROINFLAMMATORY CYTOKINES, HAPTOGLOBIN IN CHILDREN OF VARIOUS ABO BLOOD GROUP AND HEAMOGLOBIN-GENOTYPE WITH *P. FALCIPARAUM* MALARIA IN NNEWI, NIGERIA

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CERTIFICATION

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DEDICATION

This work is dedicated to my little ñangelsô who in the near future will become great scholars

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ABSTRACT

Malaria is characterized by marked changes in cytokine production from immune responses to infection (Jurgen, 2007). Genetics influences these variations in cytokine expression and ABO blood group and haemoglobin phenotype are genetic expressions (Deepa *et al*, 2011). Acute phase proteins may also be involved in cytokine induced replication of inflammatory processes (Warren (2010). This case controlled study involving children with *plasmodium falciparum* malaria (PFM) in Nnewi, Nigeria, sought to examine pro-inflammatory cytokine production in severe and uncomplicated PFM compared with healthy control group, to assess the influence of ABO blood group and haemoglobin phenotype in cytokine expression in malaria and to determine whether differences in serum cytokine levels correlated with severity.

The study group comprised of 158 children between the ages of 3 months and 12 years who were attending Paediatrics units of Nnamdi Azikiwe University Teaching Hospital, Nnewi, between the months of March and December, 2011. The study protocol involved screening children with febrile illness for possible malaria parasiteamia employing microscopic examination of Giemsa stained blood smear. A slide is considered negative after examining 100 fields with high power objective without seeing a *P. falciparum (PF)*. Those who had positive smear were recruited as subjects. Patients who had malaria in addition to other infections were excluded. 56 healthy children who attended child welfare clinics or who came for medical examinations and tested negative to malaria were used as control subjects. The following parameters were assessed; serum cytokine (TNFŨ, IL-1, IL-6) and haptoglobin testing, using ELISA test kits (Abcam company U.K), complete blood count (using Sysmex KX-21N machine), haemoglobin electrophoresis (using Shandon electrophoresis machine) and blood grouping (of Dacie and Lewis) and parasite density were determined for each patient. We identified 15 cases of severe

malaria and 143 cases of uncomplicated malaria. The mean levels of the cytokines tested (in picograms/milliliter) were for the uncomplicated malaria category: IL- value was 177.9 ±316.31 when compared with the control value of 45.6 ±37.04; ($p< 0.05$); IL-6 value was 492.3± 596.84 when compared with the control value of 48.0±35.27; ($p<0.05$), TNFŬ was 132.2± 229.42 when compared with the control value 48.9±58.98; ($p< 0.05$). In severe forms: IL-1 gave a value of 315.8±233.71 when compared with the control value 45.6±37.04; ($p<0.05$) IL-6 value was 1275.3±605.37 when compared with the control value 48.0 ± 35.27; ($p<0.05$) and TNFŬ value was 369.0 ± 453.45 when compared with the control value 48.9 ± 58.98; ($p < 0.05$). The percentage frequency of malaria in the AA phenotype was 82.3% in severe forms while 86.7% was seen in the uncomplicated category. Also the frequency of malaria in the AS phenotype was 17.7% in severe category and 13.3% recorded in uncomplicated. In the same vein, the percentage frequency in blood group O for the uncomplicated form was 66.9% while that of severe form was 60%. Lower frequency of 33.1% and 40% were seen in uncomplicated and severe forms respectivel). An association was observed between malaria and AA phenotype (($X^2 = 8.06$; $p < 0.05$) while no association was observed between malaria and ABO blood group($X^2 = 15.9$; $p < 0.05$). IL-6 and TNFŬ levels correlates with parasite density and platelet count ($p < 0.05$). IL-1 has no correlation with parasite density ($p > 0.05$). IL-6 and TNFŬ show a negative correlation with haemoglobin and haptoglobin levels ($p < 0.05$) but not with IL-1 ($p> 0.05$). This study observed an elevation in the levels of Ll-1, IL-6 and TNFŬ and these increase correlates with severity. Anaemia, thrombocytopenia, PCT, RDW, MPV, % monocytes and % lymphocytes could be considered as prognostic variables.

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CHAPTER ONE

1.0 INTRODUCTION

Malaria is a common and serious tropical parasitic disease caused by single celled protozoa (sporozoa) of the genus *plasmodium.* It is transmitted by female anopheline mosquito. Around the world, malaria is generally considered the most important parasitic infection of humans, especially in many endemic tropical and sub tropical areas in which the morbidity and mortality associated with the disease continues to increase. It claims the lives of more children worldwide than any other infectious disease (Gatti *et. al.*, 2007). It is transmitted from person to person through the bite of infected female *Anopheles* mosquito. (Hoffman *et. al*., 2004). Other methods of transmission include blood transfusion (Okocha *et. al.*, 2005; Bahram, 2005), mother to baby (congenital malaria).There are five species affecting man; *P. Falciparum, P. Ovale, P.Malariae, P.Vivax, P. knowlesi* (Figtree *et. al.,* 2010). The first three species are responsible for malaria transmission in Nigeria with *P. Falciparum* causing 98% of the infections. *(*Oshikoya, 2006) and virtually all deaths from malaria are due to *P. Falciparum* because of its high rate of complications (WHO, 2008). Globally, of the 247 million human malarial infections, 91% are caused by *P. falciparum* specie and 98% in Africa respectively (WHO, 2008). It is more prevalent in sub-Saharan Africa than in other regions of the world. In most other countries with malaria transmission, other plasmodial species predominate (WHO, 2008). Clinical malaria is defined as having symptoms of malaria (history of fever, chills, headache, or sever malaise) accompanied by a positive blood stage infection by microscopy. (Moormann *et. al.*, 2009).

PLASMODIUM LIFE CYCLE

Plasmodium specie has a complex life cycle: the asexual stage in the vertebrate host called schizogony and the sexual stage in the insect vector called sporogony (Iyabo, 2006; Ademowo and Bangus, 2010). (Figure 1.1)

ASEXUAL STAGE (SCHIZOGONY); is divided into two phases

1. The pre-erythrocytic (hepatic) phase
2. Erythrocytic phase

THE PRE-ERYTHROCYTIC (LIVER) PHASE

Infection in humans begins with the bite of an infected female Anopheline mosquito. Sporozites released from the salivary glands of the mosquitoe enter the bloodstream during feeding and quickly invade the liver cells (hepatocytes). Sporozites are cleared from the circulation within 30minutes. It is believed that the kupfer cells of the liver have strong attraction for the sporozites and therefore clear them from the blood circulation and have the ability to kill many of the sporozites (Brooks *et. al.*, 2004). During the next 14 days in the case of *P.falciparum,* the liver-stage parasites differentiate and undergo asexual multiplication (sporogony) resulting in tens of thousands of merozoites which burst from the hepatocytes. The time interval between the inoculation of the infective sporozites and the appearance of the merozites in the blood is variable, depending on the infecting species; (8-25 days for *P. falciparum,* 8-27 days for *P. vivax,* 9-17 days for *P. ovale*, and 15-30 days for *P. malariae*) and this interval is called the prepatent period. In the case of *P.vivax* and *P. ovale*, some sporozoites may go into hibernation (the cryptobiotic phase) in which they are called hypnozoites. They can be dormant for months or years and on reactivation they cause clinical relapse.

THE ERYTHROCYTIC PHASE

Individual merozoites invade red blood cells (erythrocytes) by endocytosis process and undergo an additional round of multiplication producing 12-16 merozoites within a schizont. The merozoites of different species have preferences for the age of erythrocytes they invade. The merozites of *P.vivax* have preferences for young immature red blood cells (reticulocytes). Those of *P. Malariae* invade the older ones while those of *P.falciparum* indiscriminately attack both young and old erythrocytes. This partly explains the virulence of *P. falciparum* infection in which 10% or more of the erythrocytes may be invaded. During this phase, the merozoites grow within the erythrocytes, first forming a ring like early trophozoite to late trophozoite then form a compact that covers the entire red blood cells. This asexual division leads to the formation of a schizont containing merozoites. (Figure 1.1 & 1.2). The features of this stage are highly diagnostic in malaria microscopy. At maturity, both the schizonts and red blood cells rupture, releasing merozoites into the blood stream (Kelley, 1997). The length of this erythrocytic stage of the parasite life cycle depends on the parasite species: 48 hours for *P. Falciparum, P.vivax, P. ovale* and 72 hours for *P. Malariae.*

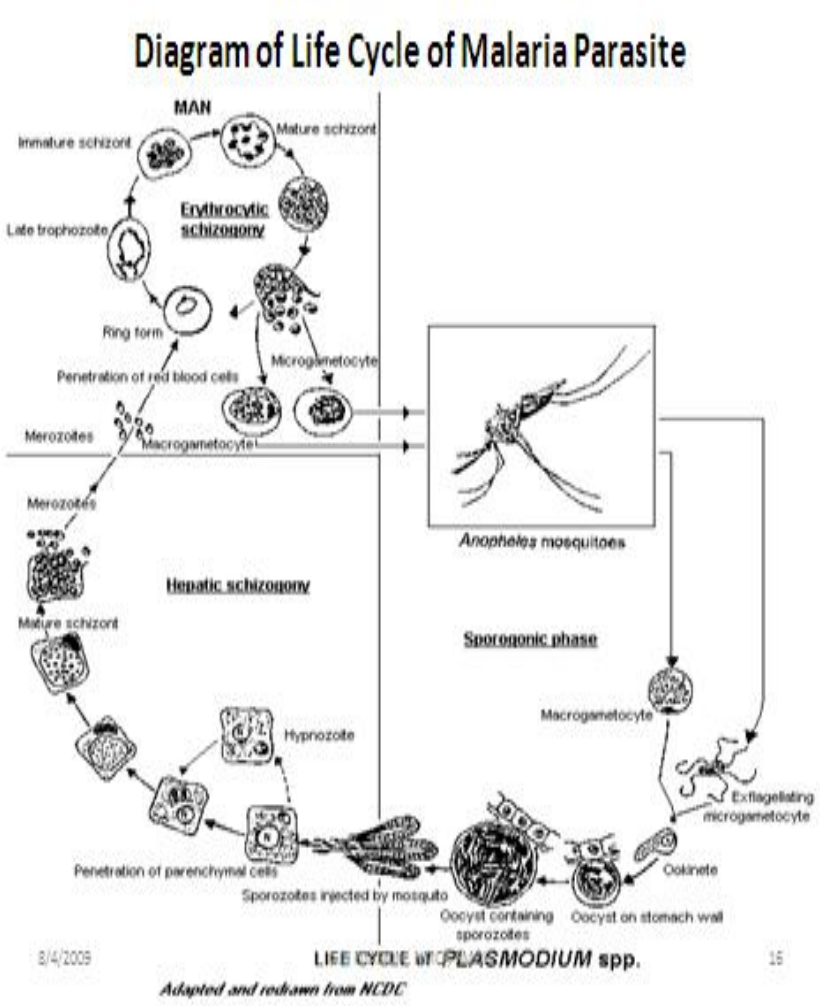


FIGURE1.1 DIAGRAM OF LIFE CYCLE OF MALARIA PARASITE

(Adapted from National Malaria Control Program Training Manual, 2009)

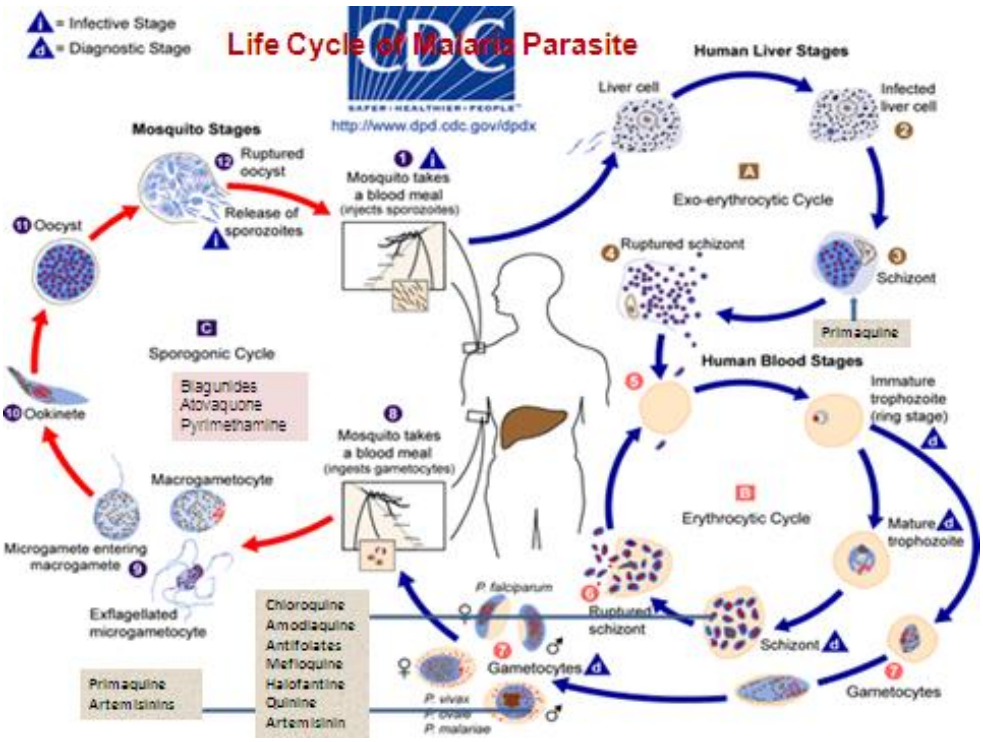


FIGURE 1.2 LIFE CYCLE OF MALARIA PARASITE

(Adapted from Society for family Health Abuja, Microscopy Training Manual 2010)

associated with the synchronous rupture of the infected erythrocyte. The released merozoites go on and invade additional erythrocytes.

Not all the merozoites divide into schizonts; some differentiate into sexual forms giving rise to male and female gametocytes. (CDC, 2007)

SEXUAL STAGE (SPOROGONY)

These gametocytes (which are the infective stage) are taken up by a female anophylean mosquito during a blood meal. Within the mosquito midgut, the male gametocyte undergoes a rapid nuclear division, producing 8 flagellated microgametes which fertilize the female macrogamete. The resulting ookinette traverses the mosquito gut wall and encysts on the exterior of the gut wall as oocyst. Soon the oocytst ruptures, releasing hundreds of sporozites into the mosquito body cavity where they eventually migrate to the mosquito salivary gland. This is the infective stage for man. The sporogony in the mosquito takes about 10-20 days and thereafter the mosquito remains infective for 1-2 months. During another blood meal the sporozites are inoculated into man thus completing the cycle.

The immune response to malaria though not well understood involves both innate (natural) and acquired (adaptive) arms (Warren, 2010). The innate arm is basically through phagocytosis by monocyte and granulocyte, the induction of acute phase reactant proteins and the activation of complements (Warren, 2010). Acute phased proteins are synthesized mainly in the hepatocytes in response to inflammation, trauma, cancer, tissue injury and infection. Their concentrations could be altered and increased in many folds beyond the normal plasma level in such disorders. Some of the acute phase proteins bind to the surface of the micro-organism or parasites and activate the complement system. Some examples of acute phase proteins are C - reactive protein (CRP), serum amyloid P component (SAA), ferritin, Ŭ-1- antichymotrypsin (ACT), Ŭ-1- acid glycoprotein (ACP), fibrinogen, haptoglobin and mannose binding protein (WHO, 2004). Haptoglobin is an alpha 2 globulin that functions to bind the globin portion of free haemoglobin in the blood (Gupta *et. al.*, 2011)

Immune responses provided by the activation of the of thymus mediated lymphocytes (T-cells) by the antigen presenting cells usually the macrophages and the B-cells are more effective. Venugopal (2007) added that adaptive immunity in malaria involves both the humoral and the

cell mediated immune (CMI) responses. The humoral immune response involves the activation of B-cells by the malaria parasites which results in synthesis of immunoglobin (Ig) molecules namely, IgM, IgG, IgA and IgE. However, the destruction of the malaria parasites by the humoral immune mechanism seems not to be effective enough because the parasite has little time to stay in the blood stream before entering into the liver coupled with the fact that in the blood stream, the parasite lives inside the red blood cell thus making it difficult for the organism to have direct contact with anti-bodies (Arora and Arora, 2010). Also this immunity is strain-specific and can be lost if the individual migrates to a region where malaria is not endemic. Furthermore, the efficacy of the humoral response is limited by the intracellular tendencies of the parasite as well as its ability to alter its surface molecules through various maturational stages. The humoral response is bolstered by a variety of non-specific effector mechanisms. The presence of excess type-1(also referred to as pro-inflammatory) cytokines, including interferon gamma (IFN-ɔ), interleukin (IL)-1, IL-2, IL-12, and tumour necrotic factor alpha (TNF)-Ŭ has been confirmed in infected individuals. Therefore following infection by *P.falciparum,* the pro-inflammatory cytokines such as TNF-Ŭ and (IFN)-ɔ are produced during the early stages of the host immune response (Scragg *et. al.*, 1999). These mediators elicit anti-parasitic activities resulting in inhibition of parasitemia and stimulation of phagocytosis to enhance parasite clearance (Gowda, 2007). At the later stages of infection, type-2 cytokines also called anti-inflammatory cytokines such as IL-10 are produced and down regulates the potentially pathogenic inflammatory responses that are important for controlling parasiteamia (Ho *et. al.*, 1998).

The pathological changes in malaria are related to the development of asexual parasites in the blood (that is the blood-stage parasites); exoerythrocytic stages, gametocytes, and sporozoites do not induce pathology in the host (Pain *et. al*., 2001). In *P. falciparum* infections, the multifaceted nature of interaction between the erythrocyte, the host immune system and the parasite is central to the pathogenesis of severe malaria and results in mechanical and rheological changes to the infected erythrocyte. These modifications lead to knob protrusions, cytoadherence, rosette formation and sequestration of the parasite into host tissues. RBCs adhere to the vasulature through sequestration process and also infected RBCs bound to uninfected RBCs (Chotivaich *et. al.*,1998) and/or to platelets (Pain *et. al.*,2001) forming rosettes. Rosetting is a process by which RBCs form clusters consequent to *P. falciparum* infestation thereby obstructing blood flow in

micro circulation. Sequestration and rosette formation impairs blood flow, causing tissue ischemia and cell death. A strong association between rosette formation and ABO blood group is said to exist, with group A and group B RBCs forming rosette more than group O RBCs (Udomsangpetch *et. al.*, 1993). Parasitized erythrocytes bind to host molecules: Cluster of Differentiation (CD) 36, intercellular adhesion molecule (ICAM-1), thrombospondin, E-selectin, Vascular endothelial adhension moecule (VCAM-1), chondoctrin sulphate (Hommel, 1993). And it is difficult to dissociate the role played by ABO sugars from the contribution of other glycoslated adhesion molecules (CD36, ICAM,).This line of thought notwithstanding, most report on this issue are contradictory (Deepa, 2011) and therefore is subject to more studies for better conclusion.

The release of malaria antigens, pigments and toxins give rise to a cascade of pathological events. Among these is the production of cytokines, particularly TNF-Ŭinduced by the release of parasite products during schizont rupture. Cytokines are small cell (5-20 KDa) signaling soluble proteins or glycoprotein molecules secreted by variety of cells in response to a foreign antigen or stimuli protein (Venugopal, 2007). Kalus, (1996) posited that they are non-antibody molecules with multiple and overlapping functions. They cause activation, proliferation, differentiation and maturation of many different immune cells, while some could be redundant or antagonistic. Most cytokines are primarily involved in inflammation and regulation of both the local and systemic cells. It has also been established that most cytokines exert full activity in haemoposiesis (Inigo and Manuel, 2002).

In malaria, pro-inflammatory (type 1) cytokine molecules such as interleukin-1, (IL1) Tumour necrosis factor alpha (TNFŬ), Interferon gamma (IFNɔ), Interleukin eight (IL-8), Interleukin twelve (IL-12) and Interleukin eighteen (IL-18) etcetera are secreted by the immune cells; phagocytes (monocytes /macrophages and neutrophiles) (Were *et. al.,* 2009). These cytokine molecules may enhance inflammation and production of toxins. On the other hand, other cytokines are also produced that are anti-inflammatory (type 2) and they include; interleukin four (IL-4), interleukin ten (IL-10) and transforming growth factor beta (TGFɓ) (Perkins *et. al.*, 2002). Interleukin 6 acts as both pro-inflammatory and anti-inflammatory. IL-6ôs role as anti-inflammatory is mediated through its inhibitory effects on TNFŬand IL-1 and activation of IL-1ra and IL-10.

TNF-Ŭ has been implicated as the cause of malaria fever. And appears to play a central role; complemented by the effects of other cytokines such as IL-1 and IL-6 in pathogenesis of malaria. Following infection by *P.falciparum,* the pro-inflammatory cytokines such as TNF-Ŭ and interferon (IFN)-ɔ are produced during the early stages of the host immune response (Scragg *et. al.*, 1999). These mediators elicit anti-parasitic activities resulting in inhibition of parasitemia and stimulation of phagocytosis to enhance parasite clearance (Gowda, 2007). At the later stages of infection, anti-inflammatory cytokines such as IL-10 are produced and down regulates the potentially pathogenic inflammatory responses that are important for controlling parasiteamia (Ho *et al*, 1998). The balance in ratio of IL-10 and TNF-Ŭ is therefore important in determining malaria outcome. Therefore, marked imbalance between the two cytokines could determine the severity of the infection (Perkins *et. al*., 2000; Tiago *et. al.*, 2011). And in severe, acute infections such as malaria, the ability to mount an effective innate response may mean difference between life and death (Walther *et. al.,* 2006).

Malaria pigments, hemozin (Hz) acquisition by phagocytes has been shown to suppress cellular immunity and enhance malaria severity (Lyke *et. al.*, 2003; Calsals-Pascual *et. al.*, 2006) Hz is synthesized by trophozoites and early schizonts and is acquired by phagocytic and endothelial cells (Awandare *et al* 2007).They cause dysregulation in inflammatory mediator release from human monocyte /macrophages (Ochiel *et. al.*, 2005; Awandare *et. al.*, 2006; Awandare *et. al.*, 2007). It has also been demonstrated that Hz promotes suppression of Regulated on Activation, Normal T cell Expressed (RANTES also called CCL5) in children (Were *et. al.*, 2009) and such suppression has been associated with enhanced severity of malaria anemia, suppression of erythropoiesis, and increased mortality in children with cerebral malaria (Ochiel *et. al.,* 2005; Were *et. al.*, 2006). Severe malarial anaemia accounts for the greatest worldwide proportion of malaria-associated morbidity and mortality (Breman *et. al.,* 2001). The mechanisms of severe malaria anemia development include direct and indirect destruction of parasitized and non parasitized red blood cells, ineffective erythropoiesis, and dyserythropoisis (Abdalla, 1990). Aroro and Aroro (2010) in a similar but independent report shows that anaemia could occur due to direct lyses of red blood cells as a result of the life cycle of the plasmodium, the splenic removal of both infected and non infected RBC (coated with immune complexes), the increased fragility of RBC and the decreased production of RBC due to bone marrow suppression. The association of genetic markers with malaria has been the subject of numerous investigations,

since the protection afforded by sickle-cell hemoglobin against infection by *falciparum* malaria parasite was discovered by Allison (1957).

Malaria burden is enormous, causing high mortality and morbidity particularly in the under-fives. It poses serious challenge to health care givers in a country like ours where there is a high transmission rate all year round (WHO, 2001), The malaria situation has worsened with emergence of resistant strains (Sowunmi *et. al.*, 1997; Nchinda, 1998), the poor socioeconomic status of the citizens have not helped matters. And no suitable vaccine is available at this time (Cohen, 2010). It is at this backdrop that initial interest to undertake this study lies. Though many investigators have carried out extensive studies on malaria, some attempting to identify factors that confer host protection and those that contribute to pathology or injury most acknowledge that there are still gaps in the body of knowledge particularly as it relates to cytokine expression in malaria. Inflammatory cytokines play an important role in human immune responses to malaria (Lyke *et. al.,* 2004). However, the role of these mediators in disease pathogenesis and the relationship between host protection and injury remains unclear. Though some authors (Lyke *et. al.,* 2004) have sought to further understand the complexities of cytokine responses and their role in the pathogenesis of clinical malaria by correlating cytokines responses to clinical disease, their work was limited to the severe malaria grouping, and did not have information on the uncomplicated category. Others tried to link cytokine induced replication of inflammatory processes to acute phase proteins and complement (Warren, 2010). Yet others show that parasite factors such as the parasite density and their ability to infect high percentage of the erythrocyte correlates positively to the rate of synthesis of inflammatory cytokines and can contribute to the severity of the disease (Chotivanich *et. al.*, 2010). In another development, the association of genetic markers with malaria has been the subject of numerous investigations, since the discovery of the protection afforded by sickle-cell hemoglobin against infection by *falciparum* malaria parasite. This line of thought is supported by Lyke and colleagues (2004) who proposed that host genetic variations are associated with malaria pathology Also, broad range of available evidence suggests that the origin, distribution and relative proportion of ABO blood groups in humans may have been directly influenced by selective genetic pressure from *Plasmodium falciparum* infection (Christen *et. al.*, 2007). In all these works none was able to bring all these factors together in an attempt to identify factors that confer host protection and those that contributes to pathology or injury. The choice of this study is based on these gaps. Therefore

assessing the host red cell genetics, assessment of the response of the host to the presence of the parasite and the extent of the inflammatory process may throw more light in the search of factors influencing malaria outcome.

AIMS AND OBJECTIVES OF THE STUDY

AIMS

The aim of this study is to determine the serum levels of the pro inflammatory cytokines, acute phase reactants, and influence of ABO blood group and haemoglobin types in children with *p. falciparum* malaria compared with a matched healthy (control) group in Nnewi, Anambra State, Nigeria.

SPECIFIC OBJECTIVE

This aim will be achieved in these specific ways;

1. To determine the serum levels of pro inflammatory cytokines (IL-1, IL-6, and TNFŬ) in Nigerian children with *p. falciparum* malaria based on WHO classification of severity (WHO, 2000).
2. To evaluate the level of haptoglobin (acute phase reactant) as marker of inflammation in the malaria categories.
3. To determine the haemoglobin phenotype, ABO Blood Group, complete blood count and level of parasiteamia (parasite density) of the study subjects.
4. To see if there is an association between level of cytokines expressed, haptoglobin level, parasite density, haemoglobin level, ABO blood groups, haemoglobin phenotypes in the clinical groupings of malaria in children studied.

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORY OF MALARIA

Malaria parasites have been with man since the dawn of time. It is said that the human race and malaria parasite have had a long evolutionary host-parasite association (Wiesenfeld, 1967; Joy *et. al.*, 2003). They probably originated in Africa. Fossils of the mosquitoes up to 3 million years proves that the female anopheles mosquito was present before the earliest history (Kelly, 1997) Also, advances in bioinformatics (Coluzai, 1999; Hart, 2004) largely support hypotheses inferred from changes in human ecology that around 10,000 years ago *P. Falciparum* populations rapidly expanded in Africa and spread worldwide, coincided with human population growth and subsequent diasporas facilitated by the dawn of agriculture (DeZulueta, 1987; DeZulueta, 1994). Hippocrates (400-370 BC) a physician from ancient Greece, regarded as the ófather of medicineô was the first to describe the clinical manifestations and complications of the disease in the fifth century B.C.

The name malaria, derived from the Italian word-*'Mala aria'* meaning bad or spoiled air, comes from the linkage suggested by Giovanni Maria Lancisi (1717) of malaria with the poisonous vapours of swamps in the 18th century. The speciesô name comes from the Latin *falx* meaning *sickle,* and *parere* meaning to *give birth (*Kumar and Clark, 1995). The causative organism itself was first seen by Charles Alphonse Laveran on November 6, 1880 at a military hospital in Constantine, Algeria, when he discovered a microgametocyte exflagellating in a fresh blood from a patient (Dobson, 1999). Transmission remains a mystery until some years after; precisely in 1894 when Manson discovered that filariasis was transmitted by mosquitoes and hypothesized that mosquitoes could also be the vector of malaria. This hypothesis was experimentally confirmed by two independent researchers; Giovanni Battista and Ronald Ross in 1898. Two years after Grassi proposed an exoerythrocytic stage in the life cycle. This was later confirmed by Short, Garnham, Covell Shute (1948), who found *plasmodium vivax* in the human liver. *P. falciparum* was later discovered by an Italian malariologist, Marchifara and Bignami which they associate with the most severe and dangerous form of malaria (Sherman 1998; Fantina 1999).

The native Indians of Peru and Ecuador had been using the tinctures of the cinchona bark for treating malaria as far back as 1600, because they noticed that people who had fever when they drink from pools where barks of cinchona fell in were recovered of the fever (Coker and Adesegun, 2006). In 1640, Huan de Vego employed the tincture of the cinchona bark for treating malaria. Thompson (1650) introduced this ñJesuitsô barkò to England. Its first recorded use there was by Dr John Metford of Northampton in 1656. Later in 1696, Morton presented the first detailed description of the clinical picture of malaria and of its treatment with cinchona. Gaze (1816) studied the extraction of crystalline quinine from the cinochona bark and in 1820 extraction of pure quinine alkaloids, was carried out in France by Pelletier and Caventou which they named quinine and cinchonine. Attempt to make synthetic antimalarias begun in 1891. *Atabrine,* developed in 1933, was used widely throughout the Pacific in world war 11 but was unpopular because of the yellowing of the skin it caused. Chloroquine came into use in the late 1930s through the efforts of the Germans. Nearly all cases contracted in Africa, India, and Southeast Asia has grown resistance to this medication and there have been cases in Thailand and Cambodia in which the strain has been resistant to nearly all treatments. (Jafari *et. al.*, 2003) A combination of quinine and tetracycline has been used, but there are strains of *P.Falciparum* that have grown resistance to this treatment as well. Often the strains grow resistant to the treatment in areas where the use is not tightly regulated. *Artemisinin* was discovered in the 1970s based on the medicine described in China in the year 340 BC. This new drug became known to western scientists in the late 1980s and early 1990s and is now a standard treatment according to WHO guidelines (2010); which is employed as artemisinin-based combination therapies.

The history of mosquitoes as vectors of malaria parasite started in 1800 when some biochemical scientists suspected mosquito as the vector. Various research works continued until 1898 when Ronald Ross (1857-1932) was able to work out the life cycle of malaria parasite in mosquito.

Numerous eminent individuals have been seriously debilitated or have their lives cut short by a febrile illness which probably resulted from *P, Falciparum* infection .Alexander the great (323BC), St. Augustine (First Bishop of Canterbury), (604AD), King James1, King Cardinal Wolsey and Oliver Cronwell to name but a few.

The ancient disease is not only in our midst, but is responsible for an estimated 500 million cases annually. A high proportion of these infections involves children of less than five years old and pregnant women (Okafor, 2007), HIV infected persons and non-immune visitors.

2.2 AETIOLOGY

Malaria parasites belong to Kingdom Protista and sub-kingdom protozoa of family *Plasmodidiae*, genus *Plasmodium.* The genus comprises 12 subgenera, of which five parasitize reptiles, four parasitize birds, and three parasitize mammals- namely *Plasmodium (Plasmodium), Plasmodium (Lavrerania) Plasmodium (Vinckeria).*

*S*ubgenus *Plasmodium (Plasmodium*) comprises twenty species that affect only primates; three of these, namely *Plasmodium (Plasmodium) vivax*, *Plasmodium (Plasmodium) ovale, Plasmodium (Plasmodium) knowlese* parasitize only man; and one, namely *Plasmodium (Plasmodium) malariae* is anthropo-zoonotic with a preference for man. The other 16 species infect only subhuman primates.

Subgenus *Plasmodium (Lavrerania)* comprises two species, also affecting primates exclusively: one, namely *Plasmodium (Lavrerania) falciparum*, parasitizes man, and the other parasites parasitize chimpanzee. However, *P. falciparum* can infect the chimpanzee after spleenectomy (Hickman, 1969)

Subgenus *Plasmodium (Vinckeria)* comprises 19 species, all affecting rodents exclusively; and some of these, like others that affect birds and primates, are used as experimental models in the laboratory. In summary, the malarial parasites that parasitize man are of five different species, namely *P. vivax, P. ovale, P. malariae, P. falciparum,* and *P. knowlesi* according to common nomenclature.

2.2.1 *Plasmodium vivax*

This is the agent of the so-called benign tertian fever. It is prevalent in temperate areas and to some extents in large tropical areas. It is found in Northern Africa, East and South Tropical Africa, Mexico, Turkey, Central and South Asia, Central and South America. It is not found in Nigeria, and indeed in entire West Africa (Luzzatto, 1979). The receptor for *P. vivax* is the Duffy blood group and it has been shown that Duffy-negative erythrocytes (individuals) are resistant to

invasion by *P.vivax* infection (Gupta and Chowudhuri, 1980). About 23% of Nigerians are Duffy negative (Ukaejiofo, 2009). The duration of the-pre erythrocytic phase is 8 days, and the incubation period is approximately 13 days from the time of sporogony to the time of sporozoite injection. It undergoes secondary exoerythrocytic cycle by the hypnozoites. Each mature schizont in the liver produces 8000 to 20,000 merozoites; each mature schizont in the blood stream produces 12 to 18 merozoites. The asexual erythrocytic cycle takes 48 hours to complete. The various intraerythrocytic forms are generally larger than those of other species. *P. vivax* has marked ameboid motility and produces characteristic reddish stippling of red blood cells called Schuffnerôs stippling. The gametocytes, usually round-shaped, appear in the blood about 3 days after the appearance of the asexual forms.

2.2.2 *Plasmodium ovale*

This specie causes a tertian fever similar to that of *P.vivax* only that is milder. It is found in tropical Africa and in some areas of the East. The duration of the pre-erythrocytic phase is 9 days; incubation is about 17 days. The tendency to recurrent attacks is less than with *P. vivax.* The fixed tissue schizont produces about 15,000 merozoites, the hematic schizonts produces 4 to 8. The exoerythrocytic asexual cycles takes 50 hours. The parasitized RBCs often acquired an oval shape and present Schuffnerôs stippling, the gametocytes are round.

2.2.3 *Plasmodium malariae*

This is the agent for the quartan fever. It is found in tropical and subtropical areas particularly Africa, Indian and Guiana. The duration of pre-erythrocytic stage is about 14 days. The incubation period is 26 days. The *plasmodium* specie has been known to cause relapses of malarial rigors many years after the initial attack; since no secondary exoerythrocytic cycle has ever been demonstrated; however these relapses seem to represent ñrecrudusencesò due to the long residence of erythrocytic merozoites in the blood stream. The liver schizont produces about 15,000 merozoites. The haematic schizonts produce approximately 8. The asexual erythrocytic cycle takes 72 hours and the parasitized Red blood cells have Ziemann stippling. Mature trophozoites have tendency to form band within the red blood cell. This specie also parasitizes the chimpanzee in natural conditions, so that the anthropoid ape may constitute a reservoir of infection.

2.2.4 *Plasmodium falciparum*

This specie causes malignant tertian fever. It is widespread throughout tropical and subtropical areas of Africa, Asia, Central and South America and Oceania. The duration of the pre-erythrocytic phase is approximately 5 days. The incubation is 9 to 10 days. This specie has no secondary exoerythrocytic phase. Therefore any relapse may be due to recrudescence. The liver schizont produces over 30,000 merozoites, the haematic schizonts produces as many as 32. Blood parasitization is generally more severe than with other species because maturation to haematic trophozoite takes place in the deep tissues since the parasite has the tendency to be ñsequeresteredò out of the peripheral blood in the tissues. Therefore only the early trophozoite stage (ring) and occasionally the gametes are seen in peripherial blood. The rings are often located in the periphery and often multiple, the erythrocytes shows characteristic Maurer cleft. These features are highly diagnostic. The asexual erythrocytic cycle takes 48 hours.

2.2.5 *Plasmodium Knowlesi*

P. knowlesi is a primate malaria parasite that causes malaria in a long-tailed (*macaca fasicularia*) but may also infect humans both in natural and artificial means (Wellcome Trust Sanger Institute 2007). It was first described in a case acquired in Indonesian Borneo that was imported into Australia (Figtree *et. al.*, 2010).Commonly found in the forest areas of Southeast Asia (Figtree *et al*,2010) *P. knowlesi* and *P. malariae,* are often indistinguishable morphologically. Features of *P. knowlesi* by light microscopy include early trophozoites with fine ring forms, double chromatin dots, and 2ï 3 parasites per erythrocyte (resembling *P. falciparum*), trophozoites with a bird's-eye appearance, mature trophozoites with a band appearance resembling *P. malariae* and mature schizonts with a higher average merozoite count (16/erythrocyte) than in *P. malariae* (12/erythrocyte) (Singh *et. al.*, 2004; Lee *et. al.*, 2009)

Distinction of P. *knowlesi* from *P. malariae* has useful management implications for patients and public health control measures. *P. knowlesi* potentially can cause severe disease and death, whereas *P. malariae* is generally benign. Deaths and severe disease caused by *P. knowlesi* result from pulmonary and hepatorenal failure (Cox-Singh *et. al.,* 2009). Severity of *P. knowlesi* infection is related to potentially high parasitemia levels produced by its rapid and unique 24-hour erythrocytic cycle and its ability to infect all stages of erythrocytes (Knowe *et. al.*, 2004).

Sequestration is not thought to occur during *P. knowlesi* infection, and neurologic complications seen during *P. falciparum* infection have not been described. Thrombocytopenia is a common feature (Daneshvar *et. al.,* 2009; Jeremiah and Uko, 2009).

2.3 EPIDEMIOLOGY

Malaria is a major international health problem, posing serious challenges to modern healthcare. Despite the impressive efforts of the WHO- Sponsored malaria eradication programmes since 1956, technical and socio-economic difficulties have led to the resurgence of the disease in many areas of the world where eradication had been achieved (Caceres, 2000).The population at risk is estimated to be 2.6 billion and each year, there are hundreds of millions of cases of this disabling disease reported worldwide. Estimates for the annual mortality vary widely. WHO estimates that each year, 300 to 500 million people contract malaria and about 1 million die most of which are children under five . In absolute number, malaria kills 3,000 children per day and an African child dies every second from malaria (Mahmood *et. al.*, 2006; Okafor, 2007). Unfortunately, malaria is endemic in greater than 100 countries in the world, and half of these are African countries (Bahram *et. al.*, 2005) especially sub-saharan Africa, which accounts for 80% of the world wide cases. The areas with high-risk include : sub-Saharan Africa, Madagascar, Sumatra, Borneo,Iran Jaya, Papua New Guinea, Vanuatu and the Solomon Islands, some border districts of Thailand, Cambodia and Myanmar and some Amazonian regions of South America. The areas with moderate to low-risk include: South America, the Indian subcontinent including Sri Lanka, parts of Southeast Asia, Northern Africa, Mauritius, Central America, Haiti, and the Near East. (Figure1.3). Residents of non-endemic areas occasionally have imported malaria because of international travels to malaria endemic areas (Gonzalez *et. al.*, 2009). Figures as high as 11,000 cases per year was recorded in European Union countries (Jelink, 2002) and a mortality rate ranging from 0.3% to2.2% globally (Jelink, 2002; Spinazzole *et. al.*, 2007).

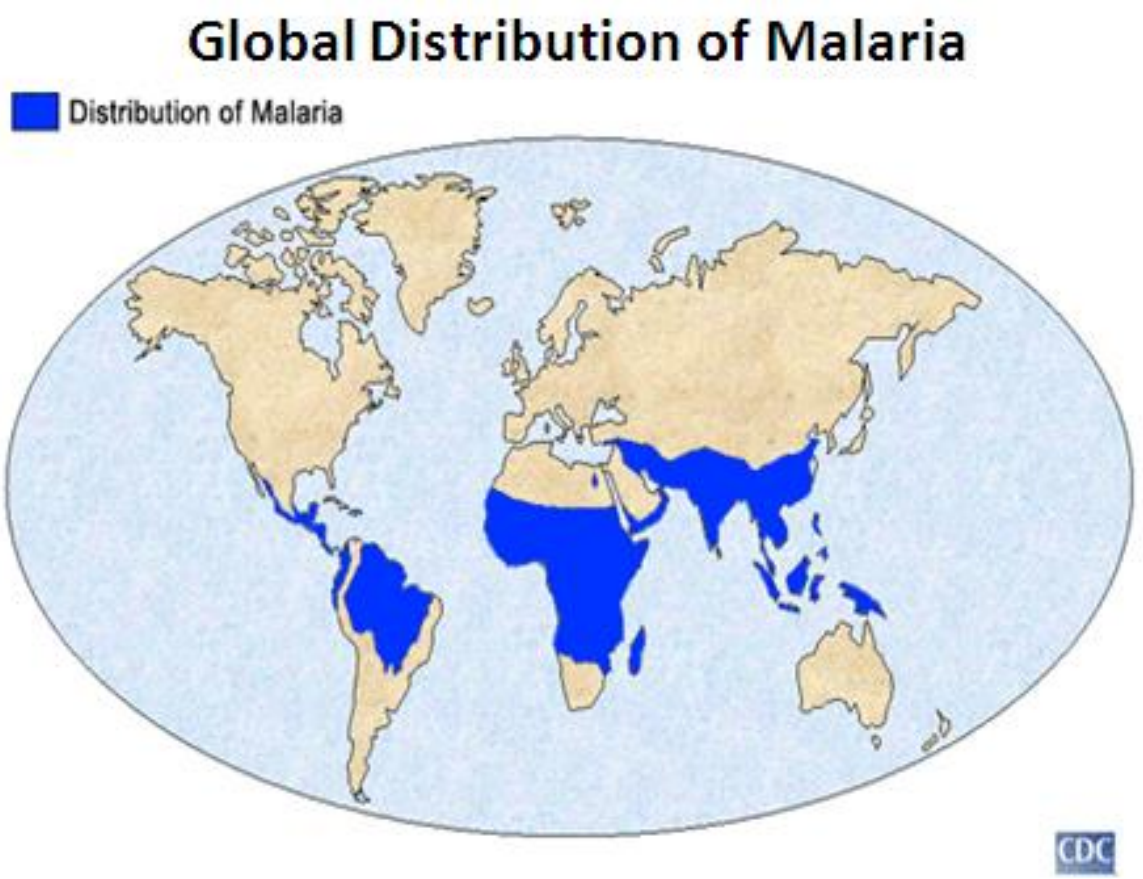


FIGURE1.3 GLOBAL DISTRIBUTION OF MALARIA

(Adapted from malaria training manual, Society for Family Health, Abuja 2010)

Nigeria has a high prevalence of malaria, and it is one of the leading causes of morbidity and mortality in the country (Federal Ministry of Health 1992; Onwujekwe *et. al.*, 2000; Federal Ministry of Health, 2001). The periodic prevalence of malaria is 919/100,000 per annum. 140 million Nigerians are at risk of having malaria and 50% of the population is likely to have at least one episode of malaria a year (WHO, 2008). It is estimated that in highly endemic areas as in Nigeria, a child under five years of age develops 5 to 6 episodes of malaria every year (Federal Ministry of Health, 1989). The toll of the disease is highest in the under-fives (Kaneko 1998). It is responsible for 25% of infant mortality and 30% of childhood mortality. It accounts for 50% of outpatient consultations/ visits and between 15% and 30% of hospital admissions in the country. The disease is endemic throughout Nigeria and more than 90% of the populations live in areas with all year round high transmission rates (WHO, 2001). However, infection rate is highest in the rainy season (Okocha *et. al.,* 2005) from May to November. Other vulnerable groups include non-immune pregnant women with risk of miscarriage (up to 60% in *P. falciparum* infection) and maternal death rates of 10ï 50%. Also in semi-immune pregnant women in areas of high transmission, malaria can result in miscarriage and low birth weight, especially during the first and second pregnancies. An estimated 200, 000 infants die annually as a result of malaria infection during pregnancy (WHO, 2010).

Semi-immune HIV-infected pregnant women in stable transmission areas are at increased risk of malaria during all pregnancies. Women with malaria infection of the placenta also have a higher risk of passing HIV infection to their newborns. People with HIV/AIDS are at increased risk of malaria disease when infected.

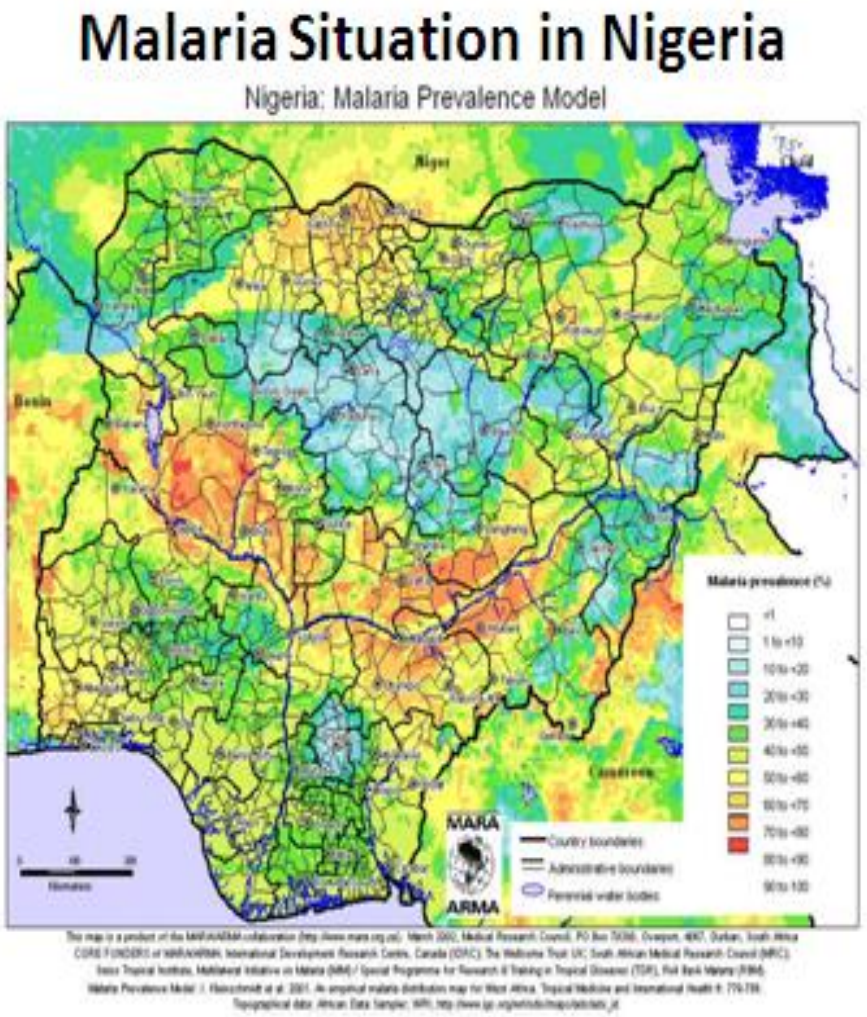


FIGURE 1.4 MALARIA SITUATIONS IN NIGERIA

(Adapted from Malaria Micrscope Training Manual, Society for Family Health, Abuja, 2010)

2.4 MODE OF TRANSMISSION

The major means of transmission is mainly through the bite of infected female anopheles mosquito vector. Because malaria is a haemoparasite, it can also be transmitted through any vehicle that is contaminated with blood example needle prick. Other potential routes of transmission are blood transfusion, congenital malaria (mother to baby) (WHO, 2000). The transmission of malaria via blood transfusion is the next second important route of transmission (Kazemi *et. al.*, 2005) in terms of frequency. It was first reported in 1911 (Bruce- Chywatt, 1982; Woolesy 1991) and it is well established that all four malaria species (*P .Falciparum, P. malariae, P. ovale and P. vivax)* may be transfusion transmitted (Wylie 1993). Malaria parasites survive for at least a week in components stored at room temperature or at 4^0C (Guerrero *et. al.*, 1983). The risk of acquiring malaria through transfusion is dependent on the incidence and prevalence of the disease at a particular area. In countries where malaria is not endemic (like USA and Canada) the incidence is very low (Slinger *et. al.*, 2001). The annual incidence reported by US Centre for disease control (CDC) is only 1-3 cases per year. Another independent researcher in United States reported about 1:4000, 000 (Guerrero *et. al.*, 1983). In Pakistan, the transmission rate is as high as 50 cases/million blood donation (Bruce-Chywatt 1982). Elsewhere in South East Nigeria a peak incidence of 55% was recorded amongst blood donors during the peak periods of the rains (April-October) and 26.5% during the dry season (Okocha *et. al.*, 2005). Another researcher, working in the South East also reported an incidence of 40.9%. Elsewhere in Iran, 33.5% incidence was reported among blood donors (Kinder- Gazard *et. al.*, 2005).

Imported malaria is an important and growing problem in many countries in which the disease is not endemic because international travels to disease endemic areas continues to increase (Gonzalez *et. al.*, 2009). The number of reported imported cases is 11,000 patients per year in Europaen Union countries with *P. falciparum* malaria episodes estimated at nearly 8,000 per year (Jetlinek *et al*, 2005). Mortality rate for imported malaria range from 0.3% to 2.2% globally (Matteelli *et. al.*, 1999; Jetlinek *et. al.*, 2005; Christen *et. al.*, 2006; Spinazzole *et. al*,.2007) and from 11% to 30% in person with severe malaria episodes (Espinosa *et.al*.,1997; Matteelli *et. al.*, 1999; Bruneel *et. al.*,2003; Corne *et. al.*,2004; Spinazzole *et. al.*,2007)

2.5 CLINICAL SIGNS AND SYMPTOMS

Malaria is an acute febrile illness. Symptoms appear seven days or more (usually 10ï 15 days) after the infective mosquito bite. The first symptoms ï fever, headache, chills and vomiting ï may be mild and difficult to recognize as malaria. If not treated within 24 hours, *P. falciparum* malaria can progress to severe illness often leading to death (Barat 1999). Children in endemic areas with severe disease frequently develop one or more of the following syndromic presentations: severe anaemia, respiratory distress in relation to metabolic acidosis, or cerebral malaria. In adults, multi-organ involvement is also frequent.

For both *P. vivax* and *P. ovale*, clinical relapses may occur weeks to months after the first infection, even if the patient has left the malarious area. These new episodes arise from "dormant" liver forms (absent in *P. Falciparum* and *P.malariae*), and special treatment ï targeted at these liver stages ï is mandatory for a complete cure.

A typical malaria fever attack starts with a ñcold stageò (rigor) in which the patient shivers and feels cold. The patient also experiences headache, muscle pain and loss of appetite. This is followed by a ôhot stageô in which the temperature rises to maximum followed by severe headache, back and joint pains with vomiting and diarrhoea. The final stage is when the patient perspires, the temperature falls, headache and other pains are relieved and the patient is exhausted usually and may fall asleep.(Hoffman *et. al.*, 2004).The fever paroxysms occurs at regular intervals of time because the parasites exhibits synchronicity. Synchronicity is the tendency for the parasites to grow in synchrony (grow and mature the same time releasing its toxins at the same time.) This occurs at regular intervals. The periodicity of malaria paroxysm depends on the type of parasite specie that caused the infection. For *P. vivax* and *P.ovale* infections, it occurs every third day (Tertian periodicity). In *P. malariae* infection, it occurs every fourth day (quarten periodicity) and, in *P. falciparum* just every third day (subtertian periodicity). However in clinical practice, the signs and symptoms are nonspecific and include continuous or intermittent fever, chills, headache and pains in the back and limbs, general malaise, anorexia, nausea (Hausler Jr and Sussana 1989). This is seen in its simple or uncomplicated form. Often time malaria is complicated with other signs or organ involvement, giving rise to the severe form. WHO in 1990 and revised in 2000 advanced criteria for defining severe malaria as :the presence of one or more of the following features in a patient with *p.*

falciparum asexual parasitemia and no other confirmed cause for their symptoms (1) Unrousable coma with a Glasgow Coma Scale score < 9;(2) anaemia with a haemoglogin level of <5g/L; (3) renal failure with a serum creatinine concentration >3mg/dL and/ or a 24-hour urine output<400mL despite rehydration;(4) pulmonary edema or acute respiratory distress syndrome;(5) hypoglycemia with a blood glucose level <40 mg/dL (6) circulatory collapse with a systolic blood pressure <80mm Hg despite adequate volume replacement (7) abnormal bleeding and/ or disseminated intravascular coagulation(DIC) (8) repeated generalized seizures;(9) acidiosis (pH<7.35 or a serum bicarbonate level<15mmol/L) (10) macroscopic haemoglobinuria if definitively related to acute malaria;(11) jaundice or total bilirubin level >2mg/dL and (12) parasitemia >5%.(WHO, 1990; WHO,2000). Elsewhere, it was reported that severe malaria could be considered when the parasitic load is >10/1000 RBCs; hemoglobin is < 6 g/dL, platelet count is <10,000/mm3, hepato or splenomegaly is present, or clinical signs of severe malaria such as fever >37.5^{0}C and other organ involvement is present (Deepa *et. al.,* 2011).

2.6 INSECT VECTORS

There are 462 formally named Anopheles species, with a further 50 provisionally designated and awaiting description (Harbch, 2009). Of these, approximately 70 have been shown to be competent vectors of human malaria (Service and Townson, 2002). However, about 20 different *Anopheles* species are said to be locally important around the world. All these important species bite at night, breed in shallow collections of freshwater like puddles, rice fields, and hoofprints (WHO, 2010).The other factors that increase overall vectorial capacity are their abundance, their propensity for feeding on humans, their mean longevity or a combination of any of these (Takken and Lindsay 2003).Transmission is intense in places where the mosquito is relatively long-lived, so that the parasite has time to complete its development inside the mosquito. The important vectors responsible for the transmission of malaria parasites are *Anopheles gambiae* (the principal vector), *Anopheles albimanus, Anopheles freeborni, Anopheles maculates, Anopheles stephensi*, *Anopheles Arabiensis, Anopheles melas , Anopheles Funestus, Anopheles Albimanus, Anopheles puntulatus* and *Anopheles nili* (Antonio-Nkondjio, *et. al*., 2009).

2.7 CLIMATIC AND ENVIRONMENTAL FACTORS INFLUENCING THE LIFE OF ANOPHELES MOSQUITO (THE VECTOR)

Climate and environment have a profound influence on the life of insectôs vector and on the development of malarial parasites; this is reflected in the severity of transmission and in the prevalence of human malaria over the seasons (Wersndorfer, 1980). Factors that influence transmission include rainfall - > 6000mm/year, temperature of 20-30^{0}C, relative humidity of 60% and above altitude of less than 2000metres above sea level. The two more important factors in this respect are temperature and humidity. *P. falciparum* and *P. vivax show* no sporogonic activity at temperatures below 20^0 C and 16^0 C respectively. At higher temperatures the duration of sporogony decreases with increasing temperature. The aquatic development of the larval stage is shorter, within limits the higher the temperature the lower the rate of development. Relative humidity influences the anophelesô longevity: the lower the humidity, the shorter the insectsô life span until survival is too short for completion of sporogony and malarial transmission drops. This becomes apparent at relative humidity values of less than about 60%. The presence of surface waters is indispensible for the larval stage of the vector. The existence of water pools depends largely on rainfall. This is why in tropical and subtropical areas, where rain is abundant the year round, malaria transmission is possible at all times: this is referred to as stable transmission, whereas in areas characterized by alternating dry and rainy seasons, malaria transmission begins with the rains and ceases shortly after the dry weather (seasonal transmission or unstable transmission) (Kiszewski and Teklehaimanot, 2004). All the factors named contribute to the degree of endemicity of malaria. A geographical area is called endemic when it shows a constant incidence of malarial cases with natural transmission continuing through the years. The degree of endemicity is variable, usually determined by several malariometric indices (spleen rates, parasite rates, sickle cell incidence, sporozoites rates, biting rates, records of disease and vector presence and absence) (Hay *et. al.*, 2004)

2.8 LABORATORY DIAGNOSIS

Symptoms of malaria are non-specific and overlap with those of other febrile illnesses; therefore a malaria diagnosis based on clinical grounds alone is unreliable. WHO recommends that malaria be confirmed by parasite-based diagnosis before instituting treatment. Results of parasitological confirmation can be available in a few minutes. Treatment solely on the basis of symptoms

should only be considered when a parasitological diagnosis is not possible (WHO, 2010). A wide range of molecular, serological and immunological techniques for malaria diagnosis are now available, however microscopy is still regarded as the ógold standardô for malaria diagnosis. (Zaman and Beg 2004)

2.8.1 Microscopic Diagnosis

Microscopic diagnosis is the only certain method of diagnosing all the species of malaria parasites. Microscopy also enables the recognition of various blood stages that is; trophozoites, schizonts, and gametocytes. The density of the parasitaemia can be assessed with reasonable accuracy. It affords opportunity to assess response to therapy since repeated counts reveals if the parasite count is dropping or not. This is much needed information as *P.falciparum* has developed resistance to many antimalarials. It is always advisable to make both thin and thick film. While the former is used for speciation the latter is ideal for the detection of light infection as it concentrates red blood cells in a small area which can be easily scanned. (Zaman and Beg 2004; Society for Family Health, 2010)

2.8.2 Fluorescence Microscopy

Acridine orange can also be used, if a fluorescent microscope is available. The parasite can easily be recognized as red cells that do not stain. The nucleus of the parasite takes a greenish colour and the cytoplasm is reddish. It is, however more expensive than the usual thick smear and species diagnosis is difficult.

2.8.3 Quantitative Buffy Coat

Becton-Dickinsonôs QBC (Quantitative buffy coat) method also uses acridine orange. In this case, the blood is centrifuged in capillaries and the buffy coat which contains both parasite and white cells are examined in a special fluorescent illuminator. It is fast and more sensitive than the thick film smear examination and can detect parasites even less than 100/ul (Wongrichanalai *et. al.*, 1991). It is, however, much more expensive than the usual thick smear and species diagnosis is difficult.

2.8.4 Haematology Cell Counters

Some haematology cell counters (automated analyzers) detect parasites by giving abnormal signals that are produced by hemozoin in white cells (Haenscheid *et. al.*, 2000). Hemozoin is the pigment produced by the parasites as a breakdown product of heamoglobin present in host red cells, which upon release is engulfed by phagocytes. Initial studies have shown good results in terms of sensitivity and specificity (Haenscheid *et. al*., 2000).

2.8.5 Rapid Diagnostic Tests (Rdts)/ Immunological Methods

Both antibody and malarial antigen can be detected in the blood. This method is based on the detection of parasite antigens in the peripherial blood using either monoclonal or polyclonal antibodies against the parasite antigen targets. The detection of antibody has been available using indirect fluorescent antibody (IFAT), indirect haemagglutination (IHA) and enzyme linked immunosorbent assay (ELISA). However, these methods are not ideal for routine use; their sensitivity is considerably less at low levels of parasiteamia and nonimmune individuals. They are valuable for assessment of malaria statues in a given population. Currently these test capture histidene-rich protein 11(HRPII), parasite specific lactate dehydrogenase (pLDH) and a pan malaria aldolase (Moody 2002). Histidene-Rich Protein 2(HRPII) is abundant in cytoplasm and membrane of infected erythrocytes. It is specific to *P. falciparum.* It is a heat soluble antigen, stable in plasma and circulates for weeks after clearance of the parasiteamia (Siggh and Shukka, 2012). Plasmodium lactate dehydrogenase (pLDH)/Aldolase are a conserved major enzyme of the glycolytic partway of *P. falciparum specie*. It is abundant and soluble in parasiteôs cytoplasm. It is specific for *P. falciparum* and pan specific and more vulnerable to heat and humidity extremes. It is usually released at the time of cell rupture and febrile episodes and is rapidly cleared from the circulation. (Oduola *et. al.,* 1997; Piper *et. al.*, 1999; Moody *et. al.*, 2000). The RDTs are simple, user friendly, not requiring skill and equipment. Antibodies specific for pLDH of *P. falciparum* and *P. vivax* cross-react with pLDH of *P. knowlesi* (McCuhan *et. al.*, 2008) and therefore cannot be used to reliably distinguish *P. knowlesi* from mixed infections.

2.8.6 Molecular Methods; Polymerase Chain Reaction (PCR)

With PCR, a number of copies of plasmodium sequence of the genome may be increased many times making this method the most sensitive available. A single parasite/20 microliter of blood could be detected which will make it 100 times better than a thick film (Tirasophon *et. al.,* 1991). It can also identify specie (Rubio *et. al.*, 2002).

2.9 CYTOKINE RESPONSE IN MALARIA

Cytokines are small cell (5-20 KDa) signaling soluble proteins or glycoprotein molecules secreted by a variety of cells in response to a foreign antigen or stimuli protein (Venugopal, 2007). Kalus, (1996) posited that they are non-antibody molecules with multiple and overlapping functions. They cause activation, proliferation, differentiation and maturation of many different immune cells, while some could be redundant or antagonistic. Most cytokines are primarily involved in inflammation and regulation of both the local and systemic cells. It has also been established that most cytokines exert full activity in haemoposiesis (Inigo and Manuel, 2002). There are two types of cytokines, the pro-inflammatory also called type 1 and the anti-inflammatory (type 2) cytokines.

In malaria, pro-inflammatory (type 1) cytokine molecules such as interleukin-1, (IL-1) Tumour necrosis factor alpha (TNFŬ), Interferon gamma (IFNɔ), Interleukin eight (IL-8), Interleukin twelve (IL-12) and Interleukin eighteen (IL-18) etcetera are secreted by the immune cells; phagocytes (monocytes /macrophages and neutrophils) (Were *et. al.,* 2009). These cytokine molecules may enhance inflammation and production of toxins. On the other hand, other cytokines are also produced that are anti-inflammatory (type 2) and they include; interleukin four (IL-4), interleukin ten (IL-10) and transforming growth factor beta (TGFɓ) (Perkins *et. al.*, 2002). Interleukin 6 acts as both pro-inflammatory and anti-inflammatory. IL6ôs role as anti-inflammatory is mediated through its inhibitory effects on TNFŬ and IL-1 and activation of IL1ra and IL-10.

It is evident that the pathological alterations and the outcome of the infections depend on the reciprocal regulation of the type 1 and type 2. Also, marked imbalance between the two cytokines could determine the severity of the infection (Perkins *et. al.*, 2000; Tiago *et. al.*, 2011). Therefore in severe, acute infections such as malaria, the ability to mount an effective innate

response may mean the difference between life and death (Walther *et. al.,* 2006). Several experimental works conducted both in man and experimental animals lead credence to this assertion. For example, the work of Fell and Smith (1998) in mice suggests that innate responses are essential to limit the initial phase of parasite replication, and there is also evidence from experimental human infections that parasite growth can be modulated very early during primary infections (Molineaux *et. al.*, 2002).This host innate response is mediated through the production of cytokines and chemokines from phagocytes (monocytes /macrophages and neutrophiles)(Were *et al,* 2009). In other experimental protozoan infections, innate responses have been shown to contribute to control of acute infection, synergize with chemotherapeutic agents, and augment partially effective vaccines (Scharton-Kersten, and Sher, 1997.) It has been demonstrated that components of blood-stage parasites, including parasite-derived glucophoshate inositol (GPI), induce macrophages to produce IL-1, IL-6, interferon(IFN)-ɔ and TNF-Ŭ, but there is evidence that IFN-ɔ levels need to be carefully balanced to avoid immune pathology (Kremsner *et. al.* 1995). In vivo, TNF-Ŭ production is associated with parasite clearance and resolution of fever (Kremsner *et. al*., 1995), but elevated levels of TNF-Ŭ (Kwiatkowski *et. al.,* 1990) and IL-6 (Wenisch *et. al.*, 1999; Torre *et. al., 2002*) have also been associated with cerebral malaria. Accordingly, associations have been reported between circulating levels of IL-12 and IL-18 and risk of severe *Plasmodium falciparum* malaria (Wenisch *et. al.,* 1999; Torre *et. al.,* 2002), and, in a prospective epidemiological study, IL-12 production was positively associated with IFN-ɔ and TNF-Ŭproduction and negatively associated with parasitemia (Doodo 2002). Although the work of Chotivanich and colleagues, (2010) showed that the parasite factor such as the density and their ability to infect high percentage of erythrocyte can correlate positively to the synthesis of inflammatory cytokines such as IFNɔ, and consequently contribute to the severity of the disease. Majority of other studies are not in favour, but rather suggests that parasite density do not correlate with clinical symptoms and by extension severity (John *et. al.,* 2005; Moormann *et. al*., 2009). It is common to define clinical malaria based on symptoms (history of fever, chills, headache or sever malaise) accompanied by a positive blood stage infection by microscopy (John *et. al*., 2005). A parasite density threshold is not used in definition of clinical malaria because asymptomatic parasiteamia is common particularly in endemic regions. Although an essential role has been shown for IL-12 in *P. falciparum*-induced IFN- production from human PBMC and NK cells (Rhee *et. al.,* 2001;

Artavanis-Tsakonas and Riley, 2001; Artavanis-Tsakonas *et. al*.,2003), it has however, proved very difficult to detect IL-12 in vitro and little is known of its cellular origins. Evidence also suggests that both TGF- and IL-10 can be produced very rapidly, from innate sources, during murine malaria infections and are required to down-regulate potentially pathogenic inflammatory responses once parasiteamia is brought under control (Walther *et. al.*, 2003); however, in both mice and humans, excessive concentrations of TGF-ϐ and IL-10 early in infection inhibit type-1 immune responses and thus facilitate parasite growth (Li *et. al.,* 2003). Conversely, in clinical human infections, failure to produce sufficient TGF-ϐ or IL-10 is associated with acute and severe malaria (Tiago *et. al.*, 2011), and severe malarial anemia (Kurtzhals *et. al.,* 1998; Othor *et. al.,* 1999). Finally, high ratios of IFN-ɔ, TNF-Ŭ, and IL-12 to TGF-ϐ or IL-10 are associated with decreased risk of malaria infection but increased risk of clinical disease in those who do become infected (Doodo 2002).

2.9.1 Biological Activity of Cytokines (IL-1, lL-6 and TNFα)

IL-1 a pro-inflammatory cytokine, is intensively produced by the tissue macrophages, monocytes, fibrocytes and dendrititic cells, but is also expressed by lymphocytes, Natural Killer (NK) cells and epithelial cells. They form an important part of the inflammatory responses of the body against infection. These cytokines increases the expression of adhesion factor on endothelial cells to enable transmigration (diapedesis) of immunocompotent cells such as phagocytes, lymphocytes and others, to the site of infection. It also affects the activity of the hypothalamus (the thermoregulatory center) which leads to the raise of body temperature ñfeverò. This is why IL-1 is called endogenous pyrogen. Besides this, IL-1 also caused hypealgesia (increase pain sensitivity) vasodilatation and hypertension (Contassol *et. al.*, 2012).

Interleukin 6 (IL- 6) acts as both pro-inflammatory and anti-inflammatory. It is encoded by the IL-6 gene in human. It is secreted by T cells and macrophages to stimulate immune response example during infection and trauma especially burns or other tissue damage leading to inflammation. IL-6 is one of the most important mediators of fever and the acute phase responses. It is capable of crossing the blood brain barrier and initiating synthesis of PGEs in the hypothalamus, there by changing the bodyôs temperature set point. In the muscle, lL-6 stimulates energy mobilization which leads to increase in body temperature. It is responsible for stimulating

acute phase protein synthesis as well as the production of neutrophils in the bone marrow. It supports the growth of B cells and is antagonistic to regulatory T cells.

Tumor necrosis factor-alpha (TNF-Ŭ) is a pleiotropic inflammatory cytokine. It was first isolated by Carswell and colleagues in 1975 in an attempt to identify tumor necrosis factors responsible for necrosis of the sarcoma Meth A (Carswell *et. al.*, 1975). The cytokine possesses both growth stimulating properties and growth inhibitory processes, and it appears to have self regulatory properties as well. For instance, TNF-Ŭ induces neutrophil proliferation during inflammation, but it also induces neutrophil apoptosis upon binding to the TNF-R55 receptor (Murray *et. al.*, 1997). TNF is secreted by macrophages, monocytes, neutrophils, T-cells, NK-cells following their stimulation by bacterial lipopolysaccharides. Cells expressing CD4 secrete TNF-alpha while CD8 (+) cells secrete little or no TNF-alpha. Stimulated peripheral neutrophilic granulocytes but also unstimulated cells and also a number of transformed cell lines, astrocytes, microglial cells, smooth muscle cells and fibroblasts also secrete TNF. Human milk also contains this factor (Swardfager *et. al.,* 2010). The production of TNF is inhibited by IL-6, TGF-beta, vitamin D3, prostaglandin E_2, dexamethasone, Cyclosporin A, and antagonists of platelet activating factor.

2.10 ACUTE PHASE PROTEIN AND MALARIA

The activation of the bodyôs immune system-mediated defense mechanisms is termed the acute phase response. Warren (2010) also showed that acute phased protein and complement may be involved in the cytokine induced replication of inflammatory processes. Acute phase proteins are synthesized mainly in the hepatocytes in response to inflammation, trauma, cancer, tissue injury and infection. Their concentrations could be altered and increased in many folds beyond the normal plasma level in such disorders. Some of the acute phase proteins bind to the surface of the micro-organism or parasites and activate the complement system. C - reactive protein (CRP), serum amyloid P component (SAA), ferritin, Ŭ-1- antichymotrypsin (ACT), Ŭ-1- acid glycoprotein (ACP), fibrinogen, haptoglobin and mannose binding protein are some examples of acute phase proteins (WHO, 2004).

Haptoglobin also referred to as suicide protein is an alpha 2 globulin, colourless, and functions to bind the globin portion of free haemoglobin in the blood. It is an acute phased reactant (Gupta *et.*

al., 2011). It is heterogenous and the monomer consists of 2 heavy chains, ɓ (40KDa) and 2 light chains Ŭ1(9 KDa) and Ŭ2 (16KDa) that are lined to disulpide bonds. The 2 major Hp types are Hp-1(monomeric, 98KDa), Hp1-2 (polymeric, 200KDa) and Kp2-2(400KDa). It is the transport protein for free haemoglobin in blood. The half-life of serum haptoglobin (unbound Hp) is approximately five days.(Mazza, 2002),but in the presence of free Hb (example, intravascular heamolysis) the Hb- Hp complex which forms is rapidly cleared from the serum by CD163 which are expressed on monocyte-macrophage system with resultant serum haptoglobin levels that are low or absent (Crichton, 2009).Binding of Hp/Hb complexes to CD163 leads to cytokine secretion by macrophages(Moestrap and Moller 2004) The Hb-Hp complex also prevents renal tubular injury by inhibiting Hb escape through the glumerulus. Thus, an increased burden of free plasma Hb rapidly causes a marked decrease in the measured serum Hp (normal levels 36-195mg/dl). An Hp level below 25mg/dl equaled to an 87% probability of predicting hemolytic anemia. It is therefore used as an acute phase marker of red blood cell destruction. Under normal conditions, it is either absent from the blood or present at a very low level. However, it can increase significantly in response to acute infection, inflammation or trauma. The rise in serum or plasma level and the continuous monitoring of this during the acute phase response gives valuable information. Previous study has shown that the range for different acute phase proteins were wider in African women compared with Western adults (Kuvibidila *et.al.*, 1994)**.** The work of Onyenekwe and collegues*,* (2005) showed an association between *P.falciparum* density and haptoglobin concentration, thus indicating that continued increase in *P. falciparum* density might affect haptoglobin metabolism and may consequently result in anaemia. In two other independent studies in an area with high intense malaria transmission, a strong association between Hp phenotype (especially Hp 2-2) and anaemia were established (Arkinson *et. al.,* 2006; Resource actimalaria net, 2013).

2.11 MALARIA AND HAEMATOLOGICAL PARAMETERS

Haematological changes are some of the most common complications in malaria and they play a major role in malaria pathology (Ladhani *et. al.,* 2002; 5^{TH} multilateral initiative on malaria, 2010; Igbeneghu *et. al.,* 2011; Akinsoglou *et. al.,* 2012). Changes in blood cell count are well-known features of malaria (Merlines 2013). These changes involve the major cell lines such as erythrocytes, leukocytes and thrombocytes. Anaemia, thrombocytopenia, leucocytosis or

leucopenia are usually common features and are well recognized. The extent of these alterations varies with level of malaria endemicity, background heamoglobinopathy, nutritional status, demographic factors and malaria immunity (Ghosh and Ghosh, 2007; Khan *et. al.*, 2012). Children infected with *P.F*. malaria in particular exhibit important changes in some haematological parameters (Igbeneghu *et. al.,* 2011; Akinsoglou *et. al*.,2012) with low platelet count (Ekanem *et al* 2005; Jeremiah and Uko, 2009) and Hb concentration (Weatherall *et al.,* 1983 ;Gosling and Hsiang, 2013) being the 2 most important predictors of malaria infection in children. Wickramasinghe and Abdalla, (2000) also reported that anaemia and thrombocytopenia are the most frequent malaria-associated haematologic complications. A study carried out in Kisumu, West district of West Kenyan reported significantly lower level in malaria infected children than their non infected counterpart in the following parameters: platelets, lymphocytes, eosinophlis, red cell counts and Hb. And higher counts in absolute monocyte, neutrophil counts and mean platelet volume (MPV) in comparison with non infected children. Alteration in leukocyte counts are often less pronounced than in other cell lineages, but in general total leukocyte counts have been found to be low to normal in malaria (McKenzie *et. al.,* 2005; Akinosoglou *et. al.,* 2012;). However, significant correlations have been found between parasitaemia and total leukocyte count (Marlies *et. al*., 2013). Lymphocytopenia is a frequent occurrence in *P. falciparum* malaria and has been severally described in malaria patients especially in endemic areas by several authors (Wyler 1976; Lisse *et. al*., 1994; Hviid *et. al.*, 1997; Kassa *et.al.,* 2006; Khan *et.al.*,2012), but is replaced by lymphocytosis a few days after initiation of drug therapy (Hviid *et al*, 1997).There is no established correlation between lymphocyte count and malaria severity as most reports on this yield conflicting results. Both lymphocytopenia (Lisse *et. al.*, 1994; Hiivd *et .al.,* 1997; Akinosoglou *et. al.*, 2012) and lymphocytosis (Ladhani *et. al*., 2002) have been reported to be associated with adverse outcome. The mechanism behind malaria-associated lymphopenia has still not been satisfactorily elucidated and remains the subject of debate [Hviid *et. al.,* 2000). Several underlining pathophysiologic mechanisms had been proposed. Some authors suggested transient sequestration to be responsible (Wyler *et. al*. 1976; Hviid *et. al.,* 1997). The relatively large drop in peripheral lymphocyte numbers would suggest this to be a non-specific effect, *e.g.* pooling in the enlarged spleens of patients (Wyler *et. al*., 1976) rather than a response by malaria-specific lymphocytes only. Others have pointed to the increased propensity of lymphocytes from malaria

patients to undergo spontaneous apoptosis *in vitro* (Hviid *et. al.*, 2000), this line of thought may be unlikely since apoptosis is also seen in healthy donors from endemic areas (Bald *et. al.* 1995; Toure-Balde *et. al.,* 1996). Yet others suggest a compromise in the hematopoietic response in malaria (Khan *et. al*, 2012).

2.11.1 Malarial Anemia (An Important Predictor of Malaria Infection)

In malaria endemic areas of Africa, anaemia is very common in childhood and pregnancy. It is defined as Hb level <10g/dl for both male and female and further classified as severe if Hb is, 5g/dl and below. Severe malaria anemia (SMA) is consequently defined as Hb <5g/dl in the presence of hyperparasiteamia (>20,000 parasites/dl) (WHO, 1990). However, SMA in children according to the World Health Organization (WHO 2000) modified definition is (Hb 5.0 g/dl with parasitemia of any density), while non-SMA was defined as Hb 6.0 g/dl and parasitemia of any density or Hb > 5.0 g/dl and parasitemia of any density (Collins *et. al.*, 2010). SMA accounts for the greatest worldwide proportion of malaria-associated morbidity and mortality (Snow *et. al.*, 1999, Breman *et. al.*, 2001, WHO, 2005). SMA is the primary manifestation of severe malaria in infants and young children in areas in which *P.falciparum transmission* is intense (Ongôecha *et. al.,* 2006; Bloland *et. al.*, 1999). It is important to note that anaemia is multifactorial with other causative factors, which includes iron deficiency and other nutritional deficiency, helminthes infections.

The pathogenesis of anaemia in malaria remains incompletely understood. However, direct and indirect destruction of parasitized and non parasitized red blood cells by the splenic macrophages (increased destruction), inefficient erythropoiesis, and dyserythropoiesis (decreased RBC production or disordered red cell development, which is at least in part, due to inflammatory cytokines acting on erythroid precursors) (Abdalla, 1990) are all important factor(Crichton, 2009; Mazza,2002). Tracking survival of ^{51}Cr-labelled RBCs *in vivo* provides direct evidence for a reduced half-life of uninfected RBCs in human SMA. (Looareesuwan *et. al.,*1991). It has been estimated that ten or more uninfected erythrocytes may be lost for each infected one (Prize *et. al*., 2001) presumably because malaria infection alters uninfected ones. The probable cause of red cell lost include oxidation of band 3(the anion transporter of the red cell membrane lipids and deposition of Immunoglobin G, complement or immune complexes on the erythrocyte surface. A study conducted in Kenya also shows that SMA among children is characterized by reduction

in erythropoietic response (Were *et. al.*, 2006). Haematological studies of individuals with malaria illustrate that bone marrow abnormalities, such as ineffective erythropoiesis, and reduced erythroblast proliferative rates, contribute to the development of severe anaemia (Srichalkul *et. al*,1969; Abdalla, 1990; Das *et. al.*,1999 Wickramasinghe and Abdalla 2000). It has also been shown that there is a marked reduction of reticulocyte response in relation to the degree of anaemia, even under conditions in which there are adequate levels of erythropoietin (a critical stimulus for erythropoiesis)(Kurtzhals *et. al*., 1997; Nussenblatt *et.al.*, 2001). The underlying biological pathway responsible for suppression of erythropoiesis in malaria-infected individuals appears to be as a result of decreased responsiveness to erythropoietin in the erythroid precursor caused by TNF and IFNɔ (Chang 2004; McDevitt, 2004). Other studies also demonstrate that dysregulation in cytokines, chemokines, and effector molecules is important for promoting inflammation derived anaemia in children with malaria (Were *et. al.*, 2006; Ongôecha *et. al.*, 2008). A lack of correlation between parasite densities and severity of anaemia is often reported (Philips and Pascal, 1992: Dandrops *et. al.,*1999)

2.11.2 Malaria and Platelets/ Von Willibrand Factor (VWF)

Thrombocytopenia is a very common association in malaria (Khan 2012). Wickramasinghe and Abdalla, (2000) also reported that thrombocytopenia and anaemia are the most frequent malaria-associated haematologic complications. These reports were supported by various work independent work conducted across the country and beyond. (Kehinde *et. al.,* 2005; Jeremiah and Uko 2007; Faseela *et. al.,* 2011) All these works suggest that thrombocytopenia is an important predictor of malaria infection (Jeremiah and Uko 2007), and a reliable diagnostic marker (Jadhav *et. al.,* 2004). The presence of thrombocytopenia in acute febrile travelers returning from tropical areas has become a highly sensitive clinical marker for malaria diagnosis (Acremont *et. al.,* 2002). Thrombocytopenia is defined as platelet count less 150,000/ml in whites and less than 75,000/ml in Africans (personal communication). However, degree of thrombocytopenia can be defined in grades thus; Platelet counts of 75,000 to 150,000/dl are defined as grade one thrombocytopenia, 50,000 to < 75,000/dl as grade two, 25,000 to< 50,000/dl as grade three and a count below 25,000/dl as grade four (Khan 2012). In a study carried out in Kenya titled ñImpact of infection on haematological parameters in children living in Western Kenyaò it was observed that thrombocytopenia was present in 49% of malaria-

infected children and was associated with high parasitemia levels, lower age, low Hb levels, increased mean cell volume(MPV) and platelet aggregate flag. Platelet aggregate were more frequent in malaria infected children and associated with thrombocytopenia rather than non malaria status. They also observed that children with platelet counts <150000/ml was 13.8 times (odd ratio) more likely to have malaria. This finding is consistent with the report of Jeremiah and Uko (2007) who worked among asymptomatic malaria group in South-South Nigeria. It is not surprising to associate high level parasitemia with low Hb level (anaemia) and thrombocytopenia since infected Red cell may bind indirectly to endothelial cells through platelets. Platelet-mediated red blood cell adhesion is especially important because CD36 is not strongly expressed on cerebral endothelial cells (Silamut *et. al.*, 1999) and therefore serves as a bridge between parasitized red blood cells and endothelium. This was deduced from one of the experiments carried out by Wassmer and colleagues (2004) who co-incubated infected red blood cells with CD36-deficient brain microvascular endothelial cells grown to confluence in vitro. They demonstrated that adherence of infected red blood cells to endothelial cells under both static and flow conditions depended on the presence of platelets (Wassemer *et. al.*, 2004). The adhesion of infected red blood cells to platelets is mediated by platelet CD36, and could be blocked by anti-CD36. Electron microscopy showed platelets localized between the infected red blood cells and the endothelium (Wassmer *et. al.*, 2004). Platelet localization to brain enthothelium was found to be higher in children who died from cerebral malaria compared with uninfected coma controls (Grau 2003)

The mechanism by which platelets promote adhesion of parasitized red blood cells is not fully understood, however, a mechanism has been proposed that tries to relate to the central role of Von Willibrand Factor (VWF). Lower levels of VWF are found in blood group O individuals compared with non O (group A and B) and may contribute to the apparent genetic advantages of group O in malaria. VWF in turn is directly related to the ADAMTS13 activity. Inflammation, endothelial activation, Nitrous Oxide (NO) depletion, Reduced ADAMTS13 activity, and procoagulant microparticles may contribute to cytoadhesion. Evidence for an important role of VWF in malaria is emerging. It has been shown that cerebral malaria is associated with increased levels of VWF propeptide and decreased levels of ADAMTS 13 activity. (Hollestelle *et. al*., 2006; Quirijn de Mast *et. al.,* 2009; Larkin *et. al.,* 2009). VWF propeptide is released from endothelial storage sites in response to endothelial damage or activation by cytokines found in

children with malaria (Gordeuk *et. al.*, 2006; Shi *et. al.*, 2004). Be it as it may, the fact that the platelet counts usually returns to normal quickly in a matter of few weeks when the malaria is treated successfully (Khan 2012) brings out another dimension in the issue of mechanism of thrombocytopenia, complicating its understanding. Other speculated mechanisms leading to thrombocytopenia are coagulation disturbance, splenomegaly, bone marrow alterations, antibody-mediated platelet destruction, oxidative stress, and the role of platetlets as cofactors in triggering severe anaemia. (Khan *et. al.*, 2008; Rasheed *et. al.,* 2008; Faseela *et. al.*, 2011). Though a number of these observational studies have confirmed the association of thrombocytopenia to malaria, the cause of the thrombocytopenia is at its best poorly understood.

2.12 MALARIA AND ABO BLOOD GROUP/ PARASITE ROSSETTING

The association of genetic markers with malaria has been the subject of numerous investigations, since the protection afforded by sickle-cell hemoglobin against infection by falciparum malaria parasite was discovered. There are numerous reports on the effects of ABO blood group on various forms of malaria from several countries, with contradictory conclusions or conflicting results (Deepa, 2011). The study conduted by Thakur and Verma (1992) suggests that ABO blood groups do not show differential susceptibility to malaria. The work of Joshi and colleagues (1987) carried out in Delhi agreed with this finding that no correlation between ABO blood groups and malaria exist. Yet other studies indicated a possible relationship (singer, 1986; Deepa, 2011). The study of Christine and colleagues (2005) supports this line of thought ñthat available evidence suggests that the origin, distribution and relative proportion of ABO blood groups in humans may have been directly influenced by selective genetic pressure from *Plasmodium falciparum* infectionò (Christine *et. al.,*2005), in similar vain, reports suggest a correlation between severity of malarial infection to the patientôs blood group. This school of thought has generated an interest especially in the quest for the answers to the factors influencing clinical course of malarial disease. Clinical reports of ABO blood groups and *P. falciparum* infection, reveals a correlation between disease severity and ABO groups, were blood group O confers resistance or protection to malaria, while blood group A and blood group B might be detrimental. (Deepa *et. al.*, 2011) However, several studies undertaken have not been able to link ABO blood groups to the incidence of malaria or to the repeat attacks of malaria (Fisher and Brown, 1997; Singer *et. al*., (1995). The observation by Miller and associates (1995) that human

erythrocytes lacking the Duffy blood group antigens are refractory to invasion by *P. vivax* parasites indicates the usefulness of studying the association of blood group with malaria. Russell (1963) had earlier suggested that the genetic makeup of individuals may cause a considerable variation in their reaction to malarial infections and blood groups are merely expression of genetic constitution. Other opinions appear to suggest that the variations may be ascribed to the feeding habit of the vector species. According to Boyd (1948) some people are more prone to mosquito bites than others. Wood and colleagues found that under laboratory conditions Anopheles gambiae seems to recognize blood groups and to feed preferentially on group O. Recent studies on the pathogenesis of malaria have shown that parasite triggered red blood cell rosette formation is associated with the severity of clinical disease and malaria (Trentiger *et. al.*, 1992: Pathirana *et. al*., 2005).

Rosetting was established as a *P. falciparum* virulence factor, the expression of which is modified by a variety of host factors. Rosetting is a process by which red blood cells form clusters consequent to faciparium infestation thereby obstructing blood flow in micro circulation. Anti-rosetting activity, presumably mediated by antibodies, was found in sera from patients in malaria endemic areas, and it was demonstrated that such activity was more abundant in individuals with uncomplicated malaria than in those with cerebral disease, suggesting that humoral immunity protects against rosette formation *in vivo*. Some studies have shown that rosetting parasites form larger, stronger rosettes in non-O blood groups (A, B or AB) than in group O red blood cells (Rowe *et, al.,* 1995). Also the percentage of infected RBCs forming rosettes is significantly lower in fresh clinical isolates derived from group O than in non ï O patients (Udomsangpetch *et al*, 1995). It appears that this is because the A and B antigens are receptors for rosetting on uninfected RBCs (Chotivamch and Udomsangpetch, 1998) being bound by a parasite protein called *plasmodium falciparaum* erythrocyte membrane protein 1 (PfEMP1) which is expressed on the surface of infected RBCs (Rowe *et. al.*, 1995)

2.13 MALARIA AND RED BLOOD CELL POLYMORPHISM

Human genetic background strongly influences susceptibility to malaria infection and progression to severe disease and death (Weatherall and Clegg, 2002). The different geographic distributions of sickle-cell disease, Ŭ-thalassaemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, ovalocytosis, and the Duffy-negative blood group are examples of the general

principle that different populations have evolved different genetic variants to protect against malaria (Campino 2006 ;Toria *et. al*., 2008). In Tanzania, strong associations have been described between malaria transmission intensity and polymorphisms of both the HbS and alpha-thalassaemia genes (Clark *et. al*., 2009). In addition to the sickle polymorphism (HbS) (Monkenhaupt 2006), G6PD (Sabeti, 2002), and ABO blood group (Rowe *et. al.,* 2007), a number of other traits have been proposed for the reduced risk of severe malaria. Consistent with the view that severe malaria disease is, at least in part, an inflammatory process mediated by disordered immune responses (Artavanis-Tsakonas, 2003), many of these traits are polymorphisms in genes that are relevant to immunity and inflammation such as the tumor necrosis factor (TNF), MHC class III region (Clark *et. al*.,2009) Toll-like receptors (TLR-4,9) (Monkenhaupt 2006), CD40 ligand (Sabeti 2002), the interferon gamma (IFNG) (Steveson *et. al*.,2004), and the Nitric oxide synthase type 2 (*NOS2*A) genes (Clark *et. al*., 1996). It has been shown that severity of several malaria infections such as asymptomatic, CM and SMA varies significantly between individuals and between populations. Several inter-ethnic comparative studies showed that some populations are more resistant to *P. falciparum* malaria than other sympatric ethnic groups (Toria *et al*., 2008). For example a study conducted among Fulani population in West Africa gave credence to this axiom were higher immune reactivity and higher resistance to malaria was obseverd among them than are other sympatric ethnic groups (Toria *et al*., 2008). The results suggest that T-regulatory cell activity could be central in the control of malaria infection in populations exposed to naturally high *P. falciparum* transmission. This study also highlights the existence of clear-cut differences in strategic pathways of the immunoregulatory network between sympatric populations differing in their genetic background and degree of susceptibility to malaria. A higher resistance against *P. falciparum* malaria could have been the driving selective force of this disorder. Polymorphisms within red cell genes therefore, reflect past and present actions between the parasite and host. Host genetics has changed many genes encoding red cell proteins rendering an advantage to the host over the parasite. However, many of these changes have come at a cost. Many of the hemoglobinopathies are lethal in the homozygous state yet presumably offer protection to heterozygotes which would explain the geographic coincidence between the thalassemias, HbS and malaria (Foote, 2004).

2.14 MALARIA PIGMENTS

The malaria pigment, hemozoin (HZ), is a hydrophobic polymer of heme molecules produced by the malaria parasite as a product of hemoglobin catabolism within the food vacuole (Rudzinska *et. al.,* 1965). It serves to protect the parasite from potentially toxic free heme, and in addition induces pathology in the infected host. When subsequently released into the blood stream, it is captured by and concentrated in the reticulo-endothelial system. When the infected red blood cell bursts, the merozoites go to infect new cells and the hemozoin is released into the blood stream where it is scavenged by macrophages. The hemozoin is extremely stable and remains undegraded in the host organism for an extended period of time (years), mostly concentrated in the liver, kidneys and spleen (Ulrich and Cerami 2004). Accumulating evidence suggests that HZ is immunologically active. (Were *et. al.,* 2009). Studies have demonstrated that hemozoin, either chemically made or isolated from the natural source, stimulates the production of tumor necrosis factor (TNF), and the macrophage inflammatory proteins, MIP-1Ŭ and MIP-1ƃ, in both murine macrophages and in human peripheral blood monocytes in vitro. However the molecular mechanism(s) through which HZ modulates the innate immune system is not clear, However, some suggestions was offered by the work carried out by Were and colleagues(2009) which demonstrated that HZ purified from *Plasmodium falciparum* is a novel non-DNA ligand for Toll-like receptor (TLR)9. Such responses were severely impaired in $TLR9^{\bar{I}/\bar{I}}$ (TLR9 negative). Recent evidence suggests that Toll-like receptors (TLRs) are involved in the innate immune responses to a variety of pathogens, not just *Plasmodium* infections. (Janeway and Medzhitov 2002; Akira and Takeda, 2004). This cytokine release is specific for hemozoin and not secondary to ingestion of particulate matter (e.g., latex beads), heme-containing compounds (hematin or hemin crystals) or to contamination with endotoxin (Sherry *et. al.,* 1995). Administration of chemically synthesized hemozoin to rats provoked a dysregulation of temperature comparable to that observed with the administration of endogenous pyrogens, such as TNF and MIP-1Ŭ.

In contrast to induction of cytokines by lipopolysaccharide (LPS) which peaks within hours and is promptly shut off, the indigestible hemozoin continues to induce TNF for 72 hours. This prolonged stimulation of cytokines is believed to be responsible for the elevated serum levels of TNF that are observed many weeks after malaria infections have been cured in children (Kwiatkowski *et. al.*,1990). The indigestible hemozoin continues to stimulate the macrophage to

produce cytokines for long periods prolonging the time needed for recovery after resolution of the infection. Also macrophages laden with hemozoin have a number of impaired effector functions (Schwarzer *et. al.,* 1992; Fiorri *et. al.,* 1993; Turrini *et. al.,* 1993). These include decreased ability to mount an oxidative burst, decreased killing of pathogens, antigen presentation and a depression of cellular immunity.

CHAPTER THREE

SUBJECTS, MATERIALS AND METHODS

3.1.0 STUDY SITE

The site for this study is Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi, Anambra State, South- East Nigeria. It is a tertiary facility that serves the entire state with a population of 4,177,828 according to 2006 census. (National Bureau of Statistics, 2009) It also serves its neighboring states; Delta and Imo states. The area lies in the tropical rain forest zone and has an all year round *P. falciparum* malaria transmission with bi-peak periods in March/April and November/December (Okocha *et .al*. 2005). These peak periods coincide with the onset and cessations of rains. The people are Ibos by ethnicity, with Christianity as the major religion. The main occupation of the people is farming, trading and civil service.

3. 1. 2. STUDY POPULATION.

The study population were children with *P. Falciparum* parasiteamia between the ages of 3 months and 12 years old who were attending children outpatient clinic (CHOP), children emergency clinic (CHER) or hospitalized patients during the period of study (March to December, 2011). Cases were classified as severe malaria based on modified criteria put forward by WHO (2000). More than one clinical criterion was possible, but cerebral malaria and severe anaemia were considered to be the primary defining features. Each index case was age, residence and ethnicity matched to a healthy control group. Age categories were defined as 3 to 5 months, 6 to 11months, 1 to 5 years and 6 to 12 years. Residence is defined as any one residing with the reach of the study site that visits NAUTH for health issues. A total number of 210 subjects comprising of 143 subjects with uncomplicated malaria, 15 with complicated malaria (defined according to the 1990 and modified 2000 WHO as stated earlier) and 52 subjects without malaria that served as the control group were recruited using this formula:

$$N = \frac{Z^2 PQ}{D^2}$$

Where:

N is the sample size

P is a maximum known prevalence of the disease.

Q is 1- p (proportion of persons free from the disease)

D is the degree of accuracy or precision expected (0.05)

$$N = \frac{(1.96)^2 \times (0.50 \times 0.05)}{(0.05)^2}$$

$$N = 150$$

3. 1. 3 INCLUSION CRITERIA AND EXCLUSION CRITERIA

All febrile patients with clinical features of malaria with parasitological evidence are enlisted. Clinical malaria is defined as having symptoms of malaria (history of fever, chills, headache, or severe malaise) accompanied by a positive blood stage infection by microscopy. (Moormann *et. al.,* 2009). A parasite density threshold is not used in the definition of clinical malaria because asymptomatic parasiteamia is common particularly in endemic regions (John *et. al.*, 2005). Patients with co-morbidity; that is malaria in addition to any of these: lobar pneumonia, meningitis, septicaemia, typhoid fever, pylonephritis, viral hepatitis, encephalitis, malignancies, HIV, sickle cell disease and those on antimalarial therapy are excluded. Reason for exclusion of co-morbidity is that these medical conditions also respond to challenge by producing varying levels of cytokines (WHO, 2000a; WHO, 2000b). Exclusion of patients with co-morbidity is to ensure that the level of the analytes observed in these patients is purely due to malaria. Since this stage of recruitment is based on clinical grounds, inclusion or exclusion is based on assessment by a paediatrician (Dr Ebenebe Joy), who is a collaborator at this stage in the study. The control groups were enrolled as healthy if they were asymptomatic for acute illness, had no evidence or history of chronic illness and were found to be aparasitemic upon examination.

3. 1. 4. RECRUITMENT PROCEDURE

This is a case controlled study. At enrollment, relevant clinical features including vital signs are assessed and documented by the attending paediatrican. Clinical information obtained is entered into standardized forms. For the patients who met the inclusion criteria based on clinical ground, a drop of blood through finger prick was obtained for rapid diagnostic testing (RDT) and peripheral blood smears for microscopy. The smear was stained with 10% Giemsia stain for 10

minutes and examined under the microscopy immediately (using x100 objective). The result was communicated immediately to the attending doctor for management/ treatment of the patient. The patients that have positive smear were enlisted into the study.

Controls were recruited from clients attending the infant welfare clinic (Immunization Clinic) of the Teaching Hospital and from apparently healthy clients who are on checkups and medical examination. Smears were also made as in the study group and examined for possible presence of asymptomatic parasiteamia.

Study protocols were reviewed and approved by Nnamdi Azikiwe University Teaching Hospital Nnewi, Ethical Committee and written permission sought from the Head of Department of Peadiatrics for the use of their patients.

Subjects and controls were recruited following due ethical consent from their parents/guardians.

3.1.5 SAMPLE COLLECTION

For all enlisted subjects who have met the clinical and parasitological criteria, and also for the control subjects, 5ml of venous blood sample was withdrawn; 2ml into tubes containing EDTA used for full blood count determination. The number of parasites per 200 white blood cells and the number of parasites per microliter of blood cells determined (parasite density), the presence of malaria pigments (haemozoins) were checked for. Differential WBC and morphological examination of cells were done. Absolute measures of cells and abnormalities in shape and size were determined. The remaining 3ml blood volume was dispensed into the plain tubes and left to stand in a vertical position for 1hour for clotting to take place. This was then centrifuged at 1000 x g for 10 minutes. The separated serum was dispensed in aliquots of four and stored frozen at ï 70^0C until cytokine and haptoglobin testing was performed (duration of storage not exceeding 3 months of collection). The red cells component from the separated clotted sample was used to determine the ABO blood group and haemoglobin phenotype of the subjects. The levels of TNFǍ, IL-I, IL-6 and haptoglobin in the serum were determined using ELISA Kit procured from Abcam company, Cambridge United Kingdom according to manufacturerôs recommendation. The reason for the choice of this method is that it is a sensitive technique that requires small (micro) quantities of samples.

3.2.0 METHODS

3.2.1 FILM MAKING (*Dacie and Lewis* 2006)

Blood films are made on clean glass slides. Films can also be made on cover glasses, but they have no advantage over that made on slides and are unsuitable for modern laboratory practice. Films may be spread by hand or by means of an automated slide spreader. Manual method was employed in this work because of the few work load involved in the daily collection of patients sample. Manual method was then described in this work.

THICK FILM

When making a thick film the slides were always handled by the edges or at a corner.

Using the corner of a spreader, the drops (6µl) of blood were spread to produce an even thick film of 10mm x 10mm diameter area. The blood was not excessively stirred but was spread in a rectangular form with 3 to 6 movements. The thick film was allowed to dry in a flat, level position protected from flies, dust and extreme heat. When thoughrouly dried, the slide was ready for staining.

THIN FILM;

Using another clean slide as a spreader, the small drop (2µl) was touched with the spreader about 1cm from one end and the blood was allowed to run along its edge. The spreader was firmly pushed along the slide, keeping at an angle of 45 degrees. It was ensured that the spreader is in even contact with surface of the slide at all times the blood was been prepared. The thin film was labelled with the soft lead pencil by writing across the thicker portion of the film the patients/number and date. This slide was then ready for staining.

3.2.2 FILM STAINING (*Dacie and Lewis* 2006)

The thin films were fixed in methanol and allowed to air dry. The freshly diluted Giemsa stain with buffered water pH7.2 (see appendix for dilution) was then poured on the slides (both thick and thin films) and allowed to stain for 45mins. The films were then gently washed with buffered water. The films were stood upright to air dry. Giemsa stain is the preferred stain because at pH7.2, the chromatin dots of the parasites are well stained which enhances morphological characterization. Also it can be applied to both thick and thin films. However, Lieshman stain or

any of the Romanskwy stains can be used for staining the thin films. Fields stain can also be employed in staining the thick film.

3.2.3 EXAMINATION

PRICINCPLE

Conventional light microscope is the established method for the laboratory confirmation of malaria. It is regarded as the ñgold standardô. The careful examination by an expert microscopist of a well-prepared and well-stained blood film remains currently the accepted standard for detecting and identifying malaria parasites. In most settings the procedure consists of collecting a finger- prick blood sample; preparing a thin and thick film, staining the smear(most frequently with Giemsa) and examining the smear through a microscope(with 100x oil immersion objective) for the presence of malaria parasites.

3.2.4 METHOD OF ENUMERATION OF PARASITE DENSITY.

1. The blood film was inspected under the microscope for the quality of staining.
2. Using 40x objectives, the films was scanned for area free of staining debris
3. A drop of immersion oil was allowed on the dry stained slide without applicator touching the slide.
4. Then it was inspected using the oil immersion objective (the objective not touching the slide).
5. The thick film was scanned for an area that has approximately 8 WBCs per high power field (HPF).
6. The slide was systematically inspected for malaria parasite using the horizontal examination method of systematic inspection (Fig 3.1). A tally counter was used to count the fields as they were examined.
7. If parasites were observed, they were simultaneously counted with WBCs up to a total of 200 WBCs.
8. The parasite count and WBCs were converted to absolute parasite density (parasites/µl) by the following formula:

$$\frac{\text{\# Malaria parasites X Patients WBC count}}{\text{\# WBC counted}} = \text{parasites/µl}$$

9. In the absence of the patients WBC count 8,000 could be used in place of the patients WBC count to obtain the parasite density with this formular.

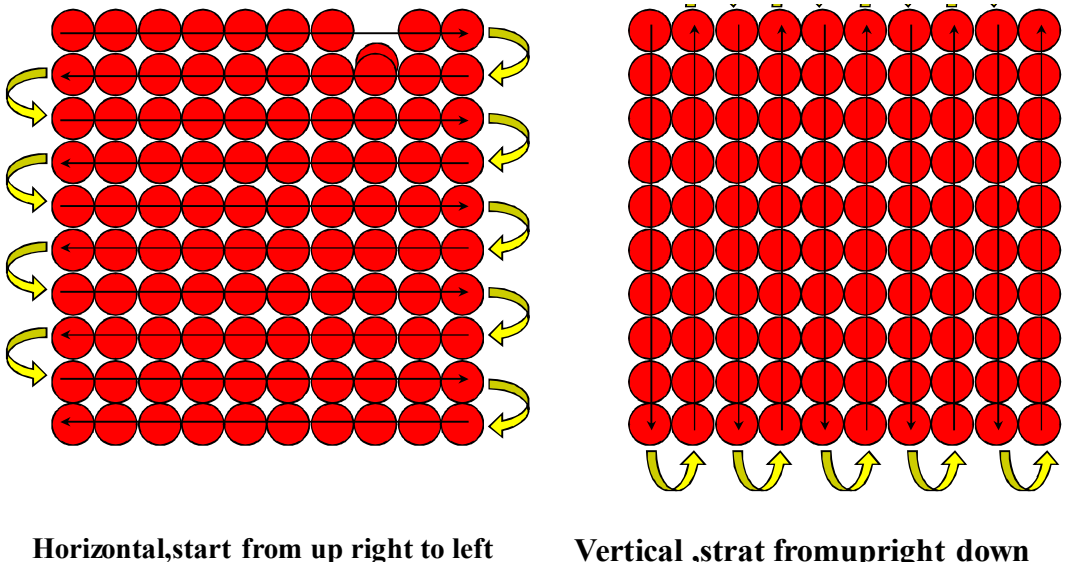
$$\frac{\text{\# Malaria Parasite X 8,000}}{\text{\#WBC counted}} = \text{Parasite/µl}$$

3.2.5 REPORTING OF RESULT

Results were expressed as the stage/s of the malaria parasites seen, the species seen, and the number seen per microlitre of blood. Asexual (rings, trophozoites and schizonts) and sexual (gametocytes) are reported separately. The asexual forms are the ones that produce clinical sympotoms in man while the presence of gametes is of public health importance.

3.2.6 EXAMINATION OF THE THIN FILM

The thin films are used both for speciation and performing differential WBC count to confirm the value obtained through automated machine. The Horinzontal method as shown in Fig 3.1 is employed. The WBCs in the fields were counted until 100 cells were counted.



Reading slide pattern

FIGURE 3.1 READING SLIDE PATTERNS

Adapted from National Malaria Control Program Training Manual, 2010

FIG 3.2 SYSMEX KN21 N MACHINE FOR CELL COUNTS IN HAEMAOLOGY

3.2 7 FULL BLOOD COUNT USING SYSMEX HAEMATOLOGY

3.2.7.1 INTENDED USE

The sysmex KX-21N is an automatic multi-parameter blood cell counter for in vitro diagnostic use in clinical laboratories. It is a bench-type instrument designed to perform nineteen parameters of haematological analysis on whole blood, either collected with anticoagulation or freshly collected from finger stick to be manually diluted before analysis.

3.2.7.2 INTRODUCTION

The Sysmex KX-21N is a 3-Part differential haematology analyzer that differentiates the white blood cells into three populations, based on their sizes, Neutrophils, Lymphocytes, and mixed population which consists of Eosinophils, Monocytes, and Basophils. KX-21N processes approximately 60 samples in an hour and displays on the Liquid Crystal Display (LCD) screen the particle distribution curves of White Blood cell Count (WBC), Red Blood cell Count (RBC) and platelets, along with the data of 19 parameters as the analysis results.

Sysmex KX-21N is a quantitative automated haematology analyzer for in vitro diagnostic use in determining 19 haematological parameters at ago. It directly measures the White Blood cell Count (WBC), Red Blood cell Count (RBC), Haemoglobin (HGB), Haematocrit (HCT), Platelets(PLT), Absolute Lymphocyte Count (LYM#), Absolute Mixed Count (MXD#) and Absolute Neutrophil Count (NEUT#), while the remaining parameters are calculated or derived: Mean Cell Volume (MCV), Mean Cell Haemoglobin (MCH), Mean Cell Haemoglobin Concentration (MCHC), Mean Platelet Volume (MPV), Percentage Lymphocyte (LYM%), Percentage Mixed (MIX%), Percentage Neutrophil (NEUT%), RBC distribution width-coefficient of variation (RDW-CV) and RBC distribution width-standard deviation (RDW-SD), PDW and P-LCR. These excellent features are what informed its use in this work.

3.2.7.3 PRINCIPLE

Blood sample collected in EDTA anticogulatant (50µl) is diluted with Cellpack in the WBC counting chamber. A fixed volume of Stromatolyser-WH solution (1 volume of stromatolyser-WH to 2 volumes of cellpack) is added to automatically obtain a final dilution of 1:500. The

addition of stromatomyser-WH lyses the RBC and so the remaining cell stroma is at a level undetectable by the instrument. They are then counted by Direct Current method. Haemoglobin is released during RBC lyses, and is converted to the methemoglobin and read photo metrically at 555nm. A portion of the diluted sample is transferred automatically to the haemoglobin detector where the absorbance of the red pigment is measured to give blood haemoglobin level.

Direct Current Detection Method: Blood sample is aspirated, measured to a predetermined volume, diluted at specific ratio, and then fed into each transducer. The transducer chamber has a minute hole called the aperture. On both sides of the aperture, there are the electrodes between which flows direct current. Blood cells suspended in the diluted sample pass through the aperture, causing current resistance to change between the electrodes. As direct current resistance changes, the blood cell size is detected as electric pulses.

Blood cell count is calculated by counting the pulses, and histogram of blood sizes is plotted by determining the pulse sizes. Also, analyzing a histogram makes it possible to obtain various analysis data.

3.2.8 CYTOKINE ASSAY

Bioassays provided the earliest methods available to characterize cytokines. While potentially more sensitive than immune-based assays, bioassays may not be able to distinguish between cytokines with cross-reacting activities. In addition, bioassays may be falsely negative in the face of inhibitors; it remains useful, however, to assay cytokines by their biological activity. Though ELISAs will not differentiate biologically active from inactive, it would pick up immunoreactive forms of the proteins. Since bioassays will detect only functional activity, they may be useful for identifying cytokine antagonists in a reaction mixture. The molecular cloning of cytokine cDNAs and the development of large-scale expression methods has made available virtually unlimited quantities of protein for biological assays. Purified proteins have also been used to develop immune-based assay techniques such as ELISAs for quantifying cytokines. ELISAs provide the best and in many cases the only mechanism for distinguishing cytokines with significant overlapping biological activities. These properties inform its choice in this work. Although the isolation of cytokine-specific cDNAs permits the use of molecular techniques to identify cytokine-specific mRNA transcripts, which is an extremely sensitive technique for detecting transcripts -the RNA-based polymerase chain reaction (RT-PCR). Biological assays for IL-1, IL-

6, and TNF are well standardized and the general techniques for the ELISA are described in this work.

3.2.8.1 BIOLOGICAL ASSAY FOR TNF alpha USING ELIZA KIT (1X96 well Plate) (ab46087)

3.2.8.1.1 PRODUCT OVERVIEW.

The human TNF-Ŭ ELISA is used for the in-vitro quantitative determination of TNF in human serum, plasma, buffered solutions or cell culture medium. The assay will recognize both natural and recombinant human TNF-Ŭ.

3.2.8.1.2 PRINCIPLE OF METHOD

A monoclonal antibody specific for TNF-Ŭ has been pre-coated onto the wells of the microtiter strips provided. During the first experimental step, the TNF-Ŭ present in the samples (or standards) is incubated with the biotin conjugated monoclonal antibody raised against TNF-Ŭ.
This is followed by a washing step, to remove all unbound biotinylated anti-TNF-Ŭ and the addition of streptavidin-HRP that binds the biotinylated antibody. After incubating and performing a further washing step, a substrate solution reactive with HRP is added. This will result in the formation of a coloured product, in proportion to the amount of TNF-Ŭ present in the sample. The reaction is terminated by the addition of acid, and absorbance can be measured at 450nm. (Product insert)

ASSAY TYPE Sandwich

TESTS 1X96 well plate

SAMPLE TYPES Cell culture supernatant, serum and plasma

SENSTIVITY <10 pg/ml

RANGE 25-800pg/ml

RECOVERY 107%

ASSAY TIME 3h 40min

COMPONENTS	TITLE	IDENTIFIER	1X96 TESTS
	12 strips		8x1 unit
	Standard	Yellow	2x1 vial
	Control	Silver	2x1 vial

Standard Diluent Buffer, x10 Concentrated	Black	1x25 ml
Standard Diluent: human serum	Black	1x7 ml
Biotinylated anti-TNF alpha	Red	1x0.4ml
Biotinylated Antibody Diluent	Red	1x7ml
Streptavin-HRP		2x5µl
HRP Diluent	Red	1x23ml
Washing Buffer.x200 Concentrated	White	1x10ml

3.2.8.1.3 PROTOCOL FOR TNF alpha Human Elisa Kit (1x 96 Well Plate). (ab46087)

HUMAN TNF alpha ELISA Kit

KIT CONTENT AND RECONSTITUTION

REAGENTS	QUANTITY	RECONSTITUTION
96-wells precoated microtiter	Plate 1	Ready to use
Plate covers	2	-
TNF alpha Standard:800pg/ml	2 vials	Reconstitute with the volume of standard diluent indicated on the vial
Control	2 vials	Reconstitute with the volume of standard diluent indicated on the vial
Standard Diluent Buffer	1 bottle	(25ml) 10x concentrate. Dilute in distilled water
Standard Diluent: human serum	1 bottle	(7ml) ready ï to - use
Streptavidin-HRP	2 vials	(5µl) Add 0.5ml of HRP Diluent before further dilutions
Biotinylated anti TNF alpha	1 vial	(0.4ml) dilute in biotinylated antibody diluent
Biotinylated Antibody Diluent	1 bottle	Ready to use
HRP Diluent	1 bottle	(23ml) Ready-to-use
Washing buffer	1 bottle	(10ml) 200x concentrate. Dilute in D/W
Substrate Solution: chromogen TMB	1 bottle (11ml)	Ready-to-use

Stop Reagent H_2SO_4 1 bottle (11ml) Ready-to-use

Preparation of Reagents:

- **Washing Buffer:** Dilute the washing buffer concentrate (200x) in a clean graduated cylinder. Mix gently to avoid foaming. Pour entire contents (10ml) of the washing Buffer concentrate into a clean 2,000ml graduated cylinder. Bring final volume to 2,000ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2^0C to 25^0C. Washing buffer may be prepared as needed according to the following table:

Number of strips	Wash Buffer (ml)	Distilled water (ml)
1-6	5	995
1-12	10	1990

- **Preparation of Standard Diluent Buffer:** Add the content of the vial (10x) to 225ml distilled water before use.
- **Preparation of TNF alpha Standards**

Depending on the type of samples we are assaying, the kit includes two standard diluents. Because biological fluids might contain proteases or cytokine-binding proteins that could modify the recognition of the cytokine we want to measure. We reconstituted the standard vials with the most appropriate standard Diluent. Since we were working with serum samples, we used standard diluents for human serum. For cells culture supernatants, standard Diluent Buffer for culture would be employed. Reconstitute TNF alpha Standard by addition of appropriate Standard Diluent. Reconstitute volume is stated on the label of the standard vial. This reconstitution produces a stock solution of 800pg/ml TNF alpha. Allow standard to stand for 5 minutes with gentle swirling prior to making dilutions. Serial dilutions of standard must be made before each assay and cannot be stored.

PREPARATION OF CONTROLS

Freeze-dried control vials also are reconstituted with the most appropriate Standard Diluent to your samples. For serum and plasma samples: use standard Diluent: human serum. For cells culture supernatants: use standard Diluent Buffer. Control has to be reconstituted with the

volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume will give a solution for which the TNF alpha concentration is stated on the vial. Allowing the control to stand for 5 minutes with the indicated volume, will give a solution for which the TNF alpha concentration is stated on the vial. Allow control to stand for 5 minutes with gentle swirling prior to distributing in control wells. This is not used after storage.

Preparation of biotinylated anti-TNF alpha

Preparation immediately before use is recommended. The biotinylated anti-TNFŮ was diluted with the biotinylated antibody diluent in a clean glass vial. Biotinylated anti-TNF alpha may be prepared as needed according the following table. Extemporaneous preparations are recommended.

Number of strips	**Biotinylated Antibody (ul)**	**Biotinylated Antibody Diluent (ul)**
2	40	1060
3	60	1590
4	80	2120
6	120	3180
12	240	6360

Preparation of Streptavidin-HRP

The streptavidin-HRP was diluted 1:100 just prior to use by adding 0.5ml of HRP diluent to the vial containing streptavidin-HRP concentrate. This dilution with HRP-diluent in a clean glass vial as needed according to the following table:

NUMBER OF STRIPS	**Pre-diluted Streptavidin-HRP (ul)**	**HRP Diluent (ml)**
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

ASSAY PROTOCOL

a. All the reagents were thoroughly mixed without foaming before use.

b. All the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards were determined. Each sample, standard, blank and control samples were assayed in duplicate. **S**ufficient Microwell Strips coated with Antibody to human TNF alpha from the aluminium pouch were removed immediately prior to use**.** Any well not required for immediate assay was returned with desiccant to the pouch and sealed tightly.

c. 100ul of appropriate Standard Diluent (see preparation of reagents) was added to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, and F2.

Standard vial with the appropriate volume as described in the chapter preparation of reagents was reconstituted. 200ul of standard was pipetted into wells A1 and A2 (see plate scheme, Fig 3.4b). 100ul was transfered from A1 and A2 to B1 and B2 wells. The contents were mixed by repeated aspirations and ejections. Care was taken not to scratch the inner surface of microwells. This procedure was repeated from the wells B1, B2 to wells C1, C2 and from wells C1, C2 to D1, D2 and so on creating two parallel rows of TNFŬ standard dilutions ranging from 800 to 25pg/ml. 100ul from the content of the last microwells used was discarded (F1, F2).

Alternatively these dilutions can be done in separate tube and diluted standard pipetted directly into wells. 100uL of appropriate Standard Diluent was added in duplicate to the blank wells (G1, G2). 100uL of sample was added to sample wells in duplicate, to the designated wells and 100uL of reconstituted control vial, in duplicate, to control wells (H1, H2).

Biotinylated anti-TNF alpha was Prepared (refer to Preparation of reagents 5).

50uL of diluted biotinylated anti-TNF alpha was added to all wells.

It was covered with a plate cover and incubated at room temperature (18^0C to 25^0C) for 3 hours.

a. The cover was removed and the plate washed as follows:

- The liquid from each well was aspirated.
- 0.3ml of washing solution was dispensed into each well.

Tthe content of each well was aspirated again.

- Steps 2 and 3 two times were repected.

Streptavidin-HRP solution was prepared just before use (refer to Preparation of reagents).

100uL of Streptavidin-HRP solution was distributed to all wells, including blanks.

The plate was covered and incubated at room temperature (18^0C to 25^0C) for 30 minutes.

The cover and empty wells were removed. Microwell strips were washed according to step i and immediately proceeded to the next step. 100uL of ready-to-use TMB substrate solution was added to all wells, including the wells for control and incubated in the dark for about 12-15 minutes at room temperature. Direct exposure to light was avoided by wrapping the plate in aluminium foil.

Incubation time of the substrate solution is usually determined by the ELISA reader performances. Many ELISA readers record absorbance only up to 2.0 O.D. Therefore the colour development within individual microwells was watched carefully and the substrate reaction stopped before positive wells are no longer properly recordable.

The enzyme-substrate reaction was stopped by quickly pipetting 100uL of Stop Reagent: H_2O into each well, including the blank wells to completely and uniformly inactivate the enzyme. The results were read immediately after the Stop Reagent: H_2SO_4 is added.

The absorbance of each microwell was read on a spectrophotometer using 450nm as the primary wave length (optionally 620nm as the reference wavelength: 610nm to 650nm is acceptable). The plate reader was blanked according to the manufacturerôs instructions by using the blank wells. The absorbance of both, the samples, control and the TNF alpha standards were determined.

3.2.8.4 CALCULATION OF RESULTS

- The average absorbance values for each set of duplicate standards, samples and controls were calculated. Duplicates should be within 20% of the mean.
- A linear standard curve was created by plotting the mean absorbance for each concentration on the ordinate against the TNFŬstandard concentration on the abscissa.
- Then to determine the concentration of TNF in each sample, the mean OD value on the ordinate was first found and extended a horizontal line to the standard curve. At that point of intersection, extend a vertical line to the abscissa and read the corresponding TNFŬ concentration.

Efforts were made not extrapolate the standard curve beyond the 800pg/ml standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain.

Concentrated samples (>800pg/ml) have to be diluted with standard diluents or with ones own sample buffer. During analysis, multiply results by the appropriate dilution factor.

- A standard curve was derived for each group of microwell strips assayed.

3.2.8.5 LIMITATIONS OF THE PROCEDURE

Since this assay method has these limitations, these precursions were observerd.

- A standard curve was established for every run, since exact conditions may vary from assay to assay.
- All glass wares were washed and rinsed in distilled water and different glass wares used in preparation of each reagent since bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware were preferred, reusable glassware were washed and thoroughly rinsed of all detergents before use.
- Since improper or insufficient washing at any stage of the procedure would result in either false positive or false negative results, efforts were made to completely empty wells before dispensing fresh washing buffer filled with washing buffer as indicated for each wash cycle and also not to allow wells to sit uncovered or dry for extended periods.

3.2.9 BIOLOGICAL ASSAY FOR IL-1 USING ELIZA KIT (1X96 well Plate) Ab100560

3.2.9.1 INTRODUCTION

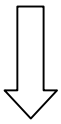
Monocytes are the main office of secreted IL1. They express predominantly IL1. They express predominantly IL1ϐ while Human keratinocytes express large amounts of IL1Ŭ. IL1 is produced also by activated macrophages from different sources (alveolar macrophages, kupffer cells, adherent spleen and peritoneal macrophages) and also by peripheral neutrophil granulocytes. IL1Ŭ and IL1ϐ are biologically more or less equivalent pleiotropic factors that act locally and also systemically.

Ab100560 IL1 alpha Human ELISA (Enyzme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the qualitative measurement of human IL1 alpha in serum, plasma, cell culture supernatants and urine. This assay employs an antibody specific for Human IL1 alpha coated on a 96-well plate and IL1 alpha present in a sample is bound to the

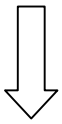
wells by the immobilized antibody. The wells are washed and biotinylated anti-human IL1 alpha antibody, HRP-conjugated streptavidin is pippetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount IL1 alpha bound. The stop solution changes the color from blue to yellow and the intensity of the color is measured at 450nm.

ASSAY SUMMARY

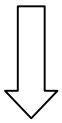
Prepare all reagents, samples and standards as instructed



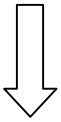
Add 100µL standard or sample to each well. Incubate at 2.5 hours at room temperature or over night at 4^{0}C



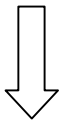
Add 100µL prepared biotin antibody to each well. Incubate for 1 hour at room temperature



Add 100µL prepared Streptavidin solution. Incubate for 45 minutes at room temperature.



Add 100µL TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature.



Add 50µL Stop Solution to each well. Read at 450nm immediately.

KIT CONTENTS

- IL1 alpha microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-Human IL1 alpha.
- Wash buffer concentrate (20x) (item B): 25ml of 20x concentrated solution.
- Standards (item C): 2 vials, recombinant Human IL1 alpha.
- Assay diluent A (item D): 30ml, 0.09% sodium azide as preservative. For standard/sample (serum/plasma) diluent.
- Assay diluent B (item E): 15ml of 5x concentrated buffer. For standard/sample (cell culture medium/urine) diluent.
- Detection Antibody IL1 alpha (item F): 2vials of biotinylated anti-Human IL-1 alpha (each vial is enough to assay half microplate).
- HRP-Streptavidin concentrate (item G): 8µL of 25,000 x concentrated HRP-conjugated streptavidin.
- TMB One-Step Substrate Reagent (Item H): 12ml of 3,3' .5,5'-tetramethylbenzidine (TMB) in buffered solution.
- Stop solution (item 1): 8ml of 2M sulfuric acid.

STORAGE AND HANDLING

Ab100560 may be stored for up to 6 months at 2^0C to 8^0C from the date of shipment. Standard (recombinant protein) should be stored at -20^0C or -80^OC (recommended at -80^OC) after reconstitution. Opened Microplate wells or reagents may be stored for up to 1 month at 2^OC to 8^OC. All unused wells were returned to the pouch containing desiccant pack, resealed along

entire edge. The kit can be used within one year if the whole kit is stored at -20^{O}C and repeated freeze-thaw cycles were avoided.

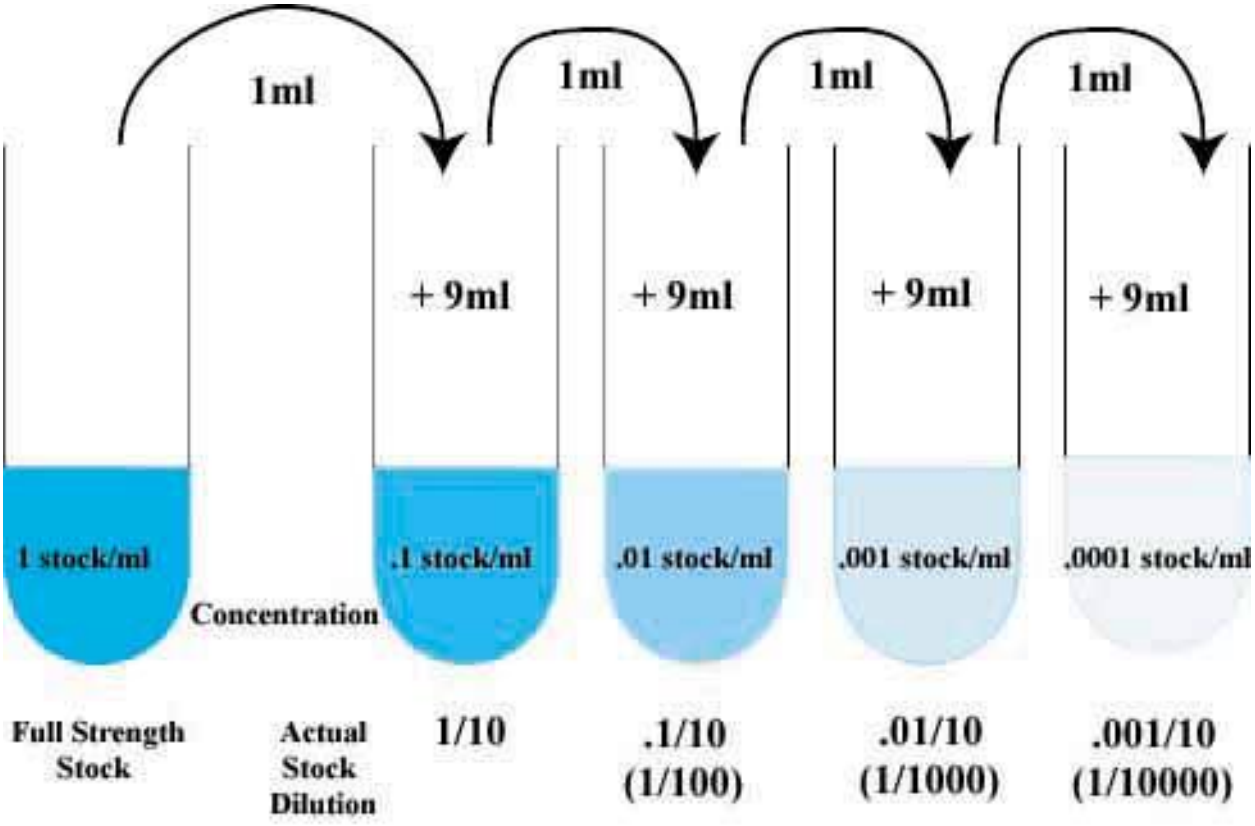
ADDITIONAL MATERIALS REQUIRED

- 1 microplate reader capable of measuring absorbance at 450nm.
- Precision pipettes to deliver 2µL to 1ml volumes.
- Adjustable 1-25ml pipettes for reagent preparation.
- 100ml and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionzed water.
- Log-log graph paper or computer and software for ELISA data analysis.
- 8 tubes to prepare standard or sample dilution.

PREPARATION OF REAGENTS

- All reagents and samples were brought to room temperature (18-25^{0}C) before use.
- Sample dilution: when sample needs to be diluted, Assay Diluent A (Item D) was used for dilution of serum/plasma since Assay Diluent B (item E) is for dilution of culture supernatants and urine.
- Assay Diluent B should and was diluted 5-fold with deionized or distilled water.
- Preparation of standard: The vial of item C was briefly spun. 400µL of Assay Diluent (for serum/plasma samples) was added into item C vial to prepare a 100ng/ml standard. The powder was thoroughly dissolved by a gentle mix. 5µL IL-1 alpha standard from the vial of item C was added into a tube with 828.3µL Assay Diluent A to prepare a 600pg/ml stock standard solution. 400µL Assay Diluent A was pipetted into each tube. The stock standard solution was used to produce a dilution series

(shown below). Each tube was mixed thoroughly before the next transfer. Assay diluent A served as the zero standard (0pg/ml).



(FIG 3.3: DIAGRAM SHOWING SERIAL DILUTION OF THE STOCK SOLUTION).

- If the wash concentrate (20x) (item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20ml of wash Buffer Concentrate into deionized or distilled water to yield 400ml of 1x Wash Buffer.

- Briefly spin the detection antibody vial (item f) before use. Add 100µL of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate should be diluted 80-fold with 1x Assay Diluent B and used in step 4 of part 7 Assay method.

- Briefly spin the HRO-Streptavidin concentrate vial (item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 25,000-fold with 1x Assay diluent B. For example: add 2µL of HRP-Streptavidin concentrate into a tube with 198µL 1x Assay Diluent B to prepare a 100-fold diluted HRP Streptavidin solution (don't store the diluted solution for next day use). Mix through and then pipette 60µL of prepared 100-fold diluted solution into a tube with 15ml 1x Assay Diluent B to prepare a final 25,000 fold diluted HRP-Streptavidin solution.

ASSAY METHOD

- All reagents and samples were brought to room temperature (18-25^{0}C) before use. It is recommended that all standards and samples be run at least in duplicate.

- 100µL of each standard and sample (see preparation of reagents step 2) were added into appropriate wells. Covered well and incubated for 2.5 hours at room temperature (over night at 4^{0}C with gentle shaking could also be employed).

- The solution was discarded and washed 4 times with 1X wash solution. Washed by filling each well with Wash Buffer (300µL) using a multi-channel pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last was, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted against paper towels.

- 100µL of 1x prepared biotinylated antibody (see preparation of reagents step 6) was addede to each well. Incubated for 1 hour at room temperature with gentle shalking.
- The solution was discarded, and then washed as in step 3.
- 100µL of prepared Streptavidin solution (see preparation of Reagents step 7) was added to each well. Incubated for 45 minutes at room temperature with gentle shaking.
- The solution was discarded. Repeat the wash as in step 3
- 100µL of TMB One-Step Substrate Reagent (item H) was added to each well. Incubated for 30 minutes at room temperature in the dark with gentle shaking.
- 50µL of stop solution (item I) was added to each well and read at 450nm immediately.

SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested (e.g., Human Angiogenin, BDNF, BLC,ENA-78, FGF-4, IL-1B, IL-2, IL-4, IL-5, IL-7, IL-8, IL-9, IL-11, IL-12 p70, IL-12p40, IL-13, IL-15, IL-309, IP-10, G-CSF, GM-CSF, IFN, LEPTIN, TARC, MIP-1a.

3.2.10. BIOLOGICAL ASSAY FOR IL-6 USING ELIZA KIT (1X96 well Plate) (ab46042).

3.2.10.1 PRODUCT OVERVIEW:

The human high sensitivity IL-6 ELISA is to be used for the in-vitro quantitative determination of interleukin-6 (IL-6) in human serum, plasma, buffered solutions or cell culture medium. The assay will recognize both natural and recombinant human IL-6. This kit has been configured for research use only and is not to be used in diagnostic procedures.

3.2.10.2 PRINCIPLE

The IL-6 is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for IL-6 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known IL-6 concentrations, control specimens and unknowns are pipetted into these wells. During the first incubation, the IL-6 antigen and a biotinylated monoclonol antibody specific for IL-6 are simultaneously incubated. After washing, the enzyme (streptavidin-peroxydase) is added. After incubation and washing to remove the entire unbound enzyme, a substrate solution which is acting on the bound enzyme is added to induce a coloured reaction product. The intensity of this coloured product is directly proportional to the concentration of IL-6 present in the samples.

Recovery: 93%

Species Reactivity: Reacts with Human

Product Name: High sensitivity IL-6 Human ELISA kit (1 x 96 well plate).

3.2.10.3 PROTOCOL FOR HIGH SENSITIVITY IL-6 HUMAN ELISA KIT

Human IL-6 High Senstivity ELISA Kit

Material Required:

- Distilled Water
- Pipettes
- Vortex mixer and magnetic stirrer

Procedural Notes/Lab, Quality Control

- When not in use, kit components were stored refrigerated or frozen as indicated on the vials or bottles labels. All reagents should were warmed to room temperature before use. Lyophilized standards are discarded after use.

- Once the desired number of strips has been removed, the bag is immediately resealed to protect the remaining strips from degradation.
- All reagents were cover or capped when not in use.
- Reagents between different lots were not interchange or mixed.
- Reagents were not used beyond the expiration date of the kit.

A clean disposable plastic pipette tip was used for each reagent, standard or specimen addition in order to avoid cross-contamination for the dispensing of H_2SO4 and substrate, avoid pipettes with metal plates.

- A clean plastic container was used to prepare the washing solution.
- The reagents and samples were thoroughly mixed before use by agitation or swirling.
- All residual washing liquid were drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Absorbent paper was never inserted directly into the wells.
- The TMB solution is light sensitive, therefore prolonged exposure to light.awas avoided. Also, contact of the TMB solution with metal to prevent colour development was also avoided. TMB is toxic therefore direct contact with hands was avoided. And dispose of properly
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. The absorbance was read within 1 hour after completion of the assay.
- When pipetting reagents, efforts were made to maintain a consistent order of addition from well-to-well. This would ensure equal incubation times for all wells.
- Incubation times described in the assay procedure was respected.
- The TMB solution was dispensed within 15min, following the washing of the microtiter plate.

SPECIMEN COLLECTION, PROCESSING AND STORAGE

- Serum: Pyrogen/endotoxin free collecting tubes were used to avoid any unintentional stimulation of the cells by the procedure. Sera were removed rapidly and carefully from the red cells after clotting and centrifuged at approximately 1000xg for 10 mins.

- Plasma: EDTA, Citrate and heparin plasma could also be used for the assay. Spin samples at 1000xg for 30min to remove particulates if plasma were to be used. Harvest the plasma, however, serum was preferred in this study.

- Storage: when the samples were not analyzed shortly after collection, they were aliquoted (250-500uL) to avoid freeze-thaw cycles and stored frozen at -70^0C. Multiple freeze-thaw cycles of frozen specimens were avoided. Badly hemolyzed or lipaemic sera were avoided. Large amounts of particles where present were removed, prior to assay by centrifugation.

- Efforts were made to thaw at room temperature and samples were completely thawed and homogenous before assaying.

REAGENT PREPARATION:

- Standard Buffer Diluent 10x concentrate: dilute 10 times with distilled water before use.

- Standards: Depending on the type of samples being assayed, the kit includes two standard diluents. Because biological fluids might contain proteases or cytokine-binding protein that could modify the recognition of the cytokine you want to measure, you should reconstitute standard vials with the most appropriate standard diluent. For serum and plasma samples use standard diluent: human serum. For cell culture supernatants use Standard diluent buffer. Standards have to be reconstituted with the volume of standard buffer diluent indicated on the vial. The reconstitution produces a stock solution of 50pg/ml IL-6. Allow standard to stand for 5 minutes with gentle swirling prior to making dilutions. Serial dilutions of standard must be made before each assay and cannot be stored.

- **CONTROLS:** Freeze-dried control vials were reconstituted with the appropriate standard diluent to the samples. For serum (and plasma) samples use standard diluent: human serum and for cell culture supernatants use standard diluent buffer. Controls have were reconstitited with the volume of standard buffer diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution for which the IL-6 concentration is stated on the vial. Controls were allowed to stand for 5 minutes with gentle swirling prior to distribution in control wells. Do not store after use.

- **Dilution of biotinylated and IL-6**

  Preparation immediately before use is recommended. The biotinylated anti-IL-6 was diluted with the biotinylated antibody diluent in a clean glass vial according to the number of wells to be used. See tables for volume pipetted.

Number of strips	Biotinlated antibody concentrate (ul)	Biotinylated antibody diluent (ul)
2	40	1060
3	60	1590
4	80	2120
6	120	3180
12	240	6360

- Dilution of Streptavidin-HRP: 0.5ml of HRP diluent was added to 5ul vial of Streptavidin-HRP. This was discarded and not kept for further experiments. Dilutions were made immediately before use. Following the number of wells to be used, further dilutions of Streptavidin-HRP were made with HRP diluent in a clean glass vial. Please see hereafter the table for volumes to pippette:

Number of strips	Pre-diluted Streptavidin-HRP (ul)	HRP Diluent (ml)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

- Washing Buffer 200x concentrate: Dilute 200 times in distilled water.

ASSAY PROCEDURE

- Before use, all reagents were mixed thoroughly without making any foam.
- The number of microwell strips required to test the desired number of samples, plus appropriate number of wells needed for running blanks and standards were determined.

  Each sample, standard, blank and optional control sampleswere assayed in duplicate. Remove sufficient microwell strips from the pouch.
- 100ul of appropriate standard diluent (see preparation of reagents) were added to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, and F2. Reconstitute standard vial with the appropriate volume as described in the chapter reagents preparation. 200ul of standard were pipetted into wells A1 and A2. 100ul from A1 and A2 were transfered to B1 and B2 wells. The contents were mixed by repeated aspirations and ejections. Care was taken not to scratch the inner surface of microwells. This procedure from the wells B1, B2 to wells C1, C2 and from the wells C1, C2 to D1, D2 and so on were repected creating two parallel rows of IL-6 standard dilutions ranging from 50 to 1.56pg/ml. 100ul from the content of the last microwells used (F1,F2) were discarded .Note: Alternatively these dilutions can be done in separate tube and diluted standard pipetted directly into wells.
- 100ul of appropriate standard diluent was added to the blank wells (G1-G2)

- 100ul of sample was added to sample wells and 100ul of the reconstituted control vial added to the control wells (H1, H2).
- Preparation of biotinylated and IL-6 (see preparation of reagents).
- 50ul of diluted biotinylated anti-IL-6 was added to all wells.
- These were covered with a plate cover and incubate for 3 hours at room temperature (18^0C-25^0C).
- Then the cover was removed and the plate washed as follows:

  A. The liquid from each well was aspirated.

  B. 0.3ml of washing solution was dispensed into each well.

  C. The content of each well were aspirated again.

  D Steps B and C were repeated two times.
- 100ul of HRP solution were prepared into all wells, including the blank wells. Put back the cover.
- 100ul of HRP solution were spensed into all wells including the blank wells. And the cover put back.
- The microwell stripswere incubated at toom temperature for 30 minutes.
- Plate cover and empty wells were removed. Microwell strips were washed according to Step-9. The next step was proceeded immediately to.
- 100ul of ready-to-use TMB substrate solution was pipetted into all wells, including the blank wells and incubated in the dark for 12-15 minutes at room temperature. Direct exposure to light was avoided by wrapping the plate in aluminium foil.
- Incubation time of the substrate solution is usually determined by the ELISA reader performance: Many ELISA readers record absorbance only up to 2.0 OD. The O.D. Values of the plate were monitored and the substrate reaction stopped before positive wells are no longer properly readable.

- The enzyme-substrate reaction was stopped by quickly pipetting 100ul of H2SO4: stop reagent into each well, including the blank wells, to completely and uniformly inactivate the enzyme. Results were read immediately after the addition of H2SO4: stop reagent.
- Absorbance of each well was read on a spectrophotometer using 450nm as the primary wavelength and optionally 620nm (610nm to 650nm is acceptable) as the reference wavelength.

FIG 3.4a Microplate reader

FIG 3.4b Plate scheme 96 wells

PLATE SCHEME

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50										
B	25	25										
C	12.5	12.5										
D	6.25	6.25										
E	3.12	3.12										
F	1.56	1.56										
G	blank	blank										
H	CTRL	CTRL										

Efforts were made not to extrapolate the standard curve beyond the 50pg/ml standard curve point, since dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples (>50pg/ml) were diluted with standard diluent or sample buffer. During analysis, the results were multiplied by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, and jaundiced) were reported not have not been investigated, also rate of degradation of native IL-6 in various matrices has not investigated (product insert).

Sensitivity

The minimum detectable dose of IL-6 is less than 0.8pg/ml. This has been determined by adding 3 standard deviations to the mean optical density when the zero standard was assayed 32 times (product insert).

3.2.11 SERUM HAPTOBLOBIN TESTING

Column chromatograph or electrophoresis can be employed in analysis of blood samples for haptoglobin. Direct measurement of haptoglobin is also possible by turbidimetry or nephelometry and by radial immunodiffusion (Burtis and Ashwood 2000). Bioassays can be employed also; it provides the earliest methods available to characterize haptoglobins. Enzyme-linked immunosorbent assay is available. It is sensitive, exhibits no cross reactivity and is well standardized. However since this work is not centered on characterization of haptoglobin, ELISA is preferred, and the general techniques described in this work.

3.2.11.1 PRINCIPLE

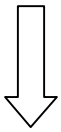
Ab108856 is designed for detection of Human haptoglobin in plasmaand serum. This assay employs a quantitative competitive enzymeimmunoassay technique that measures Human haptoglobin in lessthan 2 hours. A polyclonal antibody specific for Human haptoglobin has been pre-coated onto a 96-well microplate with removable strips. Haptoglobin in standards and samples is competed by a biotinylated haptoglobin sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase

enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

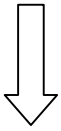
3.2.11.2 Assay Summary

- All reagents, samples and standards were prepared as instructed.

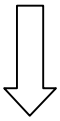
Add 25 εl standard or sample to each well



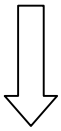
Immediately add 25 εl prepared biotin antibody to each well.



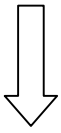
Incubate for 1 hour at room temperature.



Wash the microplate 5 X with wash buffer.



Add 50 εl of Streptavidin-Peroxidase Conjugate.

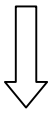


Incubate for 30 minutes at room temperature.

Wash the microplate 5X with wash buffer.

Add 50 εl of Chromogen Substrate to each well.

Incubate 10 minutes or till the optimal blue colour density develops.



Add 50 εl Stop Solution to each well. Read at 450 nm immediately.

Kit Contents

- Human Haptoglobin Microplate: A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against Human Haptoglobin.
- Sealing Tapes: Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- Human Haptoglobin Standard: Human Haptoglobin in a buffered protein base (lyophilized).
- Biotinylated Haptoglobin Globulin: 1 vial
- Diluent Concentrate (10x): A 10-fold concentrated buffered protein base (30 ml).
- Wash Buffer Concentrate (20x): A 20-fold concentrated buffered surfactant (30 ml).
- Streptavidin-Peroxidase Conjugate (SP Conjugate): A 100-fold concentrate (80 εl)
- Chromogen Substrate: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- Stop Solution: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage and Handling

- Components of the kit were stored at 2-8°C or -20°C upon arrival until used.
- Standard and biotinylated protein were stored at 2-8°C before reconstituting with diluent and at -20°C after reconstituting with diluent.

Reagent Preparation:

1. All reagents were freshly diluted and brought to room temperature before use. Crystals, when observerd in the concentrate were mixed gently until the crystals have completely dissolved.

2. Diluent (10X): The Diluent was diluted 1:10 with reagent grade water. This could store for up to 1 month at 2°C to 8°C.

3. Standard Curve: The Haptoglobulin Standard was reconstituted with the appropriate amount of Diluent to generate a solution of 40 εg/ml and allowed stand for 10 minutes with gentle agitation prior to making dilutions. Duplicate or triplicate standard points were prepared by serially diluting the standard solution (20 εg/ml) 1:2 with Diluent, then 1:4 to produce 20, 5, 1.25, 0.313, and 0.078 εg/ml solutions. Diluent serves as the zero standard (0 εg/ml).

Any remaining solution was frozen at -20°C and used within 30 days.

Standard

Point Dilution [Haptoglobin] (εg/ml)

P1 1 Part Standard (20 εg/ml) 20.000 P2 1 part P1 + 3 part Diluent 5.000 P3 1 part P2 + 3 part Diluent 1.250 P4 1 part P3 + 3 part Diluent 0.313 P5 1 part P4 + 3 part Diluent 0.078 P6 Diluent 0.000

4. Biotinylated Haptoglobulin (2X): Biotinylated Haptoglobulin was diluted with 4 ml of Diluent to produce a 2-fold stock solution and allowed to stand for 10 minutes with gentle agitation prior to making dilutions. The stock solution should be further diluted 1:2 with the Diluent. Any remaining solution was frozen at -20^0C and used within 30 days.

5. Wash Buffer Concentrate (20x): The Wash Buffer Concentrate was diluted 1:20 with reagent grade water.

6. SP Conjugate (100X): The SP Conjugate was spun down briefly and diluted with the desired amount of the conjugate to 1:100 with Diluent. The remaining solution was frozen at -20°C.

Assay Method

1. All reagents, working standards and samples were prepared as instructed and brought to room temperature before use. The assay was performed at room temperature (20-30°C).

2. Excess microplate strips were removed from the plate frame and return immediately to the foil pouch with desiccant inside. The pouch was resealed securely to minimize exposure to water vapour and stored in a vacuum dessicator.

3. 25 εl of standard or sample was added per well and immediately 25 εl of Biotinylated Haptoglobin added to each well (on top of the Standard or sample) and mixed gently. The wells were covered with a sealing tape and incubated for one hour. The timer was started after the last sample addition and allowed to stand for I hour.

4. The plate was washed five times with 200 εl of Wash Buffer manually. The plate was inverted each time to decant the contents; then tapped 4-5 times on absorbent paper towel to completely remove the liquid.

5. 50 εl of Streptavidin-Peroxidase Conjugate was added to each well and incubated for 30 minutes. The microplate reader was tuned on to set up the program in advance.

6. Microplate was washed as described above.

7. 50 εl of Chromogen Substrate was added per well and incubated for about 10 minutes or till the optimal blue color density develops. The plate was gently tapped to ensure thorough mixing and the bubbles in the well breaked with pipette tip.

8. 50 εl of Stop Solution was added to each well. The color would change from blue to yellow.

9. The absorbance was read on a microplate reader at a wavelength of 450 nm immediately.

Data Analysis

The mean value of the duplicate or triplicate readings for each standard and sample was calculated .A Standard Curve was generated by plotting the graph using the standard concentrations on the X-axis and the corresponding mean 450 nm absorbance on the Y-axis. The best-fit line was determined by regression analysis using log-log or four-parameter logistic

curve-fit. The unknown sample concentration was determined from the Standard Curve and the value multiplied by the dilution factor.

3.2.12 ABO GROUPING (DACIE AND LEWIS 2006)

Introduction

There are several techniques available for ABO and Rhesus D grouping of red cells.These includes tube, slide, microplate, columns and flow cytometry (Roback *et.al*. 2003). Tube method was employed in this work out of choice and the various methods are brifly described.

Tube and slide tests: spin tube tests are commonly used, particularly for urgent testing where small numbers of tests are performed at once. Slide or tile techniques are widely used in under-resoured countries for ABO and D grouping. Spin test tubes are performed in 75x10 or 72x12mm plastic tubes. Immediate spin test tube may be used in an emergency, whereas routine tests are usually left for 15min at room temperature before centrifugation for 1min at 150g. Equal volumes (1 or 2 drops from either a commercial reagent dropper or a Pasteur pipette) of liquid reagents or serum/plasma and 2% cell suspensions are used. The patients red cells are tested against monoclonal anti-A and anti-B grouping reagents. The patientôs serum is tested against A_1 and B regentsô red cells (reverse grouping). In addition, the serum is tested against either the patients own cells or group O cells (negative control) to exclude rections with A and B cells as a result of patients sample.

Liquid- phase microplate methods. Microplate technology provides a cheap and secure method for batch testing when semiautomation is utilized for dispensing and reading: it is the grouping technique most commonly used in the United Kingdom. ABO and D grouping may be performed in a single microplate if monoclonal reagents are used. Column aggulutnation techniques using either a sephadex gel(DiaMed ID) or a glass microbead matrix (Ortho BioVue) are increasingly being used for grouping, especially where automated systems are in place; these should always be performed in accordance with the manufacturerôs instructions.

The principle of the tube method (employed in this study) is that the patientôs red cells are grouped separately using potent anti-A, anti-B and anti-A+B plus adequate controls. Also the patientôs serum is grouped using appropriate standard (known) cells-A_1,B and O cells. The A_2 cells are used to ensure that the antiserum can pick also weaker forms of A. The tube method is preferred in this work because of these reasons

- It is suitable for handling large number of samples(as in my work)
- The long incubation period keeps the cells and serum together for the long periods that weak reactions are more likely to be picked up than if the tile was used.
- It prevents the rapid drying up of the cells which sometimes result in false positive agglutination.

3.2.12.1. METHOD

The test and control are set up as in the protocol below, using Pasteur pipette.

- One drop of washed 2% appropriate red cell was added to the tubes in each row marked A cells, B cells, and O cells. This served for the serum grouping.
- One drop of the patients 2% washed red cells was added to each tube in the row marked anti-A, anti-B, and anti-A+B. this served for the cells grouping.
- Adequate controls were also set up.
- The metal rack was shaken to mix the serum and cells and left to incubate at room temperature for $1^1/_2$- 2 hrs
- Auto control consisting of patientôs serum and 2% washed red cells were also set up.
- The content of the tubes were inspected microscopically starting with the controls, by transferring the contents onto a microscopic slide and inspected using a low power microscope objective.

3.2.13 HAEMOGLOBIN ELECTROPHERESIS (Dacie and Lewis 2006)

There are some many separation techniques that can be used to separate haemglobin. Some of them include; Haemoglobin electrophoresis at pH8.4-8.6 using cellulose acetate membrane, the use of Citrate Agar at Ph6.0, (Schneider 1974a), Agarose Gel Electrophersis, and automated High-Performance Liquid Chromatogragh which are adapted for laboratories with a high work load (Wild and Stephens, 1997). Other includes, isoelectric focusing (IEF) and Globin chain Electropheresis (Schneider 1974b) used to establish which chain is affected. This information is useful in predicting the nature of the variant and possible interaction. However, Haemoglobin electrophoresis at pH8.4-8.6 using cellulose acetate membrane was preferred for this work. Its choice is based on the advantage of the technique being simple, reliable, and rapid. It is also satisfactory for the detection of most common clinically important haemoglobin variants

(International Committee for Standardization in Heamatology (ICSH)1988; ICSH1978a; ICSH1978b).

3.2.13.1. CELLULOSE ACETATE ELECTROPHERESIS AT AKALINE pH

PRINCIPLE

At alkaline pH, haemoglobin is a negatively charged protein and when subjected to electrophoresis will migrate toward the anode (+). Structural variants that have a change in the charge on the surface of the molecule at the molecule will separate from HbA. Haemoglobin molecules that have amino acid substitution that is internally sited will not separate, and those that have an amino acid substitution that has no overall charge will not separate by electrophoresis.

EQUIPMENTS

- Electrophoresis tank and power pack. Horizontal electrophoresis tank that will allow a bridge gap of 7cm. A direct current power supply capable of delivering 350V at 50mA is suitable for both cellulose acetate and citrate agar electrophoresis.
- Wicks or filter or chromatography paper.
- Blotting paper.
- Applicators. These are available from most manufacturers of electrophoresis equipments, but fine micro capillaries are also satisfactory.
- Cellulose acetate membranes. Plastic-backed membranes (7.6x6.0) are recommended for ease of use and storage.
- Staining equipments.

Reagents

- Electrophoresis buffer. Tris/EDTA/borate (TEB) pH8.5. Tris-(hydroxymethyl) aminomethane (Tris), 10.2g, EDTA (disodium salt) 0.6g, boric acid 3.2g, water to 1liter. The buffer should be stored at 4^0C and can be used up to 10 times without deterioration.
- Wetting agent. For example, Zip-prep solution (Helena Laboratories); 1 drop of Zip-prep in 100 ml water.
- Fixative/stain solution. Ponceau S, 5g, trichoroacetic acid, 7.5g, water to 1liter.
- Haemolyzing reagent, 0.5 % (v/v) triton X-100 in 100mg/l potassium cyanide.

METHOD

1. The sample was centrifuged at 1200g for 5mins. 20µl of the packed red cells was diluted with 150µl of the haemolyzing reagent. This was mixed gently and left for at least 5 min. Purified haemolysates could also be used, in which case, 40µl of 10g/dl haemolysate would be diluted with 150µl of lysing reagent.
2. With the power supply disconnected, the electrophoresis tank was prepared by placing equal amounts of TEB buffer in each of the outer buffer, and placing one along each divider/bridge support to ensure that they make good contact with the buffer.
3. The cellulose acetate was socked by lowering it slowly into a reservoir of buffer. And the cellulose acetate left to sock at least 5 mins before use.
4. The sample well plate was filled with 5µl of each diluted sample or control and covered with a 50-mm coverslip or a ñshortò glass slide to prevent evaporation. A second sample well plate was loaded with Zip-prep solution.
5. The applicator tips were cleaned immediately prior to use by loading with Zip-prep solution and then applying them to a blotter.
6. The cellulose acetate stripwere removed from the buffer and blotted twice between two layers of clean blotting paper. The cellulose acetate was not allowed to dry.
7. Load The applicator was loaded by pressing the tips into the sample wells twice and appling this first loading onto some clean blotting paper. The applicator was then reloaded and the samples applied to the cellulose acetate.
8. Acetate plates were placed across the bridges, with the plastic sides uppermost. Two glass slides were placed across the strip to maintain a good contact. Electrophorese carried out at 350V for 25mins.
9. Immediately transfer the cellulose to Ponceau S to fix and stain for 5min after electrophoresis.
10. Excess stain was removed by washing for 5min in acetic acid for 10min, blotted using clean blotting paper, and left to dry.
11. The membrane was labelled and stored in a protective plastic envelope.

3.2.14 STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS version 10; (SPS Inc., Chicago 111). Descriptive statistics (mean, standard deviations) were determined. Inferential statistics were determined using these test statistics; Analysis of Variance (ANOVA) was used for testing differences in cytokines and haptoglobin levels between clinical groups. The level of statistical difference was set at ($p < 0.05$), were differences were observed among groups, a post-hoc Bonferoni multiple comparative analysis was performed to test (compare) intra group differences, set at a significant level of ($p < 0.05$). Categorical data were expressed as percentages and Chi square analysis was employed for comparism of 2 variables set at level of significance of $p < 0.05$. Associations were tested using Pearsonòs linear regression for bivariate correlation set at a coefficient of $p < 0.05$.

CHAPTER FOUR

RESULT

Five hundred and fifty-three (553) children with febrile illness presenting at the peadiatrics unit of Nnamdi Azikiwe University Teaching Hospital, Nnewi were screened for possible presence of malaria parasiteamia according to the study protocol between the months of March to December 2011. One hundred and fifty-eight (158) patients tested positive by microscopic examination of a Giemsa stained blood slide, giving a prevalence of 35% (158/553). *P. falciparum* was the only specie observed in all the patients studied (158/158) giving 100% prevalence for *P. falciparum* specie in Nnewi and itôs environ and 5 patients with mixed infection of *P. falciparum* and *P. malariae* species (5/158) placing a prevalence of 3% for *P. malariae* species in Nnewi. No other species were seen. Using the criteria for severe malaria which are; anemia with Hb level, 5g/L, acute respiratory distress, renal failure, prostration, shock, abnormal bleeding and/or disseminated intravascular coagulation (DIC), parasiteamia >20,000/ul of blood, repeated generalized seizures (WHO, 2000), 15 were identified to have severe malaria; 10 had hyperparasitemia, 10 had severe malaria anaemia (however, some of the severe malaria cases had more than one defining criterium) and 5 met other defining criteria (i.e. respiratory distress, shock, cerebral malaria etcetera). The mean duration of symptom until presentation was 3 ± 2 days in severe malaria category and 2.2 ± 3days in the uncomplicated group.

Table 1 shows the demographic characteristic and hematological parameters of the subjects studied according to their categories. The study group consisted of n = 143 children with uncomplicated malaria, n = 15 with complicated malaria and n = 52 of healthy control group. The mean age of the children with uncomplicated malaria was 33.9 ± 34.37 months, that of the severe group was 40.9 ± 25.77 months and the control group was 29.5 ± 39.28 months. Analysis of variance (ANOVA) indicated lack of significant differences ($p > 0.05$) in age. Thus the study groups were aged matched. There are also no significant differences ($p > 0.05$) in values for these parameters: RBC, MCH, MCHC and PDW amongst the three groups. The mean value for WBC increased with malaria severity: control group was 4.8 ± 1.29, uncomplicated malaria group was 9.2 ± 5.02 and severe malaria group was 13.2 ± 12.22, indicating a significant difference ($p<0.05$) amongst the three categories. The mean values for haemoglobin concentration (Hb Conc) and hematocrit (HCT) decreased with malaria severity: control group

for Hb Conc and HCT were 11.8 ± 1.23 versus 21.0 ± 10.0 respectivly, uncomplicated malaria group were10.4 ± 1.92 versus 32.6 ± 5.36, and severe malaria group 5.9±3.63 versus 18.2 ± 9.5.8 respectivly, indicating a significant difference ($p < 0.05$) amongst the three categories. The mean platelet count did not differ between the control and uncomplicated (267.9 ± 84.55 versus 278.4 ± 142.9 ($p > 0.05$)), but a difference was observed between the control and complicated (267.9 ± 84.55 versus 125.7 ± 54.01($p < 0.05$). The same trend was also seen with MCV were the mean values did not differ between the control and uncomplicated (74.1 ± 4.80 versus 72.5 ± 10.03, $p > 0.05$), but a difference was observed between the control and complicated (74.1 ± 4.80 versus 58.2 ± 14.53($p < 0.05$)). The control group also indicated significantly ($p < 0.05$) lower values in: PCT, RDW, MPV, and % monocyte count but higher % lymphocyte count ($p < 0.05$) compared to the uncomplicated malaria group. In contrast, the values of these variables (PCT, RDW, MPV, and % monocyte and also % lymphocyte) did not differ between the control and the complicated malaria group.mThe post hoc analysis did not indicate any significant differences when % neutrophil values were compared between groups.

Table4.1. Demographic and haematological parameters of children with complicated and uncomplicated malaria compared with healthy control group.

VARIABLES	CONTROL GROUP (n = 52)	UNCOMPLICATED MALARIA GROUP (n = 143)	COMPLICATED MALARIA GROUP (n = 15)	F-STAT	P VALUE
Age (months)	29.5 ± 39.28	33.9 ± 34.37	40.9 ± 25.77	0.67	0.51
WBC ($x10^9$/L)	4.8 ± 1.29	9.2 ± 5.02	13.2 ± 12.22	20.03	0.000*
RBC ($x10^{12}$/L)	4.8 ± 0.52	4.8 ± 4.20	3.0 ± 1.12	1.78	0.17
Haemoglobin(g/dl)	11.8 ± 1.23	10.4 ± 1.92	5.9 ± 3.63	51.71	0.000*
Haematocrit (l/l)	35.4 ± 3.65	32.6 ± 5.36	18.2 ± 9.58	59.52	0.000*
Platelet ($x10^9$/L)	267.9 ± 84.55	278.4 ± 142.9	125.7±54.01	9.92	0.000*
PCT (%)	21.0 ± 10.07	14.3 ± 14.54	3.6 ± 5.17	27.13	0.000*
MCV (fl)	74.1 ± 4.80	72.5 ± 10.03	58.2 ± 14.53	17.43	0.000*
MCH (pg)	24.6 ± 1.71	24.6 ± 16.34	19.8 ± 5.99	0.85	0.43
MCHC (g/l)	33.1 ± 1.53	32.2 ± 5.10	33.6 ± 2.75	1.38	0.25
RDW	16.6 ± 5.99	31.7 ± 23.67	19.8 ± 5.99	11.87	0.000*
MPV	8.5 ± 0.79	9.3 ± 1.23	8.3 ± 0.94	10.48	0.000*
PDW	11.3 ± 1.79	12.3 ± 3.75	11.2 ± 2.40	1.89	0.15
% Lymphocytes	54.9 ± 24.61	42.9 ± 16.51	48.1 ± 15.29	7.91	0.000*
% Monocytes	9.0 ± 3.10	11.2 ± 4.69	7.1 ± 5.06	8.57	0.000*
% Neutrophils	38.9 ± 10.72	45.7 ± 19.80	36.8 ± 22.69	3.59	0.029*

Data is expressed as mean ± standard deviation. * Significant difference ($p < 0.05$). Abbreviations: WBC = White blood cell; RBC = Red blood cell; PCT = Plateletcrit; MCV = Mean cell volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; RDW = Red cell distribution width; MPV = Mean platelet volume; PDW = Platelet distribution width.

Table 4.2 shows the mean serum levels of the pro-inflammatory cytokines in control, uncomplicated malaria and complicated malaria groups of subjects. The mean values (expressed in picogram/ml) showed increased values in uncomplicated group compared with control in all the cytokines assayed: IL-1 (177.9 ± 316.31 versus 45.6 ± 37.04), IL- 6(492.3 ± 596.84 versus 48.0 ± 35.27), TNFŬ(132.2 ± 229.42 versus 48.9 ± 58.98) and progressively increased value in complicated group with mean IL-1(315.8 ± 233.71 versus 45.6 ± 37.04), IL-6(1275.3 ± 605.37 versus 48.0 ± 35.27), TNFŬ(369.0 ± 453.45 versus 48.9 ± 58.98). Analysis of variance (ANOVA) indicated significant ($p < 0.05$) differences in all the pro-inflammatory cytokines amongst the control, complicated malaria and uncomplicated malaria groups. Further analysis using Bonferoni multiple comparison test indicated that the control group had significantly ($p < 0.05$) lower mean IL-1 and IL-6 compared to uncomplicated malaria group ($p < 0.05$) and the complicated malaria group. Mean TNF-Ŭ was lower in control group compared to the complicated malaria group ($p < 0.05$) but did not indicate significant difference ($p >0.05$) with the uncomplicated group value. Furthermore, IL-1 did not differ between uncomplicated and complicated group ($p> 0.05$).

Table4.2a. Mean serum levels of the pro-inflammatory cytokines in control, uncomplicated malaria and complicated malaria groups of subjects.

VARIABLES (pg/ml)	CONTROL GROUP (n = 52)	UNCOMPLICATED MALARIA GROUP (n = 143)	COMPLICATED MALARIA GROUP (n =15)	F-STAT	P VALUE
IL-1	45.6 ± 37.04	177.9 ± 316.31	315.8 ± 233.71	7.46	0.001*
IL-6	48.0 ± 35.27	492.3 ± 596.84	1275.3 ± 605.37	34.97	0.000*
TNF-Ŭ	48.9 ± 58.98	132.2 ± 229.42	369.0 ± 453.45	11.78	0.000*

Data is expressed as mean ± standard deviation. * Significant difference ($p<0.05$).
Abbreviations: IL-1 = Interleukin - 1; IL-6 = Interleukin - 6; TNF-Ŭ= Tumor Necrotic Factor - Alpha.

Table4.2b. Bonferonni Post-Hoc multiple comparison test

VARIABLES	Control verses Uncomplicated P - value	Control verses complicated P-value	Uncomplicated verses Complicated P-value
IL1	0.008	0.002	0.183
IL6	0.000	0.000	0.000
TNF-α	0.071*	0.000	0.000

Abbreviations: IL-1 = Interleukin - 1; IL-6 = Interleukin - 6; TNF-Ŭ= Tumor Necrotic Factor - Alpha.* Non significant difference (P> 0.05)

Figure 4.1 shows the Mean haptoglobin levels (expressed in gram/litre) in the control, uncomplicated malaria and complicated malaria groups. The mean value for the uncomplicated malaria categoty was 108.7 ± 65.5 versus 68.9 ± 28.7 and 32.6 ± 35.7 versus 68.9 ± 28.7 in the severe malaria category. ANOVA indicated significant ($F = 18.56$; $p < 0.05$) difference in haptoglobin amongst the groups. However, post hoc Bonferoni analysis further showed that mean haptoglobin level is significantly higher in the uncomplicated malaria group compared to the control group ($p < 0.05$), also a significant difference was observed between the complicated and the uncomplicated group ($p < 0.05$). In contrast, no significant difference was observed between the control group and the complicated malaria group ($p > 0.05$).

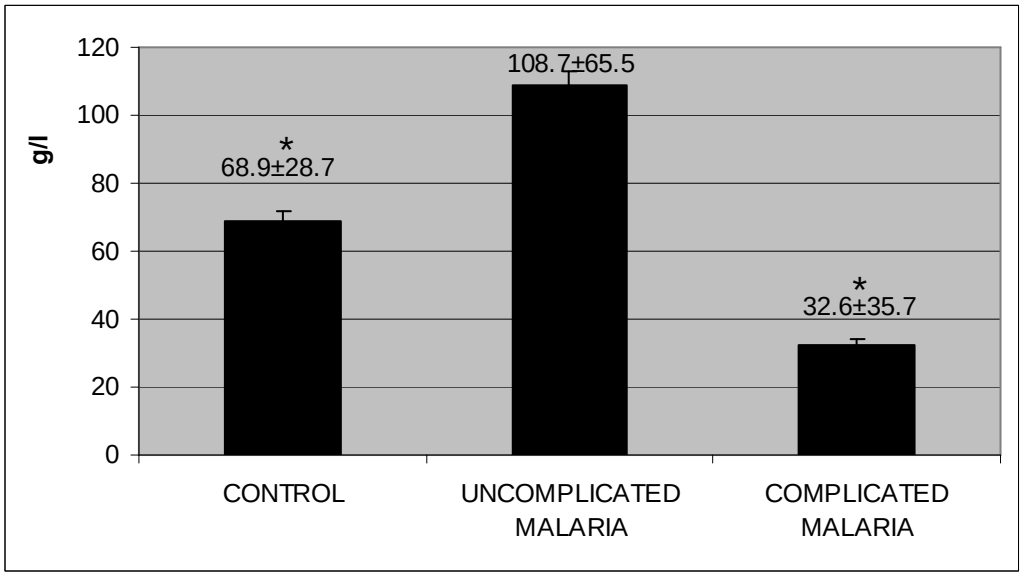


Figure4.1. Mean haptoglobin levels in the control, uncomplicated malaria and complicated malaria groups. Data is mean ± standard deviation. * Significantly lower than the uncomplicated malaria value.

Figure 4.2 shows the frequency distribution of the hemoglobin phenotype of the study subjects: uncomplicated and complicated malaria groups.

82.3% of the uncomplicated malaria group had AA Hb phenotype while 17.7% had AS Hb phenotype. Amongst subjects with complicated malaria, 86.7% had AA Hb phenotype, while 13.3% had AS. Chi square analysis indicated that the frequency of AA was significantly greater compared to AS in both uncomplicated malaria ($X^2 = 58.7$; $p < 0.000$) and complicated malaria ($X^2 = 8.06$; $p = 0.005$) groups. In addition, more of the AA patients had higher proportion of severe forms of malaria (13/129 = 10%) than the AS subjects (2/27 =7.4%), p<0. 05.

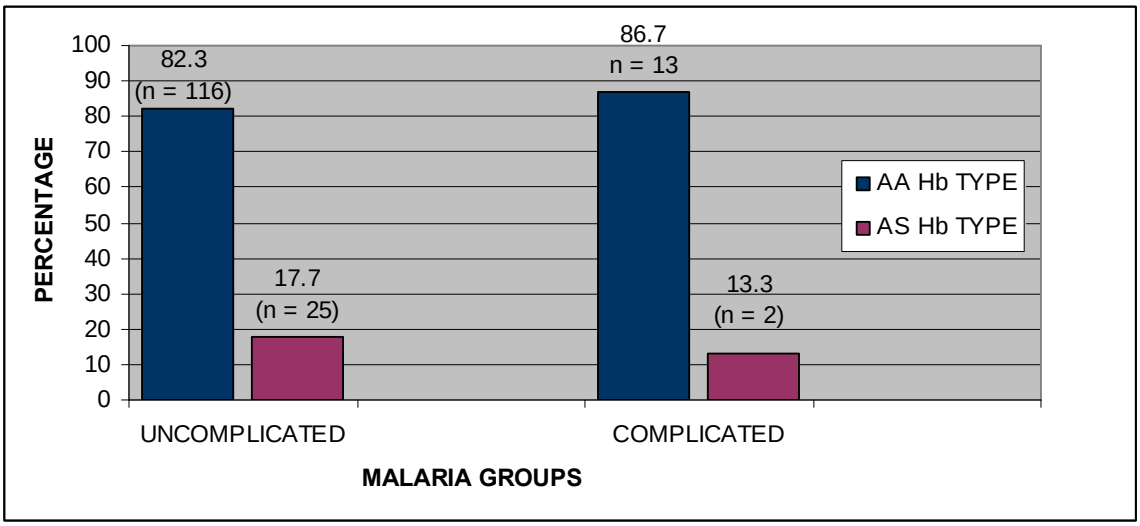


Figure4.2. Frequency distribution of the hemoglobin phenotype (AA and AS) of the study subjects: uncomplicated and complicated malaria groups.

Figure4.3 shows the frequency distribution of the blood groups of the study subjects (uncomplicated and complicated malaria groups).

66.9% of the uncomplicated malaria group had O blood group while 33.1% had Non-O blood groups. Amongst subjects with complicated malaria, 60% had O blood group, while 40% had Non-O blood groups. Chi square analysis indicated that the frequency of O blood group was significantly greater compared to Non-O groups in uncomplicated malaria ($X^2 = 15.9$; P=0.000) but not in complicated malaria group ($X^2 = 0.60$; P = 0.44).

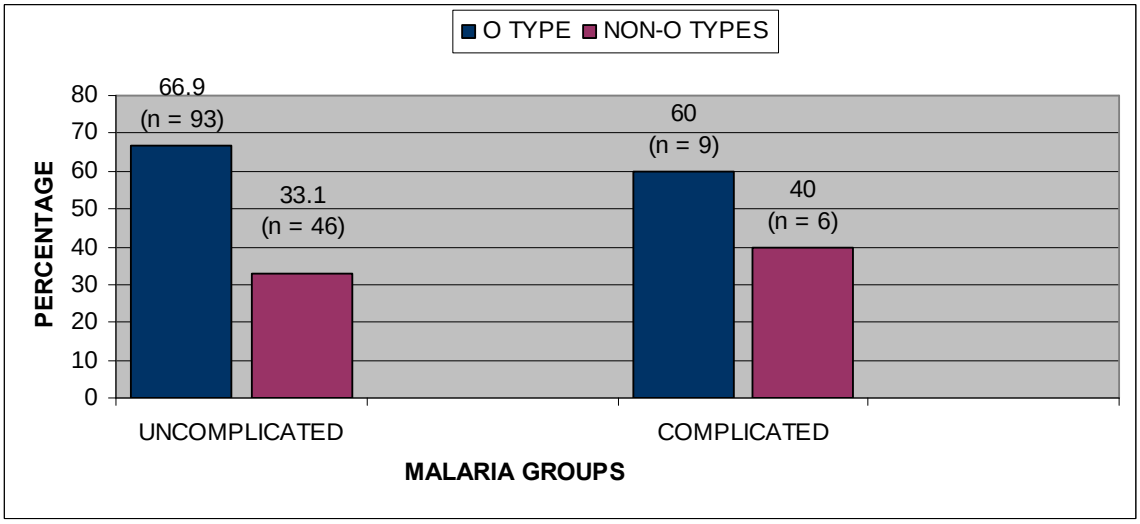


Figure 4.3 Frequency distribution of the blood groups of the study subjects (uncomplicated and complicated malaria groups).

Table4. 3 Association between blood group and malaria status of test subjects

BLOOD GROUPS	**MALARIA STATUS**		
	UNCOMPLICATED	**COMPLICATED**	**Total**
O blood Group	93 (91.2)	9 (8.8)	102 (100.0)
Non-O blood Group	46 (88.5)	6 (11.5)	52 (100.0)
Total	139 (90.3)	15 (9.7)	154 (100.0)

. $\chi 2 = 0.289$; $P = 0.591$

Chi-square analysis indicated no significant ($P = 0.591$) association between blood group and malaria status of subjects.

Table 4.4 shows the mean values of other markers of the study subjects (uncomplicated and complicated malaria groups). The mean parasite density (expressed in cells/µl) for the uncomplicated malaria group was 4096.4 ± 169.54 while the mean parasite density of the complicated group was 55008.2 ± 941.12.

Independent sample z-test indicated that subjects with uncomplicated malaria had significantly higher haemoglobin concentration ($p < 0.05$), platelet count ($p < 0.05$) but significantly lower WBC ($p < 0.05$) and parasite density ($p < 0.05$) compared to the complicated malaria group.

Table4.4 The mean values of other markers of the study subjects (uncomplicated and complicated malaria groups).

VARIABLES	UNCOMPLICATED MALARIA	COMPLICATED MALARIA	z-STAT	P VALUE
Haemoglobin Conc. (g/dl)	10.4 ± 1.92	5.94 ± 3.63	7.66	0.000*
White Blood Cell ($x10^9$/L)	9.2 ± 5.02	13.2 ± 12.22	-2.46	0.015*
Platelet Count ($x10^9$/L)	278.4 ± 142.99	125.6 ± 54.01	4.09	0.000*
Parasite Density (Cells/µL)	4096.4 ± 169.54	55008.2 ± 941.12	-14.42	0.000*

Data is mean ± standard deviation. * Significant difference. Z- score test other wise called standard normal deviate.

Table4.5 shows bivariate correlation between haptoglobin level and the pro-inflammatory cytokines in study subjects.

Pearsonôs linear regression indicated significant negative correlations between haptoglobin level and IL-6 (r=-0.286; p < 0.001) and TNF-Ŭ(r=-0.237; p < 0.01) respectively in the combined data of ółlô subjects. In contrast, no significant association was observed between haptoglobin and IL-1(r=-0.122; p > 0.05). The same trend was observed in the uncomplicated malaria group, in which significant negative associations were observed between Haptoglobin and IL-6 (r=0.032; p < 0.05) and TNF-Ŭ(r=0.028; p < 0.05) but not with IL-1. In the complicated malaria group, no significant correlations were observed between haptoglobin and all the cytokines (p > 0.05)

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Table 4.5 Bivariate correlation between haptoglobin level and the pro-inflammatory cytokines in study subjects.

VARIABLES	ALL TEST SUBJECTS		UNCOMPLICATED MALARIA GROUP		COMPLICATED MALARIA GROUP	
	Coefficient	P	Coefficient	P	Coefficient	P
Haptoglobin verses IL-1	-0.122	0.128	-0.069	0.411	-0.451	0.092
Haptoglobin verses IL-6	-0.286	0.000*	-0.180	0.032*	-0.371	0.174
Haptoglobin verses TNF-Ŭ	-0.237	0.003*	-0.184	0.028*	-0.122	0.665

Abbreviations: IL-1 = Interleukin - 1; IL-6 = Interleukin - 6; TNF-Ŭ= Tumor Necrotic Factor - Alpha.* Significant difference ($p < 0.05$).

Table4.6 shows bivariate correlation between parasite density level and the pro-inflammatory cytokines in study subjects.

Pearsonôs linear regression test indicated significant positive correlations between parasite density level and IL-1(r=0.161; $p < 0.05$), IL-6 (r=0.433; $p < 0.05$) and TNF-Ŭ (r=0.170; $p < 0.05$) respectively in the combined ółlô subjects data. The same trend was observed in the uncomplicated malaria group, in which significant positive associations were observed between parasite density and IL-1 (r=0.169; $p < 0.05$), IL-6 (r=0.308; $p < 0.05$) and TNF-Ŭ(r=0.268; $p < 0.05$). In the complicated malaria group, no significant correlations were observed between parasite density and all the cytokines ($p > 0.05$).

Table 6. Bivariate correlation between parasite density level and the pro-inflammatory cytokines in study subjects.

VARIABLES	ALL TEST SUBJECTS		UNCOMPLICATED MALARIA GROUP		COMPLICATED MALARIA GROUP	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Parasite Density vs IL-1	0.161	0.043**	0.169	0.043**	0.077	0.784*
Parasite Density vs IL-6	0.433	0.000**	0.308	0.000**	0.441	0.100*
Parasite Density vs. TNF-Ŭ	0.170	0.033**	0.268	0.001**	-0.345	0.207*

Abbreviations: IL-1 = Interleukin - 1; IL-6 = Interleukin - 6; TNF-Ŭ= Tumor Necrotic Factor - Alpha.* Non significant difference ($p > 0.05$) ** significant difference ($p < 0.05$).

Table4.7 shows bivariate correlation between haemoglobin concentration and the pro-inflammatory cytokines in study subjects. Significant negative correlations were observed between haemoglobin level and IL-6 ($p < 0.05$) and TNF-Ŭ ($p < 0.05$) respectively in the combined ǿallô subjects data. In contrast, no significant association was observed between haemoglobin and IL-1. The same trend was observed in the uncomplicated malaria group, in which significant negative associations were observed between haemoglobin and IL-6 ($p < 0.05$) and TNF-Ŭ ($p < 0.05$) but not with IL-1. In the complicated malaria group, significant correlations were observed between haemoglobin, IL-1 ($p < 0.05$), and IL-6 ($p < 0.05$) but not with TNF-Ŭ.

Table 4.7 Bivariate correlation between haemoglobin conc and the pro-inflammatory cytokines in study subjects.

VARIABLES	ALL TEST SUBJECTS		UNCOMPLICATED MALARIA GROUP		COMPLICATED MALARIA GROUP	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Hb conc verses IL-1	-0.080	0.318	0.066	0.432	-0.604	0.017
Hb conc verses IL-6	-0.425	0.000*	-0.250	0.003*	-0.599	0.018
Hb conc verses TNF-Ŭ	-0.343	0.000*	-0.233	0.005*	-0.300	0.278

Abbreviations: IL-1 = Interleukin - 1; IL-6 = Interleukin - 6; TNF-Ŭ= Tumor Necrotic Factor - Alpha.* significant difference ($p < 0.05$).

Table4.8 shows the bivariate correlation between haemoglobin phenotypes and the pro-inflammatory cytokines in study subjects. Data indicated no significant association between haemoglobin phenotypes and all the cytokines in all the study groups. These results indicate lack of significant variations or differences in IL-1, IL-6 and TNF-Ŭ between the AA haemoglobin type and AS type.

Table4.8 Bivariate correlation between haemoglobin phenotypes and the pro-inflammatory cytokines in study subjects.

VARIABLES	ALL TEST SUBJECTS		UNCOMPLICATED MALARIA GROUP		COMPLICATED MALARIA GROUP	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Haemoglobin phenotypes verses IL-1	-0.035	0.659	-0.012	0.885	-0.319	0.247
Haemoglobin phenotypes verses IL-6	0.026	0.741	0.085	0.310	-0.442	0.099
Haemoglobin phenotypes verses TNF-Ŭ	0.045	0.573	-0.020	0.817	0.487	0.065

Table 4.9 shows a bivariate correlation between blood groups and the pro-inflammatory cytokines in study subjects. Significant positive correlation was observed between blood group types and IL-6 ($p < 0.05$) but no significant association existed between blood group types and IL-1 and TNF-Ŭ respectively in the óallô test subjects data. No significant association was observed between blood group types and all the cytokine in the uncomplicated malaria group. In the complicated malaria group, significant positive correlations were observed between blood groups and IL-1 ($p < 0.01$), and IL-6 ($p < 0.05$) but not with TNF-Ŭ.

Table 4.9 Bivariate correlation between blood groups and the pro-inflammatory cytokines in study subjects.

VARIABLES	ALL TEST SUBJECTS		UNCOMPLICATED MALARIA GROUP		COMPLICATED MALARIA GROUP	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Blood groups verses IL-1	0.154	0.057	0.108	0.205	0.683	0.005
Blood groups verses IL-6	0.162	0.044	0.117	0.172	0.533	0.041
Blood groups verses TNF-Ŭ	0.087	0.281	0.136	0.110	-0.176	0.530

Significant associations were observed between parasite density and haptoglobin (r=0.236; P<0.01) and hemoglobin concentration (r=-0.378; p < 0.001) respectively in all subjects. In uncomplicated malaria group, significant association was observed between parasite density and hemoglobin (r=0.028; P<0.05) but not with haptoglobin (r=-0.014; p = 0.868). In complicated malaria group, no significant relationship existed between parasite density and haptoglobin (r=0.230; p = 0.410) or hemoglobin (r=0.249; p = 0.370)

Table 4. 10 Bivariate correlation between parasite density and the haptoglobin and hemoglobin concentration of study subjects.

VARIABLES	ALL TEST SUBJECTS		UNCOMPLICATED MALARIA GROUP		COMPLICATED MALARIA GROUP	
	Coefficient	**P**	**Coefficient**	**P**	**Coefficient**	**P**
Haptoglobin vs Parasite density	-0.236	0.003	-0.014	0.868	0.230	0.410
Hemoglobin vs. Parasite density	-0.378	0.000	-0.184	0.028	0.249	0.370

CHAPTER FIVE

DISCUSSION

P. falciparum malaria is characterized by marked changes in cytokine production from immune responses to infection. Genetics plays crucial role in immune response and affects cytokine expression. The very definition of malaria is complicated with manifestations varying from mild clinical illness to sever illness (Lyke et al 2004). Thus this study involving aged matched Nigerian children with *P. falciparum* malaria (severe and uncomplicated malaria) in Nnewi, Anambra State Nigeria, sought to examine cytokine production, influence of genetic on cytokine expression and to determine whether differences in serum cytokine levels correlated with varied disease manifestation. The study document elevated serum levels of pro-inflammatory cytokines (IL-1, 1L-6 and TNFŬ in malaria infection. It also found distinct differences in cytokine production correlating with parasite density and invariably disease severity. The results pattern suggests that cytokine production is a dynamic process and insights could be gained from the differences observed in the levels of the three cytokines (IL-1, 1L-6 and TNFŬ) tested, between those with severe malaria and those with uncomplicated malaria compared with the healthy controls. It found significantly elevated mean serum levels of pro-inflammatory IL-6, IL-1 and TNFŬ in the study group compared with aged matched control group. However, the mean level expressed in the severe malaria is significantly higher than the mean level expressed in the uncomplicated group. The Bonferoni post-Hoc analysis indicated a significant elevation in the mean IL-1 and IL-6, both in the severe and uncomplicated group when compared with the control group. Howbeit, in case of TNFŬ, a significant mean elevation was only observed in the severe group when compared with the control but non between the uncomplicated and control group. Several workers had demonstrated that TNF-Ŭ production is associated with parasite clearance and resolution of fever (Kremsner *et. al*., 1995) as observed in uncomplicated cases, but elevated levels of TNF-Ŭ (Kwiatkowski *et. al.,* 1990) have been associated with cerebral malaria. Thus excess production of TNF-Ŭ may be responsible for the clinical and pathology seen in severe malaria. Also Doodo (2002) had poised that high ratios of TNF-Ŭ and other inflammatory cytokines are associated with decreased risk of malaria infection but increased risk of clinical disease in those who do become infected.

Previous studies had shown IL-6 as an important pro-inflammatory cytokine that is upregulated by TNFŬ and acts in concert with other inflammatory mediators to control parasitemia (Uroquhart, 1994). In a similar vein, Kremsner and collegues (1995) have earlier demonstrated that components of blood-stage parasites, including parasite-derived glucophoshate inositol (GPI), induce macrophages to produce IL-1, IL-6, interferon(IFN)-ɔ and TNF-Ŭ, but there is also evidence that these levels need to be carefully balanced to avoid immune pathology. Chotivanich and co-workers (2010) had also in their study shown that parasite factors such as the parasite density and their ability to infect high percentage of the erythrocyte correlates positively to the rate of synthesis of inflammatory cytokines and can contribute to the severity of the disease (Chotivanich *et. al.*, 2010), and consequently result in fatal outcome (Lyke *et. al*., 2004; Riley *et al,* 2006) when overproduced (Finney *et.al.*, 2009). Else were it was demonstrated that increasing cytokine concentrations were coincident with rise in asexual parasitemia, suggesting also that there is a casual relationship between onset of blood stage infection, initiation of the immune response and subsequent parasite growth (Walther *et al* 2006). However, Walther and colleagues also noted that expression of these pro-inflammatory cytokines that brings about parasite control on the parasite growth comes at the cost of developing clinical symptoms, suggesting that the initial innate response may have far reaching consequences on disease out come. This brings out the question of balance proposed by a number of scholars (Kresmer *et al*., 1995; Perkins *et al*., 2000; Tiago *et. al*., 2011). It is important to note that IL-6 as both pro-inflammatory and anti-inflammatory cytokines and although this study did not determine the levels of type 2 cytokines(anti-inflammatory), it is evident that the pathological alterations and outcome of the infection depend on the reciprocal regulation of type 1 and type 2. Also, marked imbalance between the two cytokines could determine the severity of the infection also (Perkins *et al*., 2000; Tiago *et. al*., 2011). Therefore in severe, acute infections such as malaria, the ability to mount an effective innate response may mean difference between life and death (Walther *et al*., 2006).

It is also noted in this study that the mean levels of three cytokines tested (IL-1, 1L-6 and TNFŬ) hava a correlation with the parasity density. This finding is in agreement with that of Chotivanich and co-workers (2010) who in their study showed that parasite factors such as the parasite density and their ability to infect high percentage of the erythrocyte correlates positively to the rate of synthesis of inflammatory cytokines. However, this study discovered that is correlation

holds only in uncomplicated cases of malaria. The relationship is lost in severe cases of malaria. It is noted that some reports had suggested that parasity density do not correlate with clinical symptoms and therefore do not determine severity (John *et. al.,* 2005; Moormann *et. al.,* 2009) in avariance with the report of Chotivanich and co-workers (2010). This present study observed that those previous studies did not categorize their malaria cases based on clinical groupings as proposed by WHO (2000) on criteria for defining severity (WHO, 2000). The study therefore report that parasite density correlates positively to the rate of synthesis of inflammatory cytokines only in uncomplicated (simple) malaria. And that this correlation is lost in severe cases of malaria. One would therefore tend to alingn one self to the thought that indeed the pathology seen in malaria is not directly as a result of the activities of the invading organism, but response of the individual to it (Finney *et.al.*2009), suggesting therefore that more than one pathological process may been involved (Jurgen et al 2007), and probably the exaggerated cytokine response in addition (Kyle, 2004).

Another key finding in this study is that Hb/PCV decreases with severity. Anaemia has been reported eleswere as the most frequent malaria-associated haematologic complications, important predictors of malaria infection and a reliable diagnostic marker (Jadhav *et al* 2004). However, it is noted that this association is lost in TNFŬexpression in the severe malaria category probably suggesting that the molecular mechanism involved in cytokine-mediated anaemia is not too clear, or probably could be due to small sample size of the severe malaria group in this study. Small sample size not withstanding , evidence suggest that in increasing levels of IL-6, the liver produces increasing amount of hepcidin which in turn causes increased internalization of ferroportin molecules on the membrane which prevents release from iron stores (Nemeth *et. al*., 2004). It is acknowledged that IL-6 is an important pro-inflammatory cytokine that is upregulated by TNFŬand that IL-6 and other inflammatory cytokines also appear to affect other important elements of iron metabolism including decreasing ferroportin expression and probably directing blunting erythropoiesis by decreasing the ability of the bone marrow to respond to erythropoietin (Nemeth *et al*., 2004). Some recent publications have demonstrated the direct effect of pro-inflammatory cytokines on cell differentiation aganist erythroid partway without erythropoietin defect. This suggests that pro-inflammatory cytokine-mediated signaling partway affect erythropoietin activity. They could also interfere with erythroid-mediated signaling partway, inducing apoptosis and inhibiting the expression and regulation of specific transcritory

factors involved in the control of erythropoiesis differenciation (Morceau *et. al*., 2009). Another probable mechanism through which anaemia develops can be deduced from observance in haptoglobin metabolism in this present study. Haptoglobin is an acute phased protein that functions to bind the globin portion of free haemoglobin in the blood (Gupta *et. al.,* 2011). In the presence of free Hb (for example, intravascular heamolysis as in malaria) the Hb- Hp complex which forms is rapidly cleared from the serum by CD163 which are expressed on monocyte-macrophage system with resultant serum haptoglobin levels that are low or absent (Crichton 2009). This could explain the low value seen in the complicated category in this work. The significantly elevated values observed in uncomplicated malaria naturally indicates that mild intravascular haemolysis was present and with low parasite load and at higher level of parasiteamia the levels of haptoglobin drops as a result of mopping up of the free haemglobin (Abdalla, 1990; Mazza, 2002). Also, that no significant difference was seen in the mean haptoglobin level between control and complicated malaria ($P<0.09$) indicated overwelming intravascular haemolysis that the haptoglobin level almost crashed to vey low level (Crichton 2009). In addition, an association was observed between *P. falciparum* density, degree of anaemia and haptoglobin concentration in this study. This finding agrees with Onyenekwe, and co-worker (2005) who also reported an association between *P. falciparum* density, degree of anaemia and haptoglobin concentration, thus indicating that continued increase in *P. falciparum* density might affect haptoglobin metabolism and may consequently result in anaemia (Onyenekwe *et al*., 2005). Therefore these two factors viz: altered haptoglobin metabolism consequent to continued increase in *P. falciparum* density and disordered erythropoietin response may be major factors in the etiology of anemia in malaria infection. Though this study could not assess reticulocyte index, it has elsewhere been documented that there is a marked reduction of reticulocyte response in relation to the degree of anaemia, even under conditions in which there are adequate levels of erythropoietin (Kurtzhals et al 1997; Nussenblatt et al., 2001). The underlying biological pathways responsible for suppression of erythropoiesis in malaria-infected individuals appears to be as a result of decreased responsiveness to erythropoietin in the erythroid precursor caused by TNFŬ(Chang 2004; McDevitt, 2004) as has been observed earlier.

This study also noted that leucocyte count increases with malaria severity. This finding is in agreement with previous independent reports of various scholars as reported earlier (Ghose and Ghose 2007; Khan *et. al*., 2012; Marlies *et al*., 2013). A consideration of the differential leucocytes count reveals that there is absolute monocytosis in the uncomplicated group but not in the severe group. Halim and co-workers in 2002 observed in their work that monocytosis may enhance the predisposition to a favourable clinical outcome (Halim *et. al*., 2002). The mechanism through which monocytosis confers a favourable outcome may not be unconnected with its phagocytic and secretory role of pro-inflammtory cytokine molecules such as IL-1, TNFŮ etcetera (Were *et. al.*, 2009). Lymphocytopenia was also noted in this study. Existing literature gave conflicting report on this cell line (Hivid *et. al*., 2000; Kassa *et. al*., 2005; Khan *et. al*., 2012; Marline *et. al.,* 2013; van Wolfswinkel *et. al.,* 2013), both lymphocytopenia (Lisse *et. al.,* 1994; Hviid *et. al.,* 1997) and lymphocytosis (Ladhani *et al* 2002) were said to be associated with adverse outcome. This work shows that lymphocytopenia, but not lymphocytosis is associated with adverse outcome. The mechanism behind malaria-associated lymphopenia has still not been satisfactorily elucidated and remains the subject of debate (Hviid *et. al*.,2000). Some authors suggest transient sequestration during malaria to be the responsible molecular mechanism (Hviid *et. al*., 1997; Wyler 1976). However, the relatively large drop in peripheral lymphocyte numbers would suggest this to be a non-specific effect, *e.g.* pooling in the enlarged spleens of patients (Wyler 1976) rather than a response by malaria-specific lymphocytes only. Others have pointed to the increased propensity of lymphocytes from malaria patients to undergo spontaneous apoptosis *in vitro* (Balde *et. al*., 1995; Toure-Balde *et. al.,* 1996). Interestingly, increased apoptosis is also seen in healthy donors from endemic areas, although to a lesser extent (Balde *et. al*., 1995; Toure-Balde, 1996; Kemp *et. al.*, 2001), suggesting that chronic stimulation of lymphocytes by environmental micro-organisms may be contributing through activation-induced cell death.

Also a statistical difference was seen between the complicated and severe malaria in these parameters also: PCT, RDW, and MPV. In addition to percentage monocytes and percentage lymphocytes (already discussed) could be considered as prognostic variables. Therefore, were there are no facilities for accurate parasite detectation, these simple haematological value could aid in malaria diagnosis and in monitoring prognosis.

Thrombocytopenia is another important haematological blood cell changes observered in this study. This finding is in agreement with reports of various independent works. (Wickramasinghe and Abdalla, 2000; Jadhav *et. al.,* 2004; Jeremiah and Uko, 2007; Khan, 2012). However, the cause of the thrombocytopenia is at its best poorly understood from the report of those studies. This study, though also lacked the facility to study the molecular mechanism behind thrombocytopenia in malaria, however, brings to fore the need to consider the central role of von Willibrand Factor (VWF). Evidence for an important role of VWF in malaria is emerging, and is the future plan of the researchers involved in this work to investigate. Hollestell and colleagues (2006) showed that cerebral malaria is associated with increased levels of VWF propeptide and thus is released from endothelial storage sites in response to endothelial damage or activation by cytokines found in children with malaria (Gordenk *et. al,*. 2006; Shi *et. al*., 2004). It has been proposed that platelet is the binding site between parasitized red cells and endothelium. CD-36 is not strongly expressed on cerebral endothelial cells (Silamut *et. al.,* 1999) and therefore serves as a bridge between parasitized red cells and endothelium. This was deduced from one of the experiments carried out by Wassmer and colleagues (2004) who co-incubated infected red cells with CD-36 deficient brain micro vascular endothelial cells grown to confluence in vitro. They demonstrated that adherence of infected red blood cells to endothelial depended on the presence of platelet.

It is also observed in addition that significant number of the study subjects had haemoglobin phenotype AA and the severe forms of malaria than AS phenotye. This present study is consitistent with prevaling reports that haemoglobin AS phenotype confers resitance to malaria (Allison, 1950; Campino, 2006; Jurgen *et. al.*, 2007; Toria *et. al.,* 2008). It is therefore evident that human genetic background strongly influences susceptibility to malaria infection and progression to severe disease (Wealtherall and Clegg, 2002). The different geographic distributions of sickle-cell disease, Ŭ-thalassaemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, ovalocytosis, and the Duffy-negative blood group are examples of the general principle that different populations have evolved different genetic variants to protect against malaria (Campino 2006 ;Toria *et. al*., 2008). Also genetic variation involving red cells structure (HbS, HbC, and HbE alleles) and the rate of synthesis of haemglobin chains (thalaseamia) are frequent where malaria is highly endemic. These are classical examples of genetic variations in which homozygosity is unfavourable for the host, while heterozygosity may confer a selective

advantage (Allison, 1950; Jurgen *et. al.*, 2007). This study sets out to see whether ABO blood groups do show differential susceptibility to malaria. The study however, observed a high percentage of malaria occurring in individuals of blood group O than non ï O blood group type in different group (both complicated and uncomplicated forms), but chi-square analysis indicated no significant association between the blood group and malaria status of the subjects. The high frequence seen in blood group O indiviuals may be due the higher prevalence of this blood group globally reported and even in Africa (Hamed *et. al.,* 2012). Though some reports have alluded that the ABO blood group plays a significant role in the pathogenesis of infectious disease, this study could not sustanciate this. And therefore aligns with the view that no association exists between ABO blood group and malaria (Thakur and Verma, 1992; Joshi *et al*, 1987) as against the divergent opinon (Christine *et. al.,* 2005; Deeper, 2011).

CONCLUSION/RECOMMENDATIONS

The following conclusions drawn from this study are;

- In human malaria infections elevated mean levels of pro-inflammatory cytokines (IL-1, IL-6 and TNFŬ) are expressed.
- Significant higher levels are expressed in severe malaria compared with uncomplicated and control group. These elevated values although achieves reduction in parasity count, also results in marked reduction in haemoglobin level. It was observed that TNFŬand IL-6 showed a negative association with (Hb/PCV). The study is concluding that the course of malarial anemia is multifactorial, elevation in levels IL-6 and TNFŬbeing one of the factors.
- Anaemia, thrombocytopenia, PCT, RDW, MPV, % monocytes and % lymphocytes could be considered as prognostic variables of malaria. Therefore, were there are no facilities for accurate parasite detectation, these simple haematological value could aid in malaria diagnosis and in monitoring prognosis.
- Leucocyte count increases with malaria severity, could therefore be a marker for severity.

- Altered haptoglobin metabolism consequent to continued increase in *P. falciparum* density and disordered erythropoietin response may be major factors in anemia etiology.
- Malaria is more prevalent and severe in AA haemoglobin phenotype than AS phenotye.

The following recommendation is suggested;

❖ To study erythropoetic responses in malaria using simple retics count

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APPENDIX

Giemsa stain was prepared using the following method:

Giemsa Stain formula

Giemsa powder 3.8g

Methanol 250ml

Glycerol 250ml

Glass bead 50

Large dark bottle

Preparation

a. An amber bottle is preferred but if one is not available use a chemically dry, clear, hard glass or polyethylene bottle of suitable size . You will need about 50 solid glass beads of about 5mm in diameter.
b. Put the glass beads in the bottle; pour in the measured amount of methanol and the stain powder.
c. Tightly stopper the bottle. Allow the stain powder to sink slowly through the methanol until it settled to the bottom. Shake the with a circular motion for 2-3minutes
d. Add the measured amount of glycerol and repeat the shaking process. Continue to shake for 2-3 minutes at half hourly intervals at least six times.
e. Leave the bottle unused for 2-3days; shaking it 3-4times each day until the stain is thoroughly mixed. Keep a small amount of this stock solution in a small bottle for routine use to avoid contamination of stock solution.
f. Each newly prepared batch of stain should be properly labeled, including date of preparation, and should be tested for optimal dilution and staining time and staining time. Always keep the bottle tightly stoppered, in a cool place, away from direct sunlight. Clear glass stock bottle can be covered with a thick dark paper jacket to keep out the light.

PREPARATION OF BUFFERED WATER (pH 7.2)

Reagents

Potassium dihydrogen phosphate (anhydrous)(KH_2PO_4), 0.7g

Sodium phosphate dibasic(anhydrous)(Na_2PO_4) , 1.0g

Distilled or de-ionized water 1000ml

One beaker, capacity 250ml

One conical flask, capacity 1000ml

Two filter papers, 11cm in diameter

Analytical balance

PREPARATION

a. Measure the 0.7g KH2PO4 and of Na2PO4 using an analytical balance and a filter paper. See operation of analytical balance (SOP 6.4) for instruction on how to use an analytical balance.
b. Add the 0.7g potassium dihydrogen phosphate (KH2PO4) to a beaker and add about 150ml of water. Stir with the spatula until the salt is dissolved.
c. Add the 1.0g of sodium phosphate dibasic anhydrous (Na2PO4) to the beaker and stir with the spatula until the salt is dissolved.
d. When the salt is dissolved, add the fluid from the beaker to the conical flask until it is made up to 1L.
e. Note, The pH of this buffer must be checked weekly. The pH is checked using a pH meter.

MAKING UP THE 2% CORRECTING FLIUDS

Reagents

Analytical balance

Two filter paper 11cm in diameter

Two glass-stoppered bottles (amber colour)

Potassium dihydrogen phosphate (anhydrous) (KH2PO4), 2g

Sodium phosphate dibasic anhydrous (Na2HPO4), 2g

Distilled or de-ionized water, 200ml

Two beakers, capacity 250ml

One measuring cylinder, capacity 100ml

Labels

PREPARATION

a. Weigh 2g of Na2HPO4 and add it to 100ml of water in the beaker; stir with the wooden spatula until the salt is dissolved.

b. Pour the solution into one of the glass bottle and label the bottle 2% Na2HPO4 .
c. Repeat step 1&2 above, this time weighing out 2g of potassium dihydrogen phosphate. Pour the solution into the second glass bottle & label it correctly.
d. Note: when not being used, the bottle should be stored in a cool place, away from sunlight.
e. The pH of this buffer must be checked weekly using PH meter or a colour indicator such as the Lovibond comparator.

pH METER

Measure the pH of the buffered water using the pH meter. To alter the pH you will need to add small quantities of one of the correcting fluids,2%Na2HPO4 if the pH is below 7.2 (too acid) or KH2PO4 if the Ph is above 7.2(too alkaline).

LOVIBOND COMPARATOR

Equipment

One conical flask containing the buffered water

Correcting fluids (2% Na2HPO4 and 2% KH2HPO4)

One Lovibond glass cells

One bottle of bromothymol blue indicator

One pipette, capacity 1 ml

RESULT APPENDIX

Rapid diagnostic test (RDT) was also performed along side microscopy and 43 tested positive giving a prevalence of 7.78% (43/553). A sensitivity of and specificity of was obtained when compared with the gold standard (microscopy).