SOME IMMUNOLOGICAL PROFILE OF INDIVIDUALS WITH *PLASMODIUM FALCIPARUM* INFECTION IN ANAMBRA AND LAGOS STATE OF NIGERIA

BY
ONWUACHUSI, GINIKA LOVELYN
NAU/PG/PhD/2013587001F

DEPARTMENT OF PARASITOLOGY AND ENTOMOLOGY, FACULTY OF BIOSCIENCES, NNAMDI AZIKIWE UNIVERSITY, AWKA.

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DISSERTATION SUBMITTED TO THE DEPARTMENT OF PARASITOLOGY AND ENTOMOLOGY, FACULTY OF BIOSCIENCES, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY (PhD) IN PUBLIC HEALTH PARASITOLOGY OF NNAMDI AZIKIWE UNIVERSITY.

SUPERVISOR: PROF. C.A. EKWUNIFE

MARCH, 2019

CERTIFICATION

I hereby certify that this work is a product of my own research efforts, undertaken under the supervision of Professor C.A. Ekwunife, and has not been presented elsewhere for the award of a degree or certificate. All sources have been duly distinguished and appropriately acknowledged.

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Onwuachusi, Ginika Lovelyn Date

NAU/PG/PhD/2013587001F

APPROVAL

This research, Some Immunological Profile of Individuals with *Plasmodium falciparum* Infection in Anambra and Lagos State of Nigeria was carried out by Onwuachusi, Ginika Lovelyn (Reg. No: NAU/PG/PhD/2013587001F) of the Department of Parasitology and Entomology, Faculty of Biosciences, Nnamdi Azikiwe University, Awka.

_____	_____
Prof. C.A. Ekwunife (Project Supervisor)	Date
_____	_____
Dr. E.N. Nwankwo (Head of Department)	Date
_____	_____
Prof. C.A. Ekwunife (Faculty PG Sub Dean)	Date
_____	_____
Prof. M.U. Orji (Dean FBS)	Date
_____	_____
External Examiner	Date
_____	_____
Prof. P.K. Igbokwe (Ag. Dean SPGS)	Date

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DEDICATION

This research is dedicated to, my husband, and my beautiful son.

ABSTRACT

Human malaria which is caused by *Plasmodium spp.* is a disease of public health importance. It is known as the world's most prevalent parasitic disease. Following *Plasmodium* infection, specific antibodies are produced and cytokine activities heightened of which the balance between the pro and anti-inflammatory cytokines helps to regulate immunological status. Profiling such immune markers are therefore necessary for vaccine production, hence the study. The aim of this study was to determine the Immunological Profile of Individuals with Malaria Infection. Ethical approval was obtained from ethics board of Nnamdi Azikiwe University Teaching Hospital (NAUTH) and Anambra State Ministry of Health. Participants were selected from both hospitals and community. Five hundred and forty four (544) individuals were consented; 350 from health facilities while 194 individuals were from Aguleri community. In Anambra State, participants were recruited from NAUTH (43), Iyienu Mission Hospital (135) and Aguleri community. In Lagos participants were recruited from Regina Mundi Catholic Hospital (41) and Agura Health Centre (131). Samples from facility for immunology profile included 58(45.3%) male and 79 (54.7%) female, while community samples included 26 (56.5%) male and 20 (43.5%) female. Axillary temperature was measured using digital thermometer. Venous blood sample was collected. Malaria microscopy was done by two independent readers following WHO standard. Parasite density was computed using patient's actual White blood cell (AcWBC) count, assumed WBC (AsWBC) of 8000cells/mL and 6000cells/mL. ELISA was used for serology to determine immune profile. AcWBC was determined using haematology analyzer. The malaria prevalence for the study was 35.5% in Lagos facility, 9% in Anambra facility and 9.8% from Aguleri community, Anambra state. Infected individuals had single infection of *Plasmodium falciparum* with symptomatic parasitaemia ranging from 15-451,440 with a GMPD of 8,009 and asymptomatic 63 – 13,084 with a GMPD of 953. There was no significant discrepancy between parasitaemia obtained with AcWBC and AsWBC of 8,000 cells/mL (p=0.2892) and 6,000 cells/mL(p=0.8858). The parasitaemia discrepancy between the actual parasitaemia (ACP) and assumed parasitamia(ASP) with WBC of 8,000 cells/mL is 1.5%, 4.4%, 13.2%, 5.9%, 9.0% and 66.2% at 0-5%, >5-10%>10-15%, >15-20% >20-25% and>25 respectively; while parasitaemia discrepancy between the ACP and ASP with WBC count of 6,000 cells/mL is 5.9%, 10.3%, 11.8%, 16.2%, 5.9% and 54.4% at 0-5%, >5-10%>10-15%, >15-20% >20-25% and>25 respectively. ASP using a fixed WBC of 8,000 cells/mL and 6,000 cells/mL can be used in place of ACP calculated using AcWBC where AcWBC is not available. Mean IgG plasma level was seen to be higher among the parasitemic asymptomatic group (122.409ng/ml) than parasitemic symptomatic group (85.206ng/ml), there was strong and significant association between IgG with fever and malaria microscopy among the symptomatic group. TNF−α had a higher concentration in parasitaemic than aparasitaemic individuals. IL-8 association was seen to be significant in symptomatic age groups 2 and 3 (p=0.001: ϕ =0.355; p=0.001: ϕ =0.282respectively) of which the highest concentration (600.078mg/L) was seen in the age group 1-5. TNF−α and IL-8 concentration was seen to be higher in symptomatic than asymptomatic participants. Fever was identified in elevating the plasma level of IgG and TNF−α which could in turn have major roles to play in parasite clearance and immunity as well as disease pathogenesis. Malaria disease severity based on symptoms was found to be independent of parasitemia, sex, and age. With the proposed shift from prevention to elimination phase of malaria control, there is need for more studies on these identified immune markers which may serve as prognostic markers. Further comprehensive test using some of these immune markers may aid malaria vaccine development.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACP	Actual Parasitaemia
AcWBC	Actual White Blood Cell
ALP	Alkaline Phosphatase
ASP	Assumed Parasitaemia
AsWBC	Assumed White Blood Cell
BSA	Bovine Serum Albumn
CRF	Case Report Form
ELISA	Enzyme linked Immunosorbent Assay
GMPD	Geometric mean parasite density
IFN–γ	Interferon Gamma
IgG	Immunoglobulin G
IL-8	Interleukin 8
PBS	Phosphate Buffer Saline
RDT	Rapid Diagnostic Test
TNF-α	Tumor Necrosis Factor Alpha
TGF-β	Transfer Growth Factor Beta

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria which is caused by *Plasmodium species* is a disease of public health importance in Nigeria including Anambra and Lagos state (Agboola *et al.,* 2010; Onyido *et al.,* 2014) due to its prevalence, incidence, morbidity and mortality (Yilgwan, 2011; WHO, 2017). Malaria is known as the world's most prevalent parasitic disease (Angulo and Fresno, 2002). There are five species that infect man which include *Plasmodium falciparum*, *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi* which is a zoonosis (Ndonwi *et al.,* 2011; Brooks *et al*., 2010; Singh *et al.,* 2004; Strickland, 2000; Collier and Albert, 1998). Of all these species, *P. falciparum* is the most predominant in Africa (WHO, 2011).

Following infection with *Plasmodium spp.*, specific antibodies are produced and cytokine activities heightened, one or two weeks after initial infection and persist for three to six months after parasite clearance. These antibodies and cytokines may persist for months or years in semi-immune patients in endemic countries like Nigeria where re-infection is frequent (Doderer *et al*., 2007), offering a form of partial immunity.

Various studies have been carried out on the immune responses against *Plasmodium spp.* of which most are based on murine experiments; these experiments resulted in a resolution that both cell-mediated and antibody-dependent immunity are required for adequate protection against malaria (Mohan and Stevenson, 1998; Rovira-Vallbona *et al*., 2012). The innate immune system is believed to play a crucial role in clearing *Plasmodium spp.* from parasitized hosts, of which most of these clearances occurs in the spleen and liver (Dockrell *et al.,* 1980; Alves *et al.,* 1996).

Cytokines has been seen to be a relevant factor in patients with clinical malaria, both in severe and mild cases. Interferon γ (IFN-γ), IL-2, IL-5, IL-6, and IL-12 levels were significantly increased during infection with malaria though, the increase were predominant in patients with mild malaria while, TGF-β, TNF-α, IL-10, and IL-1β levels was identified to significantly increase with disease severity, particularly in patients with severe malaria (Prakash *et al*., 2006). It has been recorded that the lower levels of antibodies found in children with severe malaria compared to children with uncomplicated malaria were not an attribute of lower exposure to malaria parasite but rather, an imbalanced pro-inflammatory cytokine response may exacerbate the severity of infection (Rovira-Vallbona *et al*., 2012).

It has been demonstrated that *P. chabaudi* infection in γ/δ T-cell-deficient mice have significantly worsened their early and chronic parasitaemia (Seixas and Langhorne, 1999), which could be inferred that early production of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) both to splenic γ/δ T lymphocytes and to natural killer (NK) cells (Choudhury, *et al*., 2000) could be responsible for this situation. Also, Immunoglobulin G and Interferon γ (a pro-inflammatory cytokine) have been implicated to play specific roles in protection (Aucan *et al.,* 2000; Tran *et al.,* 2012).

Thus, balance between the pro- and anti-inflammatory cytokines helps to regulate the immunological status of patients with malaria (Rovira-Vallbona *et al.,* 2012).This immune regulation is dependent on the functionality of the antibodies produced. Kinyanjui *et al.,* (2004) demonstrated that anti-VSA IgG required the presence of an asymptomatic infection for protection against clinical disease caused by parasites bearing heterologous variant surface antigen (VSA).

Malaria control programme aims to move from a control phase to an elimination phase (The malERA Consultative Group on Basic Science and Enabling Technologies, 2011). With this shift in focus, there are the possibilities of the rise in other issues like the increase in susceptibility with a reduced infection rate which could give rise to severe or complicated cases of malaria hence the need for more grounded preparation during this phase. Therefore, the development of an effective malaria vaccine and accurate diagnosis is widely viewed as the way towards sustenance of malaria control and possible elimination in Nigeria. One of the factors that hinder the design of a lifelong successful vaccine against *Plasmodium* is possibly the current incomplete knowledge on protective immunity and how it can be induced (Angulo and Fresno, 2002). Therefore, a better understanding of the functionality of the immune system in malaria parasite clearance and immunity will help in better case management and possible eradication of malaria.

1.2 Statement of Problem

During a parasitic infection like malaria, the host's immune system's ability to effectively clear malaria parasite is dependent on the host's immune reaction or response (Iriemenam *et al.,* 2009a; Medina *et al.,* 2011). In human malaria infection, the immune response exerted by the human immune system can either result in the parasite clearance or immunopathology (Medina *et al.,* 2011). Cases of immunopathology in malaria infection have been identified to be mediated majorly by the cytokines and disease responses are dependent on the balance between the anti and pro- inflammatory cytokines (Iriemenam *et al.,* 2009b) which could be useful as a diagnostic marker (Bostrom *et al.,* 2012).

Malaria disease manifestations differ in different individuals and could be regulated by the age of the host and immune acquisition, the parasite's genetic polymorphisms and its variations in various regions (Lyke *et al.,* 2004).Therefore, the understanding of the human-malaria immune

responses in relation to age and how this immunity can be sustained is the key to a better case management and possible eradication of the disease; one way of understanding the intricate process of the immune activity is to profile the biomarkers related to immune responses in relation to age and defining their relative functions.

1.3 Justification

Clearance and elimination of malaria infection is hinged on the better understanding of the human-malaria immune response, integrated control measures and the production of an effective life-long malaria vaccine. The integration of these facts is important in Nigeria since the country aims to move from a control phase to an elimination phase which could result in a situation where citizens have low or no immunity and an increased susceptibility to the infection.

The clearance of malaria parasite in the blood stream is dependent on the balance between the pro-inflammatory cytokine and anti-inflammatory cytokine (Iriemenam *et al.,* 2009a; Medina *et al.,* 2011; Bostrom *et al.,* 2012). Therefore, an understanding of how certain immunological markers function could help in achieving this goal. Thus, the need to profile serum levels of Immunoglobulin G, pro-inflammatory cytokine like IL-8 and TNF-α in Individuals with Symptomatic and Asymptomatic Malaria Infection. Determining the level at which they are found in immune and non-immune individuals will go a step further to help us understand the balance needed in the body to increase the chances of a sustained Malaria free nation.

1.4 Significance of Study

An individual's response to an infection or presentation of a disease is largely a factor of the relationship between the disease-causing organism and the host; this situation also applies to people infected with the malaria parasite. Over the years efforts have been put into the control and elimination of the disease by employing vector control measures and the use of drugs (The malERA Consultative Group on Basic Science and Enabling Technologies, 2011; WHO, 2011). Though a lot has been achieved but the goal of having a malaria free nation by the year 2020 seems to be a far fetched dream. The missing link that could tip the balance in the war against malaria could be related to the host immune activity. So there is a need to understand the host-parasite immune relationship in all facets. One of the areas that had been identified is the cytokines, chemokine and immunoglobulin. The better knowledge of the interaction of these immune-dependent factors could be the key to achieving a sustainable malaria free nation.

1.5 Aim

The aim of the study was to determine the Immunological Profile of Individuals with Malaria Infection

1.6 Specific Objectives

The Specific objectives for this study were to determine:

1. Malaria prevalence in Lagos among symptomatic group

2. Malaria prevalence in Anambra state among Symptomatic and asymptomatic participants.

3. Discrepancy significance between the parasite density obtained with the automated WBC count, assumed value of 8,000 cells/mL and 6,000 cells/mL

4. Immunoglobulin G levels in individuals with symptomatic and asymptomatic malaria infection in relation to age and sex

5. Pro-inflammatory cytokine (IL-8 and TNF-α) levels in individuals with symptomatic and asymptomatic malaria infection in relation to age and sex

6. disease pathogenesis in relation with the serum profile of Immunoglobulin G and pro-inflammatory cytokine (IL-8 and TNF-α) in Individuals with symptomatic and asymptomatic malaria infection in relation to age and sex.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria Situation

Malaria is an acute and chronic disease caused by obligate intracellular protozoa of the genus *Plasmodium* which belongs to the family plasmodiidae. There are six species known to cause malaria in man which include: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale, P. cynomolgi* and *P. knowlesi* which the last two are zoonotic infection that causes malaria in macaque monkeys, but cases in man have been described in South Asia (Dondorp, 2005; Ta *et al.*, 2014).

Malaria is one of the most significant diseases in tropical and sub-tropical areas including Nigeria especially among children under the age of 5years and pregnant women (Mwaniki *et al*, 2010); malaria is also believed to contribute to the infant mortality in Nigeria (Osungbade and Oladunjoye, 2011; Morakinyo and Fagbamigbe, 2017).

In 2015, WHO world malaria report estimated that the number of cases of malaria rose from 233million in the year 2000 to 244 million in 2005, decreased to 216 million in 2010 (WHO, 2015a). Furthermore, of the estimated 216 million cases of malaria occurred worldwide in 2010, majority of the cases (80.55%) occurred in the African Region, followed by the South-East Asia (13.96%) and Eastern Mediterranean Regions (4.63%) (WHO, 2011). In the 45 malaria endemic countries in Africa Nigeria accounts for one quarter of all malaria cases (WHO 2011). Malaria prevalence further dropped to 214 million in 2015. The number of deaths due to malaria is estimated to have decreased from 839, 000 in 2000 to 438,000 in 2015. Decreases in malaria burden have been observed in all WHO Regions, with the largest proportional decreases noted in the European Region, followed by the Region of the Americans. The largest absolute decreases

in deaths were observed in Africa (WHO, 2015a).This disease is holoendemic in Nigeria where majority of the infections are caused by *Plasmodium falciparum* (WHO, 2017; Gallup and Sachs, 2001).

Furthermore, in 2017 there was further reduction of which, the subregion had about 111 million estimated cases about 41 million reported confirmed cases. A total of under-reported 19,000 malaria deaths were recorded but the estimated number of deaths was 224,000 of which Nigeria account for 52% of the estimated cases (WHO, 2017).

More so, the widespread implementation of artemisinin-combination therapy (ACT) and long-lasting insecticide bed nets (LLIN) has been linked to the declines in the incidence of malaria in certain areas of Africa including Nigeria (WHO, 2011; O'Meara *et al*., 2010). However, this improvement is constantly threatened by the emergence of drug-resistant *P. falciparum* parasites and insecticide-resistant mosquito vectors (Ranson *et al*., 2011; Dondorp *et al.*, 2009).

Despite the encouraging decrease in malaria burden in Africa, Nigeria still holds a bulk of the disease incidence (27% globally) (WHO, 2017), where malaria infections are mostly prevalent among children under the age of 5years and pregnant women and also immune compromised individuals (FMoH, 2005; Mwaniki *et al*, 2010). Though malaria is a frequent cause of ill-health in children in the tropics, it is believed to be rare in the newborn period (Samal, 1981; Del Punta *et al.*, 2010). This might be due to some reasons, which includes the assumption that the fetal haemoglobin would exert its protective influence during the immediate neonatal period; and the fact that the clinical signs of neonatal malaria are indistinguishable from that of neonatal sepsis (Del Punta *et al.*, 2010, Orostegui-Pinilla and Rodriguiz-Morales, 2011). These assumptions and many more lead to the fact that neonatal malaria might pass unnoticed. However, this may not be

so with a recorded prevalence of neonatal malaria of about 54.2% (Okechukwu and Olateju, 2011, Osungbade and Oladunjoye, 2011).

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2.2 Malaria in Neonates

Neonatal malaria defined as the presence of malaria parasite in the peripheral blood (smear) of a baby in the first month of life is a serious cause of morbidity and mortality in Sub-Saharan Africa (Hyacinth *et al*., 2012; Mwaniki *et al.,* 2010); though it was thought to be rare (Covell, 1950, Del Punta *et al.*, 2010) in areas with stable malaria; presumably because of the transplacental transfer of protective antimalarial IgG antibodies and a relatively high haemoglobin F content of the infant's erythrocyte (Strickland, 2000, Oyibo *et al.,* 2009), in view of the fact that a significant proportions of neonates with malaria is missed in the wards on the assumption that the disease is rare (Runsewe-Abiodun *et al*., 2006), due to the fact that malaria in neonate is confused with neonatal sepsis and other neonatal infection because of the similarity in the signs and symptoms (Runsewa-Abiodun *et al*., 2006, Del Punta *et al*., 2010, Okechukwu and Olateju, 2011, Hyacinth *et al*., 2012), this could also be attributed to the fact that local health facilities in resource limited settings often lack the capacity to diagnose malaria infection (Osungbade and Oladunjoye, 2011).

However, malaria is now being encountered in the new born period (Orogade *et al*., 2008, Hyacinth *et al.,* 2012); this higher incidence could also be attributed to an increased resistance and virulence of parasites resulting from altered antigenic determinants in addition to increased reporting (Valecha *et al.,* 2007).

Malaria in neonates was thought to be rare especially in neonates of semi-immune mothers in holoendemic areas; as a result, sick neonates admitted to newborn special care units are often presumed to have neonatal sepsis. Sepsis is an infection caused by bacteria, in most cases, malaria in neonates masquerade as sepsis (Ojukwu *et al*., 2004).

The clinical differentiation between sepsis and malaria in neonates is difficult because the signs and symptoms are usually vague, non-specific and overlapping (Ojukwu *et al*., 2004; Ekanem *et al*., 2008). The similarity between the presentation of sepsis and malaria in the neonatal period could lead to misdiagnosis. In some areas usually malaria endemic areas, sepsis is usually treated first, even when the child's condition does not improve despite the use of different antibiotic regimes; this is done with the previous conception that malaria in neonates is rare because of the maternal protective IgG (Ojukwu *et al*., 2004; Eknem *et al.,* 2008; Del Punta *et al*., 2010). This assumption is believed to be one of the reasons why neonatal malaria had been thought to be rare. Studies in recent times have shown that this is not so, thus neonates with sepsis should also be screened for malaria to avert possible undesired outcome.

A report by Ojukwu *et al.* (2004) in which 84 neonates admitted for presumed sepsis showed that 28 (33.3%) had positive blood smear for malaria parasite alone, 10 (11.9%) had septicemia alone while 4 (4.8%) has both malaria and septicemia. They further went ahead to state that the presenting features in the babies with neonatal malaria and sepsis included fever in 24 (85.7%), 5 (50%); refusal to feed 23 (82.1%), 10(100%); respiratory distress 19 (67.9%), 10 (100%) respectively (Ojukwu *et al.,* 2004). These clinical features in babies with and without neonatal malaria admitted for neonatal sepsis shows that cases of suspected to be sepsis in neonates could actually be malaria.

Therefore, neonates suspected with sepsis should also be screened for malaria parasite. Malaria in neonates could be as a result of infection with *P. falciparum, P. vivax, P. malariae* and *P. ovale*. Malaria can be grouped into three types based on the mode of parasite entry as congenital, acquired, and transfusional.

2.3 Congenital Malaria

For a long time, the frequency of the occurrence of congenital malaria remained a subject of controversy with many report before the 1970s describing it as a very rare event (Covell, 1950). Uneke (2011) reported that some other reports in the 1980s however noted that the low reported incidence of congenital malaria particularly in Africa was surprising since malaria occurs more commonly in pregnancy.

Despite the evident occurrence of neonatal malaria, it could sometimes pass unnoticed because it may be symptomatic or asymptomatic especially in babies of women with high level of immunity (Okechukwu and Olateju, 2011). Congenital malaria is generally assumed to be asymptomatic because, majority of parasites are being rapidly cleared from the neonates circulatory system as a result of passive immunity crossing the placenta, active immunity which develops from exposure to soluble malaria antigen in the uterus and the high proportion of fetal hemoglobin present in babies which retards the growth of the parasite (Kakkilaya 2011a; Strikland 2000)

Malaria parasite also crosses the placenta along with the protective immunity conferred on the new born. Malaria in neonates is considered to be congenital when asexual parasites are detected in the peripheral blood within the first week of life (Hashemzadeh and Heydarian, 2005; Orogade *et al.,* 2008, Okechukwu and Olateju, 2011). This phenomenon results from malaria parasites crossing the placenta from maternal blood to the fetal circulation (Kolawole *et al.,*

2009); this is possible because the parasites adopt ways to adhere to blood cells. *P. falciparum* among other *Plasmodium* species have the unique ability of cytoadhesion; adhesion molecules such as CD36 and intercellular adhesion molecule-1 may be involved in the development of malaria in neonates. Chondroitin sulfate A and hyaluronic acid have been identified as the adhesion molecules for parasite attachment to placental cells (Strickland, 2000).

The parasites sequester along the surface of the placental membrane, specifically the trophoblastic villi, extravillous trophoblasts, and syncytial bridges. Intervillous spaces are filled with parasites and macrophages which interfere with oxygen and nutrient transport to the fetus which contribute to the complications experienced by both mother and child (Kakkilaya, 2011a). Congenital malaria due to transplacental or peripartal infection of the fetus is being increasingly reported, and has been reported in 8–33% of pregnancies from both malaria-endemic and nonendemic areas (Kakkilaya, 2011b).

It has been reported as an aftermath of maternal infections with all four species of human *Plasmodium*, although most cases are reported following *P. falciparum* or *P. vivax* malaria (Okechukwu and Olateju 2011, Orogade *et al.*, 2008, Uneke, 2011, Valecha *et al*., 2007, Orostegui-Pinilla and Rodriguez-Morales, 2011). In non-endemic countries, *P. malariae* may cause a disproportionately higher number of congenital malaria cases due to its longer persistence in the host (kakkilaya, 2011b).

2.4 Acquired Malaria

Malaria parasite is acquired through the bite of infected female *Anopheles* mosquito, the risk of transmission or infection is increased in rural areas and varies seasonally in many locations, the highest occurrence is seen in rainy season (WHO, 2017). Acquired malaria in neonate is as a

result of infected female *Anopheles* mosquito bite anytime after delivery when asexual parasitaemia is detected within a minimum incubation period of greater than one week (Ibhanesebor, 1995; Okechukwu and Olateju, 2011).

Acquired malaria could easily be prevented by using the different intervention methods of malaria control which include: prevention of mosquito bites by making sure that individuals sleep under insecticide treated nets, wearing protective clothing at dusk to prevent mosquitoes from biting human beings and deny blood meal, the use of insect repellants in homes and clinics to repel mosquitoes, draining stagnant waters where mosquitoes breed, prompt treatment of diagnosed patients with anti-malaria drugs to reduce the spread of the disease, intermettent preventive treatment for pregnant women and individuals with no or low immunity such as tourist and breeding sterile genetically modified mosquito.

2.5 Transfusional Malaria

Several diseases can be transmitted via blood transfusion; malaria is one of the several blood borne infection that are transmitted through blood transfusion. This can be controlled by the proper screening of donated blood for transfusion by blood banks. Transfusion acquired malaria first reported by Gerhardt 1884, accounts for rare, though important cases of neonatal malaria (Doraiswamy, 2005).

The risk of acquiring an infection via transfusion is greatly increased in sick premature infants because they receive frequent transfusion. The full-term infant is not fully competent immunologically and the premature infant is less able to deal with infection (Piccoli *et al*., 1983).

Transfusional malaria is said to occur when malaria parasite is detected in the peripheral blood of a patient whose peripheral blood was negative prior to blood transfusion. Transfusion-acquired malaria is uncommon and factors such as drug resistance and concomitant Glucose-6-phosphate dehydrogenase (G6PD) deficiency an X-linked recessive hereditary disease can cause treatment difficulties (Virdi *et al.,* 2003).

2.6 Malaria in Pregnancy

Malaria infection during pregnancy is a major public health problem in tropical and subtropical regions throughout the world (Aluko and Oluwatosin, 2012, Onwuanaku *et al.*, 2012). In most endemic areas of the world, pregnant women are the main adult risk group for malaria (Oyibo *et al.,* 2009); this assertion is supported by researchers that had reported its prevalence ranging from 14% to 72% in endemic areas during pregnancy (Omalu *et al.*, 2011). This occurrence is due to the depression of cell-mediated immunity during pregnancy. The burden of malaria during pregnancy is caused chiefly by *Plasmodium falciparum,* the most common malaria species in Africa (Roll Back Malaria, 2010).

Malaria in pregnancy can lead to the death of the mother, abortion of the fetus or still birth, low birth weight (Onwuanaku *et al.*, 2012, Yakoob *et al.,* 2005) and premature delivery, all of which are associated with an increased risk of neonatal death and impaired cognitive development. The susceptibility of pregnant women to malaria is dependent on the number of pregnancies; it is seen as being higher during first pregnancy and reduces with increased gravidity (Akpiri and Agi, 2014). The primi-gravid and second-gravid are more susceptible because of the presence of a new organ (Placenta) and the fact that they had not yet developed full immunity.

Reports indicate that both *Plasmodium falciparum* and *Plasmodium vivax* can cause adverse pregnancy outcomes (Del Punt *et al*., 2010; Akpiri and Agi, 2014). The symptoms, complications and outcome of malaria during pregnancy differ with the intensity of malaria transmission and thus with the level of immunity the pregnant woman has acquired (RBM, 2010). Due to the fact that a child could acquire malaria through the mother it is necessary that proper care should be taken to reduce the effect and outcome of malaria in pregnant women thus averting the possible adverse effect of the disease on mother and child

2.7 Malaria Prevalence in Nigeria from 1995-2016

Malaria till date remains a significant disease of public health importance in Nigeria (Osungbade and Olateju, 2011). The trend of occurrence of malaria shows that there was a high prevalence in 1996, 2004 and 2005 (40.0%, 33.3% and 54.2%) which was seen to reduce in 2007 (10.5%), an increase in 2008 (32.7%) which further decreased in 2011(5.92%). However, report of prevalence from some areas such as the northern regions of Nigeria still remain relatively high (Nmadu *et al*., 2015; Saganuwan and Mohammed, 2016) (Table 1).

The malaria prevalence recorded by grouping states into zones; these zones according to the report of the National Population (2011) and the National Malaria Elimination Programme (NMEP) (2016) during the National Malaria Indicator Survey(s) (NMIS) showed that there was a gross reduction in the malaria prevalence (Fig 1) from 2010 to 2015.

The highest decrease was noted in the south west zone where prevalence reduced from 50.3% to 16.6%. The lowest rate of prevalence reduction was seen in the North East zone of Nigeria which includes states such as Adamawa, Bauchi, Borno, Gombe, Taraba, and Yobe. The reduction in

malaria prevalence across all zones could imply that the intervention methods employed to control malaria are working thereby reducing malaria prevalence in the different zones.

The grouping of the 36 states in Nigeria and the Federal Capital Territory (FCT), according to geo-political zones are as follows:

I. North Central: Benue, FCT - Abuja, Kogi, Kwara, Nasarawa, Niger, and Plateau

2. North East: Adamawa, Bauchi, Borno, Gombe, Taraba, and Yobe

3. North West: Jigawa, Kaduna, Kano, Katsina, Kebbi, Sokoto, and Zamfara

4. South East: Abia, Anambra, Ebonyi, Enugu, and Imo

5. South South: Akwa Ibom, Bayelsa, Cross River, Delta, Edo, and Rivers

6. South West: Ekiti, Lagos, Ogun, Ondo, Osun, and Oyo (NMEP *et al* 2016) (Fig. 1)

Table 1: The report of malaria Prevalence in Nigeria 1995-2016

Study authors/ References	Year of publication	Type of study	Study location	Prevalence of malaria (%)
Ibhanesebhor	1995	Case study	Benin, Nigeria	8.0
Ibhanesebhor *et al*	1996	Case study	Benin, Nigeria	40.0
Mbanugo and Ejims	2000	Cross Sectional	Awka, Anambra	58.3
Matur *et al*	2001	Cross sectional	Abuja, Nigeria	58
Aribodor *et al*	2003	Cross sectional	Anambra, Nigeria	76
Ojukwu *et al.*,	2004	Prospective Study	Ebonyi, Nigeria	33.3
Obiajunwa *et al*.	2005	Cross sectional	Ile-Ife, Nigeria	54.2
Yakoob *et al*.	2005	Retrospective Cohort Study	Karachi	33.0
Mukhtar	2006	Cross- sectional	Lagos, Nigeria	15.3
Okafor *et al.*	2006	Cross-sectional	Enugu, Nigeria	32.48
Runsewe-Abiodun *et al*.	2006	Retrospective descriptive study	Shagamu, Nigeria	24.8
Falade *et al.*	2007	Epidemiological Study	Ibadan, Nigeria	10.5 (IPTP-SP) 16.8(PYR)
Ekanem et al.	2008	Cross-sectional	Calabar, Nigeria	35.0

Orogade *et al.*	2008	Prospective Descriptive Study	Ibadan, Ilorin, Kaduna, Enugu 5.1%
Kolawole *et al*.	2009	Prospective Study	Ilorin, Nigeria 23.08
Lesi *et al.*	2010	Case Study	Lagos, Nigeria 13.6
Okechukwu and Olateju	2011	Prospective study	Abuja, Nigeria 41.7
Omalu et al.	2011	Case Study	Mina, Nigeria 5.92
Onwuanaku	2012	Case Study	Jos, Nigeria 32.7
George et al.	2013	Cross-sectional Study	Port-Harcourt, Nigeria 9.6
Afolabi et al.	2013	Retrospective Review	Lagos, Nigeria 23.9%
Ahmadu et al.	2014	Cross-sectional Descriptive Study	Maiduguri, Nigeria 44.5
Olasehinde *et al*	2015	Case study	Ogun, Nigeria 62.7
Nmadu *et al*	2015	Case study	Abuja, Nigeria 64
Elechi *et al*	2015	Case study	Maiduguri, Nigeria 27.7
Saganuwan and Mohammed	2016	Case study	Kogi, Nigeria 48.7

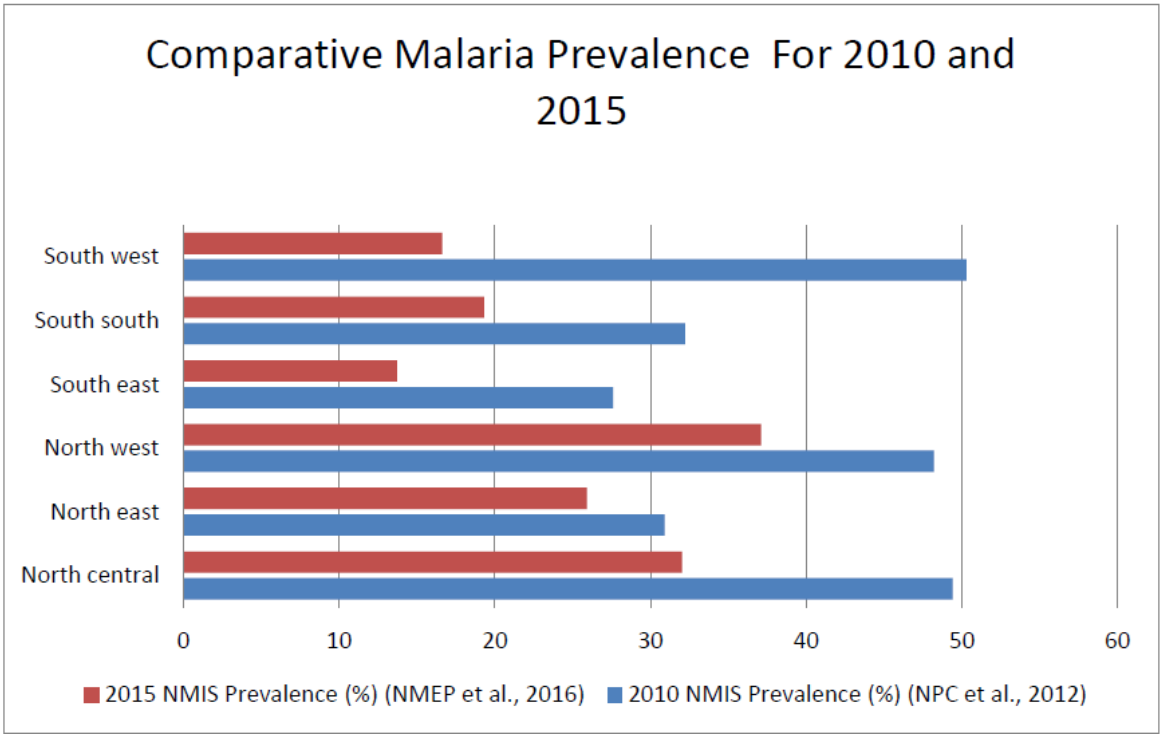

Fig 1: Nigeria Malaria Prevalence by Zones (NPC *et al.,* 2012; NMIS *et al.,* 2016)

2.8 Adaptive processes of *Plasmodium* species to parasitism

Malaria parasite is mostly transmitted through an infected female *Anopheles* mosquito (Menendez and Mayor 2007) and could lead to a disease state due to different mechanism implored by the parasite in order to evade the immune response. These parasites unlike free-living organisms have evolved special or unique means to evolved diverse adaptive features to enhance their survival in their host. However, of the five *Plasmodium species* known to infect human, *Plasmodium falciparum* is responsible for most of the severe form of human malaria and the most virulence of them all, while the *Plasmodium malariae* could result to a milder clinical manifestations compared to the other species (Brooks *et al.*, 2010).

The ability to cause disease in the host depends on the different adaptive mechanism of *Plasmodium spp.* in host, and this depends on the presence of *Plasmodium spp* in the blood of the host long enough to produce viable gametocytes at a suitable time, especially when environmental conditions are suitable for transmission (Strickland, 2000). When comparing the species, *P. falciparum* is known to have shortest incubation period through which schizonts is released 2 to 20 times as merozoites, and are known to release over 40,000 merozoites into the blood stream (WHO, 2010b), whereas for *P. malariae*, it is the only human malaria parasite that causes fever that recur at approximately three-day intervals (therefore occurring every fourth day, a *quartan fever*), longer than the two-day (*tertian*) intervals of the other malarial parasites (CDC, 2010). Although, entry into the host are achieved the same way for all the species, *P. falciparum* and *P. malariae* do not undergo relapse stage in their host, unlike *Plasmodium ovale* and *Plasmodium vivax* (WHO, 2010b).

2.9 Evasion of Host Immune System

The *Plasmodium* parasite however, faces many challenges in their life history. For instance, for *P. falciparum*, the parasite are known for their virulence in the host (WHO, 2010b) although the host tends to evolve resistance to the infections and or tolerance of their harmful effect. In vertebrate, highly developed non- specific and specific immune responses replenished by immunological memory form the main physiological barrier against parasites infections. Other defense mechanism such as avoidance of those circumstances under which infection take place have been described in several parasites in their host system (Collier and Albert, 1998). Parasite however adapts to various means by which the immune system can be evaded; these different successful mechanisms help to ensure life continuity in their environment. The evasion of the host immunity may be explained by the ability of the malaria parasite to escape host immunity by: poor immunogenicity of its antigens, sequestration, antigenic diversity and variation or alteration of the immune response.

2.9.1 Poor immunogenicity

During malaria infection immune responses are provoked due to the presence of the malaria antigens. However, these antigens may not provoke the required immune response. Because some antigens may be poorly immunogenic which makes them loose their ability to elicit an immune response either because of their intrinsic molecular structure or their analogy to host molecules or because of immune restriction, which may impair recognition (Collier and Albert, 1998, Yu *et al.,* 2004).

2.9.2 Sequestration

Sequestration is an adaptation of malaria parasite (*P. falciparum*) where the older pigmented throphozoites and schizonts temporally withdraw from the circulation and attach to deep tissues particularly liver, spleen and bone marrow (Taylor-Robinson, 2000; Moody, 2002; Sherman *et*

al., 2003; Penny *et al.,* 2006). This phenomenon usually occurs once during every asexual circle which is relatively 48 hours for *P. falciparum* (Penny *et al.,* 2006).

The ability of the malaria parasite to remain sequestered by cytoadherence to the capillary lining of certain tissues aid the parasite to evade the host immune system, since such parasites can avoid frequent passage through the spleen and thus exposure to immune effectors mechanisms, thus avoiding destruction by the immune system (Buffet, *et al.,* 2011). These parasites adhere to the host endothelium with the help of certain ligand-host-cell-receptors; *P. falciparum* isolates are able to switch rapidly from one endothelial receptor to another and this can be regarded as part of parasite survival strategy (Penny *et al.,* 2006). Sequestration does not occur in *P. malariae* or other human malaria parasites, which is another strategy used by the *P. falciparum* for their successful survival and for disease severity. When sequestration occurs, there is the possibility that the parasites might be missed during detection because of insufficient numbers of parasites. This is majorly crucial especially in the case of asymptomatic pregnant women with which parasites are sequestered in the placenta causing complications to the neonate or leading to fetal death (Penny *et al.,* 2006).

2.9.3 Alteration of the immune response

Malaria parasites also evade the immune system by alteration of the immune responses elicited. Various mechanisms of immune-suppression including polyclonal activation, macrophage dysfunction, abnormal antigenic presentation, disruption of lymphatic and splenic tissue architecture, activation of suppressor cells and antigenic competition assist the parasite in invading their host. While these mechanisms may affect the outcome of concurrent infection, they may also affect the ability of the host to mount an effective immune response to the malaria parasite itself (Collier and Albert, 1998; Brooks *et al.,* 2010).

2.9.4 Antigenic diversity

Antigenic diversity is a possible explanation of a slow development of immunity; that is strain specific immunity can ultimately be suppressed by broader species-specific immunity. In the absence of such transcending immunity, it would be difficult to understand why infections become rarer in adults, since adults continue to be exposed to new isolates (Collier and Albert, 1998; Brooks *et al.,* 2010)

2.9.5 Antigenic variation

Malaria parasites are capable of periodically changing the expression of their antigens. This provides the parasite with a powerful means for evading host immunity particularly when antigenic variation occurs in conditions where a selective pressure is exerted. Antigenic variation can result to a successive peak of infection with each new peak antigenically distinct from the previous one. Surface antigens such as Merozoite Surface Protein (MSP), which are most exposed to immune pressure, are obviously most likely exhibit antigenic variation (Collier and Albert, 1998; Brooks *et al.,* 2010)

2.10 Life Cycle Adaptation

2.10.1 Invasion of Hepatocytes

After sporozoites have entered the bloodstream, they rapidly enter the liver sinusoids and establishes itself within the hepatocytes. The membrane of the sporozoite is entirely covered by two molecules (CSP and TRAP/SSP2), which are responsible for initial attachment to the hepatocytes surface. TRAP and CSP, have such a broad specificity that recognition of different cell types is possible (Barnwell, 2001; Pradel *et al*., 2002). Also, the passage through Kupffer cells is an essential step in the sporozoites maturation, without which the apical end organelles cannot exocytose and enable invasion with the formation of a parasitiphorus vacuole. It has been

suggested that the sporozoites may, in some circumstances have to cross more than one cell before this maturation is achieved (Mota *et al*., 2002). Once inside the hepatocytes, the sporozoites differentiate, losing their apical organelles and start to grow.

2.10.2 Invasion of Erythrocytes

In order to invade the erythrocyte, the merozoites have to recognize and attach to its surface components; this is another way by the parasite evading the host immune system by occupying an immunological competent site where little or no immune responses are triggered. The nature of the erythrocyte receptor involved varies from one Merozoite to another, because each parasites species has specific host cell preferences (Collier and Albert, 1998). In *P. falciparum*, the receptor varies from one group to another.

2.10.3 Alteration of Host Cell

After infection by the malaria parasite, the host cell undergoes a variety of structural changes, which may substantially alter its function, appearance or antigenicity. The nature of the alterations induced varies from one species to another; these host cell alterations have been studied most comprehensively in the erythrocytic stage of the parasite (Sherman *et al*., 2003), but it is conceivable that they may also exist in the other intracellular stages. The alterations identified in the membrane of malaria infected erythrocyte include:

1. A visible change and shape and reduced deformability (WHO, 2010b)

2. The presence of electron- dense protrusions or knobs (Strickland, 2000)

3. The presence of small depressions or caveolae at the surface of the erythrocyte connected by a network of small vesicles and clefs (Aikawa, 1988)

4. The cytoadherence to endothelial cells or resetting with normal erythrocytes (Wahlgren *et al.*, 1989)

5. The evidence of new parasite- specific antigens associated with the red cell membrane (Hommel *et al*., 1983).

Adaptations that make parasites successful in exploitation competition might similarly be advantageous in apparent competition. That is, strategies that lead to higher parasite densities could ensure a particular parasite line access to a greater share of host resources and a greater probability of overcoming host immune responses. One example of this overlap in successful strategies is low gametocyte conversion rates in malaria. This strategy could also be advantageous in multiple infections in response to immune-mediated apparent competition and in the absence of competition for resources **(**McKenzie and Bossert, 1997).

The roles of apparent and exploitation competition in shaping gametocyte conversion rates of malaria parasites could be determined by using a model system in which the host immune response can be manipulated. Immune responses elicited by a parasite alter the host environment, and these changes have an impact on a host's ability to resist or succumb to subsequent infections by different parasite species through either suppression or facilitation of infection and growth (Collier and Albert, 1998).

2.11 Characteristics of Different *Plasmodium* species

The peripherial blood smear of *P. falciparum* characteristically contain only young ring forms and occasionally crescent shaped gametocyte, while in *P. malariae* do not distort or enlarge red blood cells, but it results in distinctive shapes of the parasite seen in the host cell; these shapes are in "bar and band forms". There are also very compact dark staining forms; its schizoits are usually composed of eight merozoites appearing in a rosette (Brooks *et al.,* 2010; WHO, 2010b).

In *P. falciparum*, multiple sporozoites can infect a single sporozoite showing multiple infections of cells with small ring forms. The trophoites are often seen in host cell in the host cell at the periphery of cell membrane at accole position. Occasionally, reddish granules known as Maurer's dots are observed while mature (large) trophozoite stages and schizoints are rarely seen in blood films, because their forms are sequestrated in deep capillaries, liver and spleen. The peripherial blood smear of *Plasmodium falciparum* characteristically contain only young ring forms and occasionally crescent shaped gametocyte (WHO, 2010b).

Plasmodium vivax is characterized by enlarged infected RBC which usually contains numerous pink granules of schuffner's dots. The throphozoite is ringed shaped but amoeboid in appearance. It contains more mature trophozoite and erythrocytic schizoints containing up to 24 merozoites and the gametocytes are usually round (WHO, 2010b).

Plasmodium malariae do not distort or enlarge red blood cells, but it results in distinctive shapes of the parasite seen in the host cell; these shapes are in "bar and band forms". There are also very compact dark staining forms; its schizoint is usually composed of eight merozoites appearing in a rosette (WHO, 2010b). The host cell of *Plasmodium ovale* is enlarged and distorted usually in an oval form. Schuffner's dots appear as pale pink granules. The infected cell border is commonly fimbriated or ragged and mature schizoint contain about 8-1 merozoites. (Assafa *et al*., 2004).

2.12 Clinical manifestation of Malaria Infection

Pathological changes due to *Plasmodium* parasitaemia are clinically reflected in the host as malaria. The clinical presentation of malaria is one of the least specific of all the major disease (WHO, 2010a), Infection by any of the malaria causing species triggers a quick response of intercellular substances and inflammatory mediators especially tumor necrosis factor α (TNFα)

responsible for fever and causes suppression of hematopoesis, thus contributing to anemia and thrombocytopenia. Although this could be as a result of the different *Plasmodium* spp having different ways of presenting their effect clinically. The clinical manifestation of malaria can be classified into asymptomatic, uncomplicated and severe malaria.

Plasmodium falciparum, are known to cause the severe phase of malaria infection, and have tendency of causing paroxysm (an increase and decrease in temperature); causing the most complications which are associated with organ dysfunctions, cerebral malaria, black water fever, prostration, pulmonary oedema, jaundice, renal failure, acute kidney injury, severe anaemia, respiratory distress, convulsion, haemoglobinuria, hyper-parasitaemia, hepatosplenomegally, acidosis and hypoglycaemia (WHO 2000). These complications can develop rapidly and progress to death within hours or days *(*Trampuz *et al.,* 2003*)*.

Plasmodium falciparum also have a unique characteristic of adhering into deep tissues and placenta; there are times when there is involvement of the brain causing cerebral malaria which could be due to an increase in the production of tumor necrosis factor during schizonts rupture following the stimulation of microphages. Liver involvement is characterized by abdominal pain, vomiting of bile, hepatosplenomegaly, severe diarrhea and rapid dehydration (Samal, 1981; Ibhanesebhor, 1995; Brooks *et al.,* 2010).

A patient infected with the *Plasmodium* parasite may experience flu-like symptoms such as head ache, muscle pains, photophobia, anorexia, nausea and vomiting. Infection with these symptoms is referred to as uncomplicated malaria (WHO, 2010a). As the infection progressed, increased numbers of rupturing erythrocytes introduce merozoites and other toxic cellular debris and hemoglobin into the blood circulation. The substance produces the typical chills, fever and

malaria rigors. These paroxysms usually appear mild or may progress to severe attack, within hours of sweating, chills, shaking persistently, high temperatures and exhaustion (Collier and Albert, 1998).

When an individual gets infected by any of the malaria causing *Plasmodium* species, the following is likely to take place: Liberation of intercellular substances and inflammatory radiators especially tumor necrosis factor α (TNFα) responsible for fever and causes suppression of hematopoesis, thus contributing to anemia and thrombocytopenia (Collier and Albert, 1998).

There is also the destruction or red blood cells accounting for anemia and to an extent jaundice seen in patients with malaria especially *falciparum* malaria. Furthermore, the intra erythrocytic substances liberated act as antigen to stimulate the immune system, and can cause hyperplasia of some organs of the reticuloendothelial system like the spleen (Collier and Albert, 1998; Brooks *et al.*, 2010), which becomes enlarged, soft and congestive with the sinus engorged, with parasitsized red blood cells.

The capillaries of the brain and kidney are also invaded by infected red blood cells during severe malaria. The infected cells become sticky due to the knobs on their surfaces and stick to vessel walls and slow down circulation in the brain resulting in ischemia. These ischemic lesions are diffused and can be reversible if managed on time. The result of ischemia on the brain and hypoglycemia account for the neurological signs seen in malaria patients. Hypoglycemia in malaria is due to liver involvement, stimulation of pancreatic β cells by *Plasmodium* to secrete insulin, anorexia and effects of quinine also contribute to hypoglycemia. It is these effects that lead to the presentation of certain clinical symptoms exhibited by neonates and non immune persons that are affected by malaria (Del Punta *et al.*, 2010).

The presenting clinical features of complicated malaria include: fever, hepatomegally, spenomegally, anemia, pallor, and jaundice (Samal, 1981; Ibhanesebhor, 1995). Some may show signs of respiratory difficulty, feeding difficulty and gastroenteritis. Others also show severe thrombocytopaenia without bleeding (Del Punta *et al.*, 2010).

The different *Plasmodium* species have their ways of presenting their effects clinically. *Plasmodium falciparum* for instance rapidly produces daily (quotidian) chills and fever a well as nausea, vomiting and diarrhea. The periodicity of the attack then becomes tertian (36-48 hours), and fulminating disease develops. There are times when the involvement of the brain causing cerebral malaria; liver involvement is characterized by abdominal pain, vomiting of bile, hepatosplenomegaly, severe diarrhea and rapid dehydration (Samal, 1981, Ibhanesebhor, 1995).

2.13 Laboratory Diagnosis of Malaria

Early and acute diagnosis of malaria is essential for effective disease management and malaria surveillance. Early, prompt and accurate diagnosis and treatment is crucial to the management of morbidity and mortality caused by malaria; this is one of the major interventions employed in the control of malaria. Some of the methods applied in the diagnosis of malaria include: Clinical diagnosis, Microscopy, Quantitative buffy coat, Polymerase chain reaction, and Rapid diagnostic test (RDT).

Among the many clinical signs and symptoms associated with malaria and used in diagnosis is fever which is often associated with chills, perspiration, anorexia, headaches, vomiting and malaise. Additional signs indicating malaria include: confusion and drowsiness. Though clinical diagnosis is not specific, it is nevertheless often the only feasible approach in many

circumstances such as in rural areas. This approach requires trained personnel, it is not expensive and it requires no specialized equipment. Its major disadvantage is related to its low specificity.
It is impossible to diagnose a condition due to malaria infection or another disease solely on the basis of the clinical presentation (WHO, 2010a). Therefore, confirmatory malaria diagnosis is essential for a successful control of malaria infection or transmission. This could be achieved by the identification of malaria parasite in blood smear, the gold standard test for malaria diagnosis. Blood films are best prepared on clean glass slides free from grease and scratches (Cheesbrough, 1999). Using a preparation of Giemsa stain and buffer solution as well as the technical skilled personnel involved in malaria microscopy (WHO, 2000; WHO 2010b). A standard white blood count of 8000 per μL is then used for the determination of parasitaemia which is counted against the malaria parasite. When viewed under the microscope, the different species are identified based on their morphological characteristics.

Microscopic examination of blood collected from patients infected with *Plasmodium* is still the most commonly used method for malaria parasite diagnosis and the estimation of parasite density due to its low cost however it requires a highly skilled personnel to carry out the test. Parasite quantification is an important aspect of malaria diagnosis since it helps to determine the severity of an infection, determine the response of patients to treatment and also determine the effectiveness of drugs during clinical trials of antimalaria drugs (WHO, 2010b).

Malaria Parasite density determination using microscope is inferred from the number of white blood cells (WBC) per mL of blood, which is automatically calculated using blood cell counters or assumed at a fixed value of 8,000 cells/mL, according to the World Health Organization (WHO) guidelines (WHO, 2010b). However, a more accurate estimate of the malaria parasite density considers the variations in WBC count occurring in different age groups and also in

malaria-endemic regions (Trape 1985; Jeremiah and Uko, 2007; Udomah *et al.,* 2016). In order to achieve this, the use of an automated WBC count in infected patients is necessary. This allows a more accurate estimation of parasite density when compared to the method that assumes a fixed value of 8,000 cells/mL. This is due to the fact that the number of leukocytes detected in a sample of patients is lower or higher than the assumed value (Jeremiah and Uko, 2007).

The equipment necessary to perform an automated WBC count is not always available in health care services located in malaria-endemic areas. Therefore, an assumed value of 8,000 cells/mL has been considered as an alternative for the quantification of parasitemia in patients infected with malaria (WHO, 2010b). The laboratory diagnosis of parasitemia requires special attention in the preparation of Giemsa stain and buffer solution used as well as the technical skill involve in malaria microscopy.

Using standard white blood count of 8000 cells per mL for determination of parasitemia as is routinely done for malaria studies according to the World Health Organization (WHO) guidelines (2010b). However, there is the assumption that WBC Count of 8,000 Cells/mL overestimates malaria parasite density (Jeremiah and Uko, 2007; Omalu *et al.,* 2008; Alves-Junior *et al.,* 2014) and a recommendation that 6,000 cells/mL be used in Nigeria which has been applied in some malaria studies (Udomah *et al.,* 2016).

Quantity Buffy coat and DNA-binding fluorochromes can be used to aid the detection and quantification by microscopy of parasites in blood smears and in layer of erythrocytes ("Buffy coat") in centrifuged blood samples. However, such modifications increase cost of microscopy and require special equipments, electricity and additional supply. Analysis of blood samples by amplification of parasite specific nucleic acid by nested and real-time polymerase chain reaction

(PCR) is reported to be highly sensitive and specific. This technique requires highly trained personnel, and is both time and resource intensive. Special equipments, liable reagents and specialized testing environments are also required.

Rapid diagnostic tests (RDTs) use immunochromatographic method to detect antigens derived from malaria parasite in lysed blood. The RDTs have been generally reported to achieve sensitivities of >90% in detection of *Plasmodium* parasites at densities at or above 100 parasites per micro meter of blood (WHO, 2010c). Tests that are currently available rely on the detection of the following: Histidine-Rich protein II(HRP-II) (Howard *et al*., 1986; WHO, 2010a), a water soluble protein produced by trophozoites and young gametocytes of *P. falciparum* only*;* Parasite lactate dehydragenase (pLDH) (Makler *et al.*, 1993) produced by sexual and asexual stages of parasites of *P. falciparum* and "non-falciparum" species and aldolase, another enzyme in glycolytic pathway that is present in all four species of malaria parasite.

Also molecular methods like utilizing polymerase chain reaction for malaria nucleotide protein (e.g. MSP1 or MSP2) detection (Orogade *et al*., 2008), Enzyme linked immunosorbent assay (Cheesbrough, 1999) can also be used for the detection of malaria parasite. These techniques are however not routinely feasible due to cost, electricity and inability to get a well trained personnel to handle the equipment.

2.14 Malaria Transmission

Vectors are organisms known to transmit pathogens from one host to another; they are usually seen as invertebrate animals, such as arthropods (Institute of Medicine, 2008). However, they may also include fomites, which are any inanimate objects that may be contaminated with disease-causing organisms and therefore could transmit diseases or rodents, which carry the

agent from a reservoir to a susceptible host thus vectors of diseases includes arthropod, rodents and even fomites (Anderson *et al*., 2004; Institute of Medicine, 2008).

Mosquitoes are insects that act as vector to various diseases including malaria: there are approximately 3,500 species of mosquitoes and about 400 species of *Anopheles,* but 40 are important vector that are able to transmit malaria well enough to cause significant human illness and death (CDC, 2015). The most important vectors in the afro tropical region (Africa south of the Sahara, Madagascar, Seychelles and Mauritius) are the *A gambiae* complex which includes *A gambiae, A. arabiensis, A. melas, A. merus, A. bwambae, A. quadriannulatus* and *A. funestus* (WHO, 2012). These species all contribute to the transmission of malaria as potential vectors.

The *Anopheles* vector, is the link between man and the malaria parasite; they prefer to lay their eggs in shallow, stagnant clean water such as ponds, marshes, swamps, floodwater, ditches and woodland pools (WHO, 2013a,b). However, they have been living successfully in many different environments; they live even in environments that are not normally theirs. These vectors grow well in hot, humid environments especially in tropical areas (Githeto *et al.*, 1993).

The *Anopheles gambiae* complex, known as the major vector system in Africa, exists only in frost free regions, or where the minimum temperature in winter remains above 5°C. They are usually active at dusk or dawn while some are nocturnal (WHO, 2010c). The vectorial capacity of the anopheline transmitters is determined by their human biting rate and sporozoites infection rate as well as developmental and survival of the parasite and vector as influenced by climatic and ecological factors (Githeto *et al*, 1993).

Mosquito borne disease is propagated by the natural environmental conditions and man-made disturbance such as clustered houses, water pollution due to lack of hygiene and socio economic status (Ani, 2004), pot holes on bad roads and gutters which will lead to the accumulation of stagnant water; thus providing a conducive environment for mosquitoes to breed (Mbanugo and Okpalaononuju, 2003).

Afoloabi *et al.,* (2013) in Ondo state reported the abundance of thirty species of mosquitos which was as a result of temperature, pH, dissolved oxygen, relative humidity, conductivity and anthropogenic factors. Also Egbuche *et al.,* (2013) within Aguleri, Anambra state malaria transmission was high due to poliferation of breeding habitate of the mosquitoe vector.

In Sokoto malaria prevalence was reported to be higher in males who engage in activities which make them more prone to be infective mosquito bites than in females who are mostly at home and protected from such infective bites (Abdullahi *et al.,* 2009). Another study carried out in Calabar where the temperature and humidity were 28.00C and 80.50C respectively indicated that the high rate of malaria transmission was as a result poor access to effective control measures such as the use of insecticide treated nets (ITNs) and the type of anti-malarial drug use in the community (Ekanem *et al*, 2008).

In Nassarawa state, the abundance of *Anopheles species* was attributed to climatic condition of the area and accumulation of water bodies in which *Anopheles gambiae* was the most abundant during the rainy season (Ayanda, 2009).

2.15 Malaria Pathogenesis

The infection of the red cells by malaria parasites, particularly *P. falciparum*, results in progressive and dramatic structural, biochemical, and mechanical modifications of the red cells that can leadto life-threatening complications of malaria. Malaria infection comes with different clinical manifestations which could range from mild to severe. The disease severity depends on the parasite species, the organ involved and the access to care, species of parasite, the immune status of the individual, pregnancy (especially primigravid and secondgravid, HIV/AIDS, some genetically inherited conditions like sickle-cell trait, ß-thalassemia, and G6PD deficiency which could have protective effect (Autino *et al.*, 2012).

At the completion of the schizogony within the red cells within 24-72 hours depending on the species of the infecting parasite, schizonts rupture to release merozoites and other substances such as red cell membrane products, hemozoin pigment, and other toxic factors such as glycosylphosphatidylinositol (GPI) which are released by the lysis of infected erythrocytes. These products, particularly the GPI, stimulate the immune system by activating macrophages and endothelial cells to secrete cytokines and inflammatory mediators such as tumor necrosis factor-α (TNF-α), interferon-γ, interleukin-1, IL-6, IL-8, macrophage colony-stimulating factor, lymphotoxin, superoxide and nitric oxide (NO). GPI is common to several merozoite surface proteins such as MSP-1, MSP-2, and MSP-4, as a key parasite toxin (Mackintosh *et al*., 2004; Chakravorty *et al.,* 2008).

The systemic manifestations of malaria such as headache, fever and rigors, nausea and vomiting, diarrhea, anorexia, tiredness, aching joints and muscles, thrombocytopenia, immune suppression, coagulopathy, and central nervous system manifestations have been largely attributed to the various cytokines released in response to these parasite and red cell membrane products. The

plasmodial DNA is also highly proinflammatory and can induce cytokinemia and fever (Fig 2) (Clark *et al.,* 2006).

Fever pathogenesis starts with the presence of *Plasmodial* DNA (pDNA) which is presented by hemozoin that is been produced during the parasite development within the red cell. The pDNA then interact intracellularly with the Toll-like receptor-9 (TLR-9), whch signals NF-Kappa B leading to protein synthesis and the release of proinflammatory cytokines. Pro inflammatory cytokines such as TNF-α and IL-1 are produced after the signaling of the immune system which in turn induce cyclooxygenase-2 (COX-2), an enzyme responsible for inflammation and pain. COX-2 then upregulates prostaglandins leading to the induction and onset of fever (Parroche *et al.,* 2007; Schumann, 2007).

Plasmodium falciparum differ from other human malarial species in that infected red blood cells do not remain in the circulation for the entire life cycle. After 24–32 hours, when young parasites mature from the ring to the trophozoite stage or gametocytes, infected red blood cells adhere to endothelial cells through a phenomena known as cytoadherance (Fig 3) (Chen *et al.,* 2000).

Plasmodium falciparum can adhere (sequester) to the vascular endothelium of organs such as lungs, heart, brain, lung, liver, and kidney, subcutaneous adipose tissues and the placenta (Fig 3) (Kyes *et al.,* 2001; Scherf *et al*., 2008) However, a study by Carvalho *et al.,* (2010) has identified that *in vitro* reticulocytes infected with *Plasmodium vivax* can sequester to some kendothelial cell lines and placental cryosections. Parasite sequestration is thought to be the pathological base of the severe manifestation of malaria, including cerebral malaria (Grau and Craig 2012).

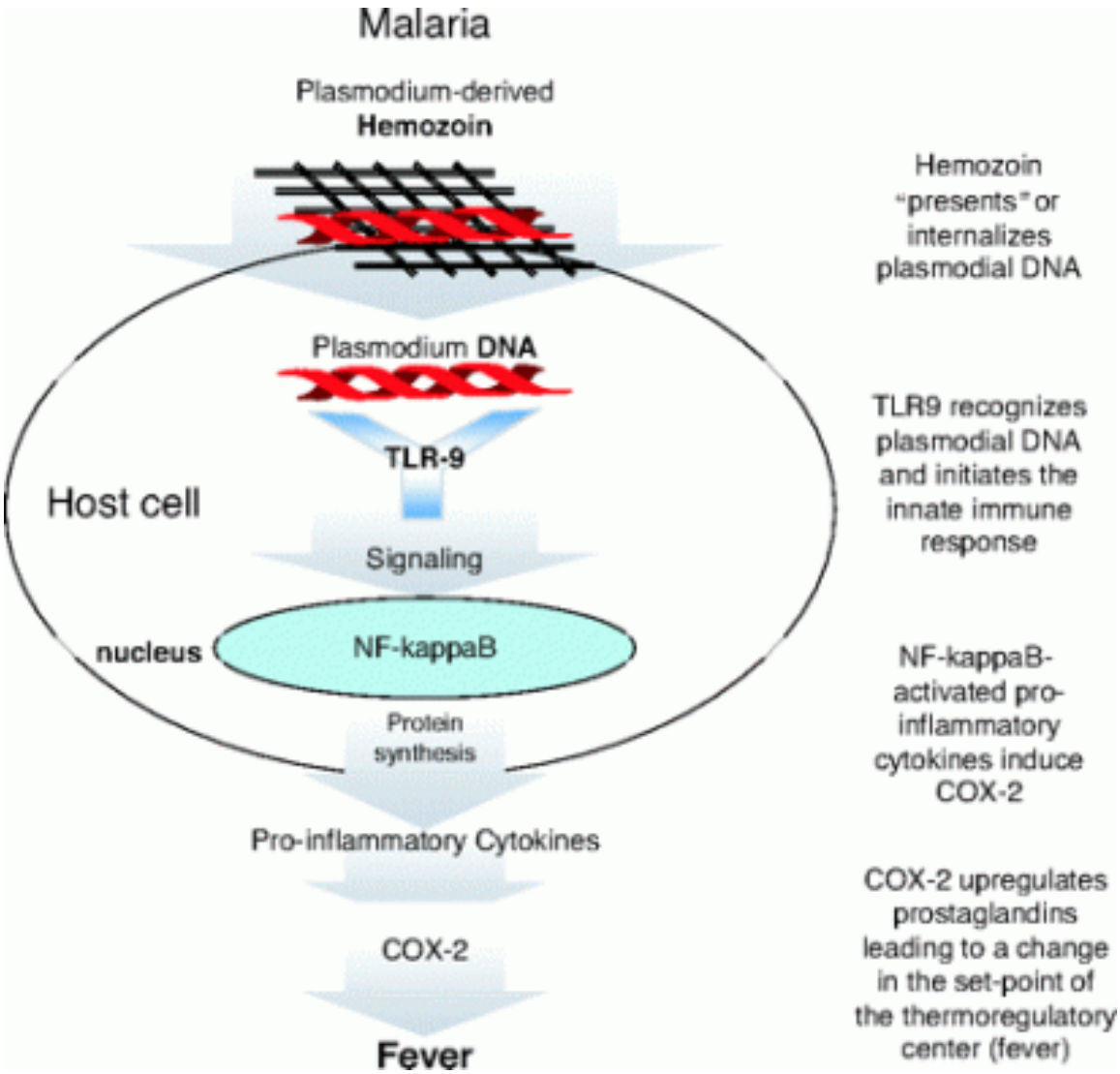

Fig 2: Pathogenesis of Fever by Malaria Parasite (Source: Schumann, 2007)

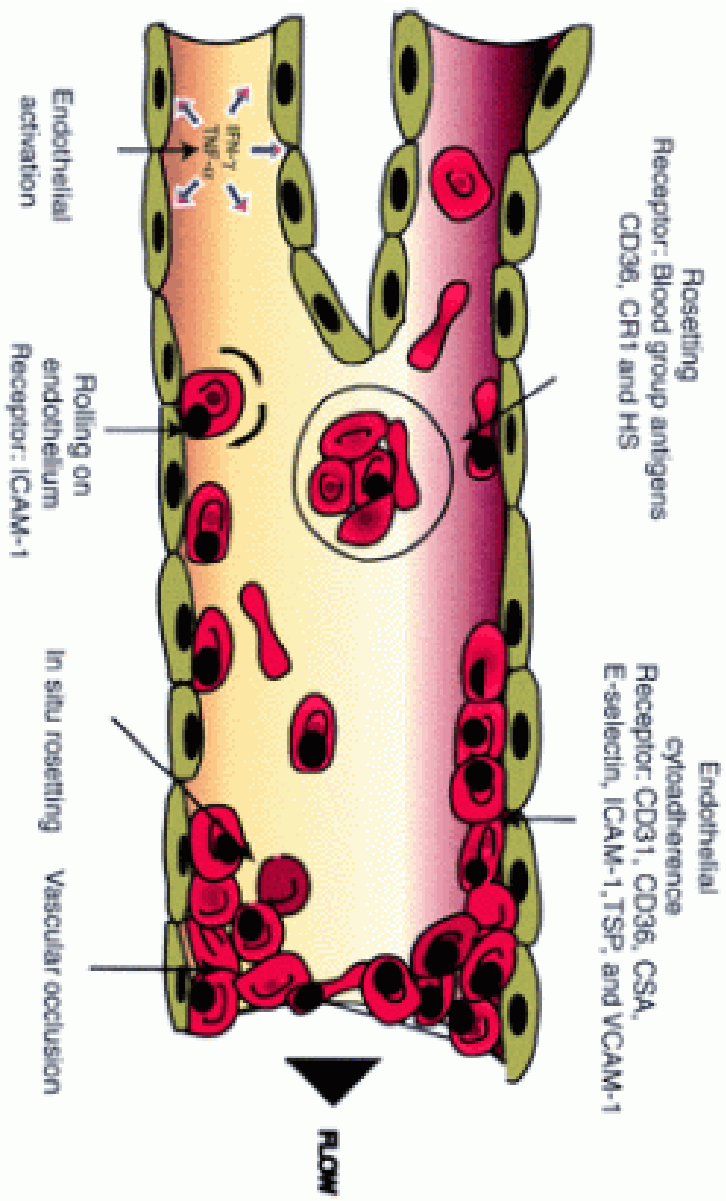

Fig 3: Cytoadherence and rosetting in Post Capillary Vasculature (Source: Chen *et al.*, 2000).

Sequestration leads to the obstruction of the flow of blood which results to local hypoxia, it promoted parasite replication sticking of infected red blood cells to non-infected red blood cells which results in rosetting. Also during sequestration, parasite activity seem to occur in the same area which increases the immune stimulation by the parasite toxins produced due to the parasitic activity, which may cause a focused production of inflammatory mediators and therefore resulting to tissue damage and possibly host cell death (Dondorp *et al.,* 2004).

Cytoadherance and resetting occur in postcapillary vasculature while **s**equestration is mostly mediated by mature parasite forms which are seen to occur approximately 20 hours after the parasite invades the RBC. The parasites produce new proteins that are exported to surface of the infected RBC which helps to increase the adhesiveness of the infected RBC to the host endothelium (Autino *et al.*, 2012).

The parasites can remain sequestered for 24 hours in the deep microvasculature during its life cycle; this is one of the parasite methods of adapting to parasitism in which, they evade clearance by the spleen, and make parasite diagnosis via microscopy more difficult since they are not seen in the peripheral blood. Sequestration of *P. falciparum* has been attributed to different class of molecules of parasite origin and ligands present on the human endothelium, among which is the *P. falciparum* histidine-rich protein (PfHRP) and the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which is a multimeric protein encoded by the *var* (variant) gene (Kyes *et al.,* 2001; Scherf *et al.,* 2008) have been implicated. The PfHRP has been associated with the establishment of knobs, symmetric membrane arrangements which appear on the surface of infected RBC, while PfEMP1 protrudes from the knobs and plays a major role in sequestration and the parasite virulence (Fig 4) (Autino *et al.*, 2012).

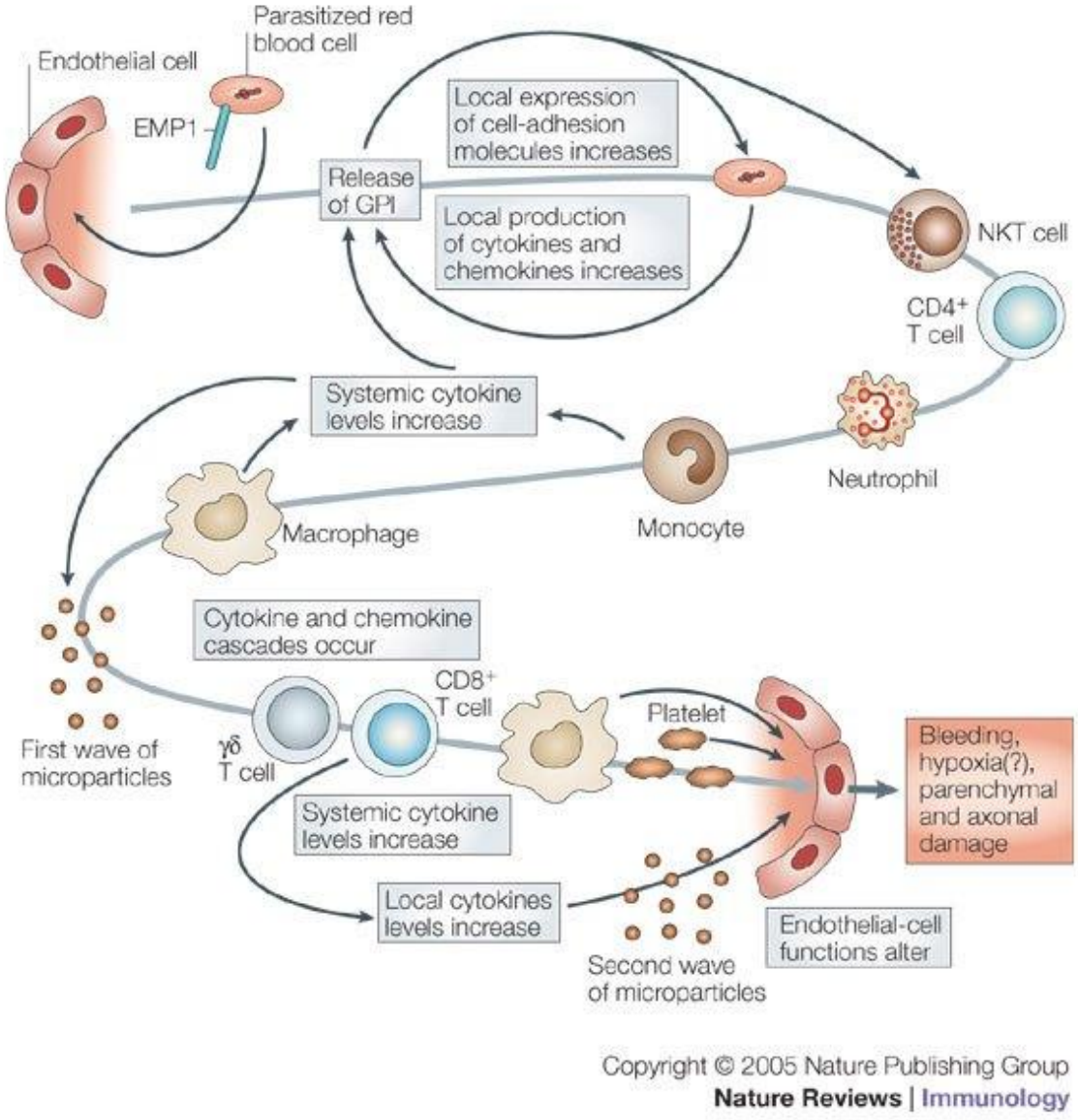

Fig 4: Schematic representation of pathogenesis of severe malaria (Source: Schofield and Grau, 2005) 41

To adhere to the host endothelium, the parasites first adhere, roll and then become firmly attached to the host endothelium adhesion molecules. Among these adhesion molecules, the intercellular adhesion molecule-1 (ICAM-1) is a major sequestration receptor which is also involved in cerebral sequestration resulting in cerebral malaria also serves as a rolling receptor. On the other hand, CD36 gives stationary and stable adherence under flow (Fig 4) (Turner *et al.,* 1994; Chakravorty and Craig, 2005; Milner, 2010).

Sequestration is also a phenomenon that is related to malaria in pregnancy, when parasites adhere to the placenta (Oyibo *etk al.,* 2009). During malaria in pregnancy, PfEMP1 is also the main adhesion receptor which adheres to the trophoblastic villous endothelium through chondroitin sulfate A (CSA) and other sugars such as glycosaminoglycans and possibly hyaluronic acid (Autino *et al.,* 2012). Malaria in pregnancy can result to complication both for the mother and the fetus especially during the first pregnancy, when women usually lack sufficient immunity against malaria parasites (Reeder *et al.,* 1999; Fried *et al.,* 2006; Rogerson *et al.,* 2007; von Itzstein *et al.,* 2008).

Furthermore, rosetting is one of the forms of cytoadherence of late stages of infected RBC to non-parasitized red blood cells or platelets (Rowe *et al.,* 2009). The infected RBC ligand which is involved in rosette formation is PfEMP1, and the receptors associated with resetting are the complement receptor 1 (CR1), heparan sulfate (HS), and the ABO blood group (Chen *et al.,* 1998; Rowe *et al.,* 2009).

PfEMP1 has been shown to bind to CR1, specifically at the C3b-binding site. The lectin-like DBL-domain of PfEMP1 can make strong adhesion with carbohydrate structures particularly A blood group antigen, favoring rosettes formation (Vogt *et al.,* 2003). Thus, non-O blood groups are considered significant risk factors for life-threatening malaria, through the mechanism of enhanced rosette formation (Barragan *et al.,* 2000; Rowe *et al.,* 2007). *P. falciparum, P. vivax*, and *P. ovale* are all able to form rosettes (Udomsanpetch *et al.,* 1995; Angus *et al.,* 1996) but only those caused by *P. falciparum* have been associated with severe malaria, and especially in African children they may enhance severe malaria. Several pathophysiological factors such as the parasite biomass; malaria toxin(s) and inflammatory response; cytoadherence, resetting and sequestration; altered deformability and fragility of parasitized erythrocytes; endothelial activation, dysfunction and injury; and altered thrombostasis have been found to be involved in the development of severe malaria

2.16 Complications of Severe Malaria

Anaemia is one of the most common causes of morbidity and mortality in malaria infection particularly in pregnant women and in children (Nussenblatt and Semba, 2002). Malarial anaemia could be acute or chronic; in holoendemic areas chronic malarial anaemia is more common. Acute malarial anaemia could occur after massive erythrocytes lysis due to elevate parasitemia or to drug-induced or immune haemolysis (Ghosh and Ghosh, 2007). Furthermore, the presence of hemozoin has also been linked to the induction of apoptosis in developing erythroid cells in the bone marrow, thereby causing anemia (Awandare *et al.,* 2007; Lamikanra *et al.,* 2009).

The potential mechanisms contributing to malarial anaemia can be divided into two categories: increased destruction of parasitized and un-parasitized erythrocytes (immune-mediated lysis,

phagocytosis splenic sequestration) and decrease of RBC production (dyserythropoiesis and bone marrow suppression,inadequate reticulocyte production, effects of inflammatory cytokines, effects of parasite factors).Co-infection with bacteremia, HIV-1 and hookworm, malnutrition and repeated malarial infections in holoendemic countries may also contribute to decrease haemoglobin levels (Nussenblatt and Semba, 2002; Haldar and Mohandas, 2009; Skorokhod *et al.,* 2010; Perkins *et al.,* 2011)

Parasitized red cells ruptured by *Plasmodium* cycles and clearance of deformed parasitized and unparasitized erythrocytes are the principal cause of malarial anaemia. Phospholipid asymmetry, membrane rigidity and reduced deformability are the mechanisms involved in the premature removal of un-parasitized red cell (Pasvol *et al.,* 1992; Dondrop *et al.,* 2004; Haldar and Mohandas, 2009). Phagocytosis and complement activation are the principal mechanisms of non-specific immune mediated clearance of erythrocytes in malaria infection (Nweneka *et al.,* 2010).

Moreover, uninfected red cells membrane proteins may be altered by reactive oxygen species (ROS) and other factors, becoming target for autoantibodies (Scherf *et al.,* 2008). It has been suggested that the spleen may play a double role in malaria infection: on the one hand it could contribute to severe anaemia, by excess removal of IRBC and uninfected RBC; on the other hand it may protect from severe cerebral malaria (Buffet *et al.,* 2009). Dyserythropoiesis plays an important role in the pathogenesis of anaemia; examination of bone marrow from children with severe anaemia showed hypercellularity, mild to normal erythroid hyperplasia and abnormal features of late erythroid progenitors (Buffet *et al.,* 2009).

Hemozoin and its phagocytosis by bone marrow macrophages has been proposed to cause dyserythropoiesis either through direct accumulation in the bone marrow and generation of

reactive toxic species or activation of the innate immune response (Skorokhod *et al.,* 2010). The immune response is central in the pathogenesis of malarial anaemia; parasitized red cells, hemozoin and malarial antigens activate monocyte and lymphocyte response (Fig 4). Pro-inflammatory and anti-inflammatory mediators, including TNF-α, IFN-γ, IL-23 and IL-1, chemokine and growth factor are produced and contribute to anaemia. On the contrary, IL-12 and IL-10 seems to be protective cytokines since low levels are found in severe malarial anaemia (Ghosh and Ghosh, 2007; Perkins *et al.,* 2011). Macrophage migration inhibiting factor (MIF) is associated with severe anaemia and bone marrow suppression (McDevitt *et al.,* 2006).

Nitric oxide is an inhibitor of erythropoiesis (Ghosh and Ghosh, 2007; Pradhan, 2009). Erythropoietin (EPO) levels are increased during malaria anaemia, but erythroid progenitors response is not adequate, particularly during chronic malaria infection, resulting in low reticulocytosis (Perkins *et al.,* 2011). It has been shown in a rodent model that exogenous EPO could stimulate splenic erythroblasts. However, their maturation is impaired due to altered iron metabolism and haemoglobin production (Chang *et al.,* 2004). Ineffective erythropoiesis, erythrophagocytosis and iron delocalisation are the most important causes of reticulopenia.

Pro-inflammatory cytokines play an important role also in iron delocalisation pathway of malarial anaemia. TNF-α induces re-localisation of ferroportin, an important protein abundant in the reticuloendothelial system that mediates macrophage iron release and intestinal iron absorption. Relocalisation of ferroportin induces decrease of iron absorption and release from macrophage cells (Nweneka *et al.,* 2010); hepcidin, a protein released during chronic disease, also induces reduction of ferroportin and its levels are increased during severe malaria anaemia (de Mast *et al.,* 2010a). STAT6, a member of signal transducer an activator of transcription family proteins, seems also to be involved in malarial anaemia through the activation of the

regulatory cytokines, in particular IL-4 and IFN-γ, resulting in erythropoietic suppression during blood stage malaria (Thawani *et al*., 2008; Robson and Weatherals, 2009).

A review study found an interesting correlation between malarial anaemia and micronutrient malnutrition: vitamin A and E, iron, zinc, riboflavin and folate deficiency may play a role in worsening the anaemia mediated by alteration of immunity, dyserythropoiesis and iron metabolism (Nussenblatt and Semba, 2002). It has also been suggested that high catecholamine concentration could alter the functions of the erythrocyte membrane, providing an additional erythrocyte clearance mechanism (Haldar *et al.,* 2007).

Thrombocytopenia is very common in malaria, usually during the early stage of P. *falciparum* and *P. vivax* infections. Incidence is high both in children and in adults (Maina *et al.,* 2010; Kochar *et al.,* 2010; Leowattana *et al.,* 2010). Thrombocytopenia in pregnant women is not well documented, but a study performed in Thailand showed that platelet counts were lower in pregnant than in non-pregnant women (Tan *et al.,* 2008). The pathogenesis of malaria thrombocytopenia is complex and may be related to coagulation disturbances, splenomegaly and platelet destruction by macrophages, bone marrow alterations, antibody-mediated platelet destruction, oxidative stress and platelets aggregation. These processes are well described in a review (Lacerda *et al.,* 2011).

A study performed in Indonesia showed that patients with *P. falciparum* and *P. vivax* malaria had lower platelet count, higher von Willebrand factor (VWF) concentration, lower ADAMTS13 activity and ADAMTS13 antigen concentrations (de Mast *et al.,* 2009). Higher VWF seems to correlate with platelet binding, leading to thrombocytopenia. In contrast, another study demonstrated that sGP1b, the external domain of platelet receptor for VWF, increased early in

the blood of malaria patients thus preventing excessive platelet adhesion (de Mast *et al.,* 2010b). Despite thrombocytopenia is very common, hemorrhagic events are rare and usually are associated with severe thrombocytopenia or disseminated intravascular coagulation (DIC) (Seshadri *et al.,* 2008; Misra *et al.*, 2011; Chaudhary *et al.*, 2011).

Deep breathing, respiratory distress and pulmonary oedema are some of the clinical feature defining severe malaria according with WHO classification (WHO, 2010c). In adults and pregnant women, rather than children, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are the most common clinical presentation burdened by an elevated mortality rate as shown in a study performed in India where severe *P. falciparum* malaria mortality rate was 35,4% and mortality was principally due to shock, acute renal failure, seizure and ARDS. Despite the fact that ARDS is mostly observed as a complication of *P. falciparum* infection, case reports due to *P. vivax,* (Saleri *et al.,* 2006; Agarwal *et al.,* 2007; Price *et al.*, 2007; Sharma and Khanduri 2009) *P. ovale* (Haydoura *et al.*, 2011) and *P. knowlesi* (Daneshvar *et al.,* 2009; William *et al.,* 2011) have been published.

Malaria associated ARDS due to *P. vivax* caused three maternal deaths in a cohort of 221 patient in India during one year of observation (Sharma and Khanduri, 2009). ARDS can occur before or after specific treatment (Saleri *et al.,* 2006) while *P. knowlesi*, infecting humans, mostly in Southeast Asia, may cause life threatening diseases, as well (Sabbatani *et al.,* 2010). A prospective study conducted in Malaysia demonstrated that the most frequent complication of *P. knowlesi* infection was respiratory distress (William *et al.,* 2011). Malaria is presently considered one of the most common risk factor of ARDS and acute lung injury (ALI) in the tropics (Mohan *et al.,* 2008) and the second cause of ARDS after sepsis (Gupta *et al*, 2001).

Little is known about the pathogenesis of malaria associated ALI/ARDS. Inflammatory mediators and increased endothelial permeability may play an important role, while parasite sequestration may take a minor role. Pulmonary manifestations of uncomplicated malaria were analysed showing that patients frequently have subclinical impairment of lung function, such as small airways obstruction, impaired alveolar ventilation, reduced gas exchange and increased pulmonary phagocytic activity (Anstey *et al.,* 2002). These features underline the activation of inflammatory pathways and could explain part of the pathogenetic mechanisms of respiratory distress occurring during severe malaria.

In Studies with rodent malaria models to understand the pathogenetic mechanisms of ALI/ARDS, it was observed that in DBA/2 mice infected by *P. berghei* ANKA (Ephiphanio *et al.,* 2010) 60% of mice had dyspnea, airways obstruction and hypoxemia; pleural effusion, pulmonary hemorrhagies and oedema were found demonstrating the role of inflammation in ALI development. Also, high levels of vascular endothelial growth factor (VEGF) were found in mice with ALI, supporting the importance of increased vascular permeability in malaria respiratory failure.

Another study which utilized DBA/2 mice infected with *P. berghei* K173, showed that proteins and inflammatory cells mainly CD4+ and CD8+ lymphocytes, neutrophils and monocytes accumulate in the lungs of infected mice (Hee *et al.,* 2011). These results were confirmed by another study which also measured the cytokines and chemokines associated with ARDS, showing an increased expression in the lungs of TNF-α, IL-10, IFN γ, CXCL10 and CXCL11, as well as monocyte and neutrophil chemo-attractant chemokines (CCL2, KC) (Van den Steen *et al*., 2010).

IRBC were also observed in the lung vessels, but at a lower extent compared with the massive sequestration in the brain. Lungs of infected mice had an increased water content, demonstrating the development of oedema (Van den Steen *et al*., 2010; Hee *et al.,* 2011), and that could be related to the decreased expression of epithelial sodium channel, ENaC, due to hypoxia, resulting from malaria associated anaemia, or to TNF-α mediated down regulation (Hee *et al.,* 2011).

In a rodent model malaria associated ARDS, dexamethasone inhibited the infiltration of macrophages and CD8 T cells into the lungs, suggesting that adjunct therapy with anti-inflammatory drugs could also be useful in the clinical setting (Van den Steen *et al*., 2010). Most studies with *P. falciparum* are based on *in vitro* models or *post mortem* observations. It was shown i*n vitro* that *P. falciparum* merozoite proteins could increase endothelial permeability, while *P. falciparum* IRBC did not show the same properties suggesting that the effects of the parasite on the pulmonary endothelium are probably mediated by the activity of Src-family kinases (Gillrie *et al.,* 2007). Morphologic changes were noted in the proteins of the tight junctions and adherent junction in association with increased endothelial permeability and development of pulmonary oedema (Gillrie *et al.,* 2007).

A post mortem study performed in children with cerebral malaria demonstrated that sequestration of *P. falciparum* -IRBC occurs in the lungs, even if to a lesser extent than in the brain, skin or intestine (Seydel *et al.,* 2006). Different pathological presentations of ARDS were observed among the different species of human malaria: the greatest severity and frequency of cases were due to *P. falciparum* and could be partially attributed to the sequestration and rosetting of infected RBC in the pulmonary microcirculation (Saleri *et al.,* 2006). Heavy parasitaemia and WBC agglutinates were associated to ARDS in *P. vivax* malaria (Sharma and Khanduri, 2009). Another study suggested that the pathogenesis of lung injury by *P. falciparum*

include cytokine-induced damage or direct effects of sequestration of parasitized erythrocytes, while ARDS caused by *P. vivax* may be due principally to dysregulation of cytokines production (Price *et al*., 2007). Elevated parasitaemia in ARDS in *P. knowlesi* infection suggests parasite-specific effects that increase pulmonary capillary permeability (Daneshvar *et al* 2009), but hypoxemia and metabolic acidosis may also contribute (William *et al.*, 2011).

According to the World Health Organization (2015a) liver dysfunction is an uncommon occurrence in malaria, while jaundice is not unusual. The incidence of jaundice and liver dysfunction in *P. falciparum* malaria varied from 5.3% to 62% and from 2.5% to 21%, respectively, in different reports (Anand *et al*., 1992; Murthy *et al.*, 1998; Mazumder *et al.*, 2002), while malarial hepatitis was rare in *P. vivax* infection (Sung and Park, 2010). Case-fatality rate in malaria-related hepatic failure is elevated, up to 40% (Murthy *et al.*, 1998; Das *et al.*, 2007; Sung and Park, 2010) when high parasite density is associated with jaundice and liver dysfunction. Liver is involved in malaria at two stages: during the preerythrocytic cycle and the erythrocytic phase.

The first step is linked to the binding of the merozoite circumsporozoite protein CSP-A and TRAP protein to the hepatocytes via the heparan sulphate glycosylaminoglycans GAG and promotes minimal liver damage. In the erythrocytic phase, jaundice is a common remark and it is directly caused by the infection (malarial hepatitis, intravascular hemolysis of parasitized RBC, septicemic hepatitis), or by indirect causes (microangiopathic hemolysis associated with DIC, G6PD-related hemolysis, antimalarial drug induced-hemolysis) or completely unrelated (coexisting acute viral hepatitis, underlying chronic hepatitis) (Anand and Puri, 2005; Gonzalo *et al.*, 2013). Intravascular hemolysis of parasitized and non parasitized RBC causes an increase of

unconjugated bilirubinemia with mild to moderate jaundice (Kochar *et al.,* 2003a); conjugated hyperbilirubinemia indicates hepatocyte dysfunction.

The pathogenesis of hepatic dysfunction is not completely known; reduction in portal venous flow as a consequence of microocclusion of portal venous branches by parasitized erythrocytes, intrahepatic cholestasis due to reticuloendothelial blockage and hepatic microvilli dysfunction, suppression of bilirubin excretion due to effect of parasitemia or endotoxemia or metabolic acidosis, apoptosis and oxidative stress are all mechanisms involved in hepatic damage (Anand and Puri, 2005; Bhalla *et al.,* 2006). Histopathological reports of congestion of hepatocytes, swollen hepatocytes, centrizonal necrosis, Kupffer cell hyperplasia, deposition of brown malarial pigment, portal infiltration with lymphocytes, steatosis, parasitized RBC, cholestasis, spotty and submassive necrosis (Kochar *et al.,* 2003a; Kochar *et al.,* 2003b; Anand and Puri, 2005; Bhalla *et al.,* 2006) are evidences of inflammatory as well as direct plasmodial effects in the damage to hepatocytes.

Kidneys in malaria are involved in two different manners: acute and chronic diseases. Acute renal failure (ARF) is one of the most challenging diseases in tropical countries and malaria plays an important epidemiological role, the mortality due to malaria ARF is high (Mishra *et al.,* 2008). A study performed in Yemen showed that malaria was the first cause of death in patient with ARF (Rohani *et al.,* 2011). Malaria acute renal failure (MARF) is more common in non-immune adults and in older children (Das, 2008). MARF is mostly associated to *P. falciparum* infection and is more frequent in low transmission areas (Ehrich and Eke, 2007; Das, 2008; Kanodia *et al.,* 2011; Basu *et al.,* 2011). Nevertheless MARF could be caused also by *P. vivax* (Kaur *et al.,* 2007; Kanodia *et al.,* 2011). *P. malariae* causes chronic and progressive

glomerulopathy, known as quartan malaria nephropathy (QMN); only few cases of MARF caused by *P. malariae* has been published (Neri *et al.,* 2008)

Quartan malaria nephropathy (QMN) is frequently seen in African children and it is clinically associated with oedema and hypertension where urine analysis shows often proteinuria and microhematuria (Barsoun, 1998; Ehrich and Eke, 2007). Pathogenesis of QMN is linked to subendothelial deposits of immune complexes containing IgG, IgM, C3 and in the 25-33% of cases also malaria antigens (Barsoun, 1998; Ehrich and Eke, 2007). It is thought to be the consequence of the activation of TH2 type T lymphocytes. The pathogenesis may also include genetic and acquired factors, such as autoimmunity, co-infection with Epstein-Barr virus and malnutrition (Barsoun, 1998). Immune complexes deposition provokes glomerular damage, resulting in proliferative glomerulonephritis; the pathology, initially focal, becomes diffuse and progressive, reaching sclerosis.

Different histopatological patterns revealed by renal biopsy induced some authors to conclude that the association between *P. malariae* infection and renal involvement was only coincidental (Ehrich and Eke, 2007). *P. falciparum* acute renal failure is more common in adults; its pathogenesis is complex and it includes mechanical and immunologic factors, volume depletion, hypoxia, hyperparasitemia and other factors (Barsoun, 1998; Ehrich and Eke, 2007; Das, 2008). High parasite density was associated with acute renal failure (Ehrich and Eke, 2007; Ali *et al.,* 2008). Parasitized erythrocyte sequestration was found in kidneys of adults who died from severe *P. falciparum* malaria (Nguansangiam *et al.,* 2007); sequestration of parasitized erythrocytes is lower in the kidney than in the brain (Nguansangiam *et al.,* 2007; Das, 2008). A study performed in Mali, showed that rosetting is present in all severe malaria clinical forms, including acute renal failure (Doumbo *et al.,* 2009).

Hemolysis, causing endothelial activation and hemodynamic alteration, can lead to acute tubular necrosis and acute interstitial nephritis (Barsoun, 1998). Hypoperfusion, due to the loss of liquids and to the absence of volume restoration, leads to renal ischemia (Das, 2008). TNFα, reactive oxygen species and inducible nitric oxide also play an important role in determining the haemodynamic alteration (Barsoun, 1998). Mononuclear cell infiltration was reported in glomerular and in peritubular capillaries of adults with ARF, while immune complexes were not reported (Nguansangiam *et al.,* 2007). It was hypothesized that the release of malaria antigens activates monocyte cells, to release proinflammatory cytokines and activate TH1 cell mediated response, causing acute interstitial nephritis (Barsoun, 1998).

The role of cytokines such us INFγ, IL-1α, IL-6, GM-CSF was studied in murine malaria infection and an association of nephritis with up-regulation of pro-inflammatory and dysregulation of anti-inflammatory cytokines was found (Sinniah *et al.,* 1999). Rarely, acute renal failure has been associated to rhabdomyolysis in *P. falciparum* and *P.vivax* infections, probably due to the sequestration of parasitized red cells in the skeletal capillaries and consequent vessels occlusion (Mishra *et al.,* 2010).

In the pathogenesis of ARF related to rhabdomyolysis, the myoglobin nephrotoxic effect play the principal role; hypovolemia, hypotension, fever, acidosis and the use of non-steroidal anti-inflammatory drugs may worsen the renal function (Reynaud *et al.,* 2005; Siqueira *et al.,* 2010). Black water fever (BWF), a rare but severe complication of severe malaria, is characterized by fever, intravascular haemolysis with haemoglobinuria, dark urines and often acute renal failure (Rogier *et al.,* 2003; Oumar *et al.,* 2007; Khan, 2009). Haemoglobin released during massive haemolysis causes renal impairment. Drugs as quinine, halophantrine and mefloquine, and G6PD deficiency, have been suggested to be the trigger of BWF.

Malaria during pregnancy is associated to high morbidity and mortality both for the mother and the child (Uneke, 2008a; Aribodor *et al.,* 2009; Bardaji *et al.,* 2011; Adam *et al.,* 2011). Mother could develop severe malaria and severe anemia, and is much exposed to obstetric adverse events (Uneke, 2008a; Agan *et al.,* 2010; Adam *et al.,* 2011; Biswajit *et al.,* 2011). Placental malaria has been associated with elevated risk of miscarriage, preterm delivery, intrauterine growth retardation, low birth weight, fetal anemia, congenital malaria and perinatal mortality (Aribodor *et al.,* 2009; Falade *et al.,* 2010; Bardaji *et al.,* 2011; Tobón-Castaño *et al.,* 2011).

Placental malaria is quite common during *P. falciparum* infection; less common in *P. vivax* malaria; *P. falciparum* and *P. vivax* placental mixed-infection can occur (Carvalho *et al.,* 2011). *P. vivax* placental malaria may lead to the same adverse events as *P. falciparum* infection even if with milder consequences (Nosten *et al.,* 1999; Carvalho *et al.,* 2011; Tobón-Castaño *et al.,* 2011). In high endemic areas, while adults are less susceptible, pregnant women are commonly susceptible to malaria infection because pregnancy causes a transient depression of cell mediated immunity.

Age under 25 years and primiparity are both risk factors for developing placental malaria (Uneke, 2008a; Falade *et al.,* 2010); moreover the risk increases in the first and second trimester of pregnancy. A meta-analysis carried out in four sub-Saharan African countries demonstrated a reduction of placental malaria in multi-gravidae women with blood group 0; however, significance were demonstrated in only two of these studies (Adegnika *et al.,* 2011). Alterations of materno-fetal blood exchange are the basis of placental malaria. During infection, parasitized red cells both from *P. falciparum* and *P. vivax* are sequestered within the placenta and they accumulate in intervillous spaces; trophozoite and schizont forms also accumulate in the placenta

(Rogerson *et al*., 2007). The presence of IRBC activates mononuclear cells which release chemokines to recruit additional phagocytic cells in the intervillous spaces.

Elevated TNF-α and IL-10 levels were also described and were associated with poor pregnancy outcomes (Rogerson *et al*., 2007). IRBC, leukocyte infiltration, fibrin and hemozoin depositions contribute to increase the thickness of the trophoblast basement membrane and to alter the intervillous and perivillous spaces, causing reduction of oxygen and nutrient transport to the fetus (Rogerson *et al*., 2007; Uneke, 2007). *P. falciparum* parasitized erythrocytes adhere to chondroitin sulphate A expressed in placenta; thus, placenta selects strains of *P. falciparum* with a CSA-binding phenotype.

Primigravidae who have not been exposed to *P. falciparum* CSA-binding phenotype are still susceptible to placental infection, while multigravidae have developed antibodies during the first successful pregnancies (Uneke, 2008a). *P. vivax* parasitized erythrocytes can also cytoadherence to the placenta but their mechanism is not clarified, yet (Carvalho e*t al.*, 2010). Molecular studies demonstrate that IRBC binding to chondroitin sulphate A (CSA), expressed on the apical membrane of the placental syncytiotrophoblast epithelium, is mediated by VAR2CSA antigen, a variant of the PfEMP1 family proteins.

There are multiple genes encoding for different VAR2CSA antigens; a study performed in Cameroon shows that the parasites infecting pregnant women living in high transmission areas have an increased copy number of *var2csa* genes compared to non-pregnant women. The multiplicity of var2csa-type genes confers to *P. falciparum* parasites a greater capacity for antigenic variation and evasion of immune response (Sander *et al.*, 2011). A study describes a new flow cytometry-based adhesion assay that use apical epithelial plasma membrane vesicles

and IRBC isolated from patients (Boeuf *et al.,* 2011). Data from this study showed that the vesicles prepared from various placental regions could adhere to IRBC in different percentage but with the same adhesion intensity; moreover, parasite molecules, other than VAR2CSA can also mediate placental adhesion, suggesting that a different molecular pathway can occur. These findings were confirmed by another study that showed that transcripts from *var* genes, different from *var2csa*, were found in 67% of placental isolates, revealing the importance of other adhesion molecules during pregnancy (Rovira-Vallbona *et al.,* 2011).

Complement activation may play an important pathogenetic role during placental malaria. C5a, a factor derived from the complement cascade, is increased in peripheral blood and placental blood of pregnant women with malaria compared with pregnant women without malaria infection (Conroy *et al.,* 2009a). Factors derived from the activation of the complement cascade are likely to have a role in inflammation during placental malaria, in particular they could stimulate the release of pro-inflammatory cytokines and chemokines by monocytes and neutrophils (Conroy *et al.,* 2009a; Conroy *et al.,* 2011). Moreover, C5a could play an important role in dysregulation of angiogenesis since it seems to stimulate monocyte production of the anti-angiogenic factor sFlt-1, a soluble variant of VEGFR-1. The sFlt-1 binds to and sequesters placental growth factor and VEGF, leading to vascular and placental alteration Conroy *et al.,* 2009a; Conroy *et al.,* 2011). Another study in Tanzanian women showed that the FLT1 genotype was associated with pregnancy loss, low birth weight, placental inflammatory gene expression and high Flt1 levels (Muehlenbachs *et al.,* 2008).

The pathogenicity of severe malaria infection is complex and it is regulated by both parasite and host factors. Cytoadherence of IRBC to the vascular endothelium and rosetting are unique features of malaria parasites which are likely to contribute substantially to the vascular damage

and the consequent excessive inflammatory/immune response of the host (Conroy *et al.*, 2009b). This can occur in many different organs, a feature that can partly explain the complexity of the clinical manifestations occurring in severe malaria. In this context, adequate clinical management of malaria patients requires first an accurate diagnosis, then appropriate antimalarial treatment, associated with adjunct supportive therapies which need to be adapted to the different clinical presentation of the disease.

2.21 Malaria and the Immune System

The immune response to infection with malaria parasite involves interplay between different cell types and cytokines. Cytokines serve as the regulators of immune response and they are the key determinants of malaria outcome (Conroy *et al.*, 2009b), they may be potential targets for therapeutic interventions. The contributions of cytokines in malaria pathogenesis include the upregulation or downregulation of postagladins, upregulation of endothelial receptor expression and redistribution on the endothelial surface, physical disturbances yo the host as seen with fever and headache, upregulation of nitric oxide production, which may cause local damage during sequestration, suppression of erythrocyte production in the bone marrow.

2.18 The Immune System

The human immune system helps to protect the body from possible infectious agents. It is known to separate the self from non-self; it is a system which is composed of two major subdivisions, the innate or non-specific immune system and the adaptive or specific immune system (Perlmann and Troye-Blomberg, 2002, Mayer *et al.*, 2011). The innate immune system is our first line of defense against invading organisms while the adaptive immune system acts as a second line of defense and also affords protection against re-exposure to the same pathogen.

2.18.1 Innate Immune System

This consists of barriers that prevent the penetration and spread of infectious agents. The innate immunity is non-specific and could be biochemical or physical. An example of its biochemical

action is seen in a situation in which Lysozyme and complements are produced to destroy any foreign body. On the other hand, protection could also be conferred via the physical barriers such as the skin, mucosa linings and phagocytes (Hamilton and Abmli, 2001). The innate mechanism of inhibiting parasite growth in the human host mostly in endemic areas is probably the reason for the cases of low parasitaemia seen in some cases of acute malaria infection (Onyenekwe *et al.,* 2004, Perlmann and Troye-Blomberg 2002).This form of immunity is present from birth and it consist on numerous types of non-specific factors that operate during infection or the development of a disease (Duah *et al.,* 2010).

The major functions of the human innate immune system include: recruiting immune cells to site of infection through the production of chemical factors, including specialized chemical radiators, called cytokines, activation of the complement cascade to identify bacteria, activate cells and to promote clearance of dead cells or antibody complexes, the identification and removal of foreign substances present in organs, tissues, the blood and lymph, by specialized white blood cells, activation of the adaptive immune system through a process known as antigen presentation, acting as a physical and chemical barrier to infectious agents. Some examples of non-specific immunity include: fever, inflammation, hydrochloric acid in stomach, mucus, boil, among others (Brooks *et al.,* 2010).

2.18.2 Adaptive Immune System

This consists of lymphocytes and immunoglobulins which help to fight infections. The lymphocytes consist of T cells and B cells that regulate immune response and impart cellular and humoral immunity to the organism. In the adaptive immune system, the B cells develop into plasma cells that secrete antibodies, while the T cells develop into effector cells that kill infected cells as well as activate macrophages and B cells. Due to the activities of the adaptive immune

system, it is further grouped into two which is the humoral and cell-mediated immunity (Brooks *et al.,* 2010).

Humoral immunity involves substances found in the humors or blood fluid; it is the aspect of kimmunity that is mediated by secreted antibodies produced in the B cells. B cells (with co-stimulation) transforms into plasma cells which secrete antibodies. The co-stimulation of the B cells can come from another antigen presenting cell, like a dendritic cell. This entire process is aided by CD4+ T-helper 2 cell, which provide co-stimulation. Secreted antibodies bind to antigens on the surface of invading microbes (such as viruses and bacteria) which flags them for destruction. Humoral immunity also involves Th2 activation and cytokine production, germinal center formation and isotope switching, affinity maturation and memory cell generation, it also refers to the effector functions of antibody which include: pathogen and toxin neutralization, classical component activation, and opsonia promotion of phagocytosis and pathogen elimination. There are five types of antibody produced in man which include: IgG, IgA, IgM, IgE, IgD (Brooks *et al.*, 2010).

Cell-mediated immunity could be specific or non-specific and is directed primarily at microbes that survive in phagocytes and microbes that affect non-phagocytic cells. It is most effective in removing virus-infected cells; it also participates in defending against fungi, protozoan, cancer and intracellular bacteria.Cell-mediated immunity is an immune response that involves the activation of microphages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. Cellular immunity protects the body by: activating antigen-specific cytotoxic T-lymphocytes that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as foreign antigen on their surface, such as viruses-infected cells, cells with intracellular bacteria, and cancer cells

displaying tumor antigens; activating microphages and natural killer cells , enabling them to destroy pathogens; and stimulating cells to secrete a variety of cytokines or biomarkers that influence the function of other cells involved in adaptive immune responses (Brooks *et al.,* 2010).

2.19 Malaria and Immune System

Humans are constantly being exposed to infectious agents on a daily basis and yet, in most cases, we are able to resist its effect. It is the immune system that enables us to resist malaria infections. During infection by malaria, the immune system responds, in the process malaria specific antibodies (Immunoglobulin) are formed by B lymphocytes (Mibel *et al.,* 2008; Brooks *et al.,* 2010). B cells display immunoglobulin molecules, these immunoglobulin serve as receptors for a specific antigen (Brooks *et al.,* 2010).

It has been noted that humans with no previous experience of malaria almost invariably become ill on their first exposure to the parasite. There is a development of febrile illness which may lead to death (Kakkilaya, 2011c). At the completion of the schizony within the RBCs, newly developed merozoites are released with the lyses of infected erythrocytes along with numerous known and unknown substances such as red cell membrane products, hemozoin pigments and glycosylphosphatidylinositol (GPI). The presence of these hemozoin the DNA of *Plasmodium* is recorgnized by the TLR9 which activates microphages and endothelial cells (by the host immune system) to secrete cytokines and inflammatory mediators. NF-kappaB-activated pro-inflammatory cytokines induces COX-2. The induced COX-2 upregulates prostagladins which leads to a change in the set point of the thermoregulatory center; thus bringing about fever.

Immunity to malaria develops relatively slowly but is often said to wane quickly if immune adults leave endemic areas; this suggest that the continued exposure to malaria antigen is required for the persistence and generation of memory cells and effector cells (Langhorne *et al.*, 2008). In addition to the roles played by antibodies, cytokines such as interleukin-12, interferon γ and Tumor necrosis factor (TNF-α) has a role to play in the boosting of the immune system (Rovira.-Vallbona *et al.*, 2012).

2.20 Immunoglobulin G (IgG) and Malaria

Studies have shown that immunity against the blood stage of *Plasmodium* parasite is associated with the ability to produce or acquire the anti-parasite antibody of the cytophilic subclasses which include the IgG1 and IgG3 (Roussilhon *et al.*, 2007; Nebie *et al.*, 2008; Iriemenam *et al.*, 2009b; Olesen *et al.*, 2010). This type of protection has not been observed for non-cytophilic classes such as the IgM (Luty *et al.*, 1994). The immunoglobulins employ different mechanisms to fight off malaria infections. These mechanisms include blockade of the RBCs by merozoites, antibody mediated killing initiated by cytophilic antibodies, and the binding of antibody to parasite-induced-molecules on the RBCs which invariably leads to the increased clearance of infected RBCs (Blackman, *et al.*, 1990; Bouharoun-Tayoun *et al.*, 1995). Malaria infection gives rise to a strongly elevated blood concentration of non-malaria-specific immunoglobulin.

An estimated 30 million women living in malaria endemic areas of Africa become pregnant each year; out of which virtually all are at risk of being infected with malaria. This assertion is supported by studies that had reported its prevalence of malaria in pregnancy ranging from 14%-72% in endemic areas (Omalu *et al.*, 2011, Agomo *et al.*, 2009, Uneke, 2008; Uneke *et al.*, 2008, Bassey *et al.*, 2007, Okwa 2003). Malarial infection during pregnancy is a major public health problem in tropical and subtropical regions throughout the world, which includes Africa south of the Sahara where 90% of the global malaria burden occurs (Roll Back Malaria, 2010). In most

endemic areas of the world, including Nigeria, pregnant women are the main adult risk group for malaria. The burden of malaria infection during pregnancy is caused chiefly by *Plasmodium falciparum*, the most common malaria species in Africa (Roll Back Malaria, 2010, Bassey *et al.*, 2007).

Pregnant women are particularly vulnerable to malaria because pregnancy reduces immunity to malaria; increases susceptibility to malaria infection, the risk of illness, severe anaemia, acute pulmonary edema, renal failure, puerperal sepsis, postpartum haemorrhage, and increases the risk of death. Chronic anemia, due to malaria may also affect a child's growth and intellectual development (Omalu *et al.*, 2011; Agomo *et al.*, 2009; Uneke *et al.*, 2008a; Bassey *et al.*, 2007; Okwa 2003).

Studies in Malaria in endemic areas in Nigeria showed an increase in parasitic concentration, fever, inflammation, and anemia among primigravidae women (Omolade, 2003). An epidemiological study conducted in several countries in Africa, revealed an interesting pattern of the infection, higher rates were observed during the first few weeks of pregnancy, which peaked during the second trimester, the rates declined in the last trimester and after pregnancy (Bassey *et al.*, 2007, Brabin, 1983). Another study carried out in the Gambia showed consistent decline in malaria antibody IgG as the pregnancy progressed. Malaria during pregnancy in most cases result to congenital malaria infections; it is associated with poor birth outcomes which include low birth weight and possible death of new born (Abrams *et al.*, 2003). Some studies have recorded that women could remain asymptomatic with malaria parasitemia (Onyenekwe *et al.*, 2004).

A new born of a mother from an endemic area acquires immunity from the mother through the trans-placental transfer of Immunoglobulin G from the mother. The transfer is made through the syncytiotrophoblasts of the placenta (Palmeira *et al.,* 2011). Placental transfer of maternal IgG antibodies to the fetus is an important mechanism that provides protection to the infant, while the humoral response is ineffective. The crossing is mediated by FcRn which is expressed on syncytiotrophoblast cells. The transfer of IgG is seen to be dependent on maternal level of total IgG; gestational age, in which it has been reported that the greatest amount of antibodies is transferred in the third trimester; placental integrity; and the IgG subclasses of which Preferential transport occurs for IgG1, followed by IgG4, IgG3 and IgG2, IgG1 is seen to occur more frequently (Bradley, 1974, Englund, 2007, Palmeira *et al.*, 2011).

However studies carried out has different views on the relationship between maternal antibodies and protection from malaria parasites; a study which was carried out in Ghana showed that children with high levels of maternal antibodies to *Plasmodium falciparum* were found to be more susceptible to the infection (Riley *et al.,* 2000). Another study in Tanzania also inferred that IgG antibodies to circumsporozoite proteins (CSP) and merozoit surface protein-1 in infants were not protective against parasitemia (Kitua *et al.,* 1999); a study in Nigeria indicated that there is a relationship between levels of maternal derived antibodies in relation to that of the neonate (Onyenekwe *et al,* 2004); while another study in Nigeria shows no level of association between levels of maternal derived antibodies which reacts with the parasitic schizont extract and the age of the first clinical episode in malaria (Achidi *et al.,* 1996). Furthermore, a study in Gambia and Kenya showed that there is a relationship between the antibodies in the maternal sera and the time of the first clinical episode (Duah *et al.,* 2010, Branch *et al.,* 1998).

Malaria is a disease that is seen to be more common in children under the age of 5years (WHO, 2011) and pregnant women; it has also been recorded to contribute to about 25% of infant mortality in Nigeria (Mwaniki *et al*, 2010, Osungbade and Oladunjoye, 2011). This is due to the fact that the maternal immunity that was passed to the child at birth has waned and the child is prone to malaria infection. At this point such a child is expected to develop his or her own immunity. In a study, Duah *et al.,* (2010) stated that the maternally transferred antibody isotopes of the IgG subclasses which were detected at birth were almost totally depleted by 4months of age. Thus at this stage the child will start experiencing malaria attack if infected and will in time develop the necessary immunity to malaria.

There is a reported association between severe disease and increased production of IgG and other immune agents or biomarkers such as the cytokines and chemokines which leads to the enhanced amounts of proinflammatory cytokine responses, including TNF-α, interleukin 1β (IL-1β), IL-6, IL-10 and IFN-γ, as well as the chemokine CCL3 (MIP-1α) and CCL4 (MIP-1β) (Rovira-Vallbona *et al.,* 2012, Langhorne *et al.,* 2008). Studies also indicate that there may be specific cytokine profiles associated with different clinical syndromes; severe malaria anemia has been associated with relatively low IL-10 responses, whereas respiratory distress is associated with abnormally large amounts of IL-10. A low amount of CCL5 (RANTES) has been associated with severe disease and mortality (Abrams *et al.*, 2003, Bostrom *et al.,* 2012, John *et al.,* 2006). Biomarkers are therefore vital in the understanding of the human immune response to malaria.

2.21 Biomarkers and Malaria

Biomarkers are indicators of molecular, biochemical or cellular changes or alterations which can be measured in biological samples such as blood or its components, saliva and urine which serve as a pointer to disease pathology, biology, or patient's response to treatment. Biomarkers are seen associated with various infections including malaria. Studies have shown that the detection

of certain biomarkers such as HRP2 and PLDH for *Plasmodium*-mediated infection at early stages of malaria can be crucial for disease management strategies and treatment regime (Prakash *et al.,* 2006).

Biomarkers are a vital aspect of public health importance which stretches from diagnose of a disease, to the identification of individuals at risk for certain infections; Also, the knowledge of biomarkers can be used to stratify patients depending on disease severity, provide prognosis of a disease, and provide guidance in the treatment and management of a disease (Lucchi *et al.,* 2011). Therefore it is necessary to understand the various malaria related biomarkers for better disease management, control, eradication and sustenance.

2.22 Malaria Related Biomarkers

Frank and Hargreaves (2003) classified biomarkers into three types: Type 0, Type 1, and Type 2. Type 0 biomarkers are measures of the natural history of disease which are correlated with clinical outcomes; Type 1 biomarkers usually determine the biological effect of a therapeutic intervention; and Type 2 biomarkers are the equivalent of "surrogacy" markers where a surrogate point has been defined as a biomarker intended to substitute for a clinical end point, with the latter being a characteristic. Malaria related biomarkers are useful for disease management (Lucchi *et al.,* 2011; Jain *et al.,* 2014) and in Nigeria where there is a goal to move from the control phase to elimination phase it would be helpful to plan or strategize for the possible dire future outcome because of the loss of immunity.

2.22.1 Histidine Rich Protein II

Histidine Rich Protein (HRP) is a polypeptide produced by *P. falciparum*; the parasite synthesizes different soluble HRPs during its asexual erythrocytic development which include: HRP I, II, and III (Howard *et al.,* 1986). HRP I protein expresses knob-like protrusion (Knob+ strain) on the cell surface and is therefore known as knob-associated protein (KAHRP-I), it is believed to help in the cytoadherence of infected erythrocytes to the venular endothelium and is

therefore possibly contributes to the high parasitaemia associated with *P. falciparum* infection (Pologe *et al.,* 1987).

2.22.2 *Plasmodium* Lactate Dehydrogenase

Plasmodium parasite takes up a large quantity of the blood glucose for its activity, to utilize the glucose produced it requires an enzyme known as the *Plasmodium* lactate dehydrogenase (PLDH) which is produced by the parasite for its metabolic activity (Jain *et al.,* 2014; Mathema and Na-Bangchang, 2015). *Plasmodium* parasite when in the red blood cell (RBC) feeds on the human blood glucose to produce Adenosine Tri Phosphate (ATP); during this metabolic process depending on the *Plasmodium* species about 60–70% of the glucose is converted to lactic acid and excreted (Roth Jr., 1990)

2.22.3 Aldolase

Aldolase is another major enzyme of the *Plasmodium* parasite which plays a major role in the parasite's glycolyticpathway, it is found in the cytoplasm of the parasite as an active and soluble form and it can also be found to be associated with the membrane fraction as an insoluble form (Jain *et al.,* 2014). Aldolase is a catalyst which helps in the cleavage of fructose-1, 6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetonephosphate in the glycolytic pathway of theparasite (Srivastava *et al.,* 1990; Mathema and Na-Bangchang, 2015). This enzyme has been used for diagnostic purposes, although reports have suggested that aldolase-based rapid diagnostic tests provide relatively lower sensitivity when compared to HRP based tests, aldolase still remains a vital target for disease diagnosis and drug development.

2.22.4 Hemozoin

When the parasite digests the blood it breaks down the hemoglobin to heme and globulin. The heme component is toxic to the parasite; therefore, the parasite initiates hemozoin formation by polymerization of the heme to form hemozoin with the help of the HRP2 (Mathema and Na-

Bangchang, 2015). Since the formation of hemozoin is a survival tactic of the parasite to survive the heme toxicity, the hemozoin has been a major target for malaria drug study.

Hemozoin has been identified to be a strong modulator of leukocyte recruitment which include neutrophil and monocyte, it was also identified to induce the expression of chemokine, macrophage-inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2, and monocyte chemo-attractant protein-1/CCL2; chemokine receptors, CCR1, CCR2, CCR5, CXCR2, and CXCR4; cytokines, IL-1βand IL-6; and myeloid-related proteins (Jaramillo *et al.*, 2004)

2.22.5 Serological Biomarkers

Several serological biomarkers associated with malaria have been identified over the years. These markers can help to serve as prognostic markers which can identify febrile malaria cases that could be mild or exacerbate into complicated cases (Jain *et al.*, 2014); they can also be used to track response to treatment given.

Studies has shown that Proinflammatory (Th1) cytokines such as tumor necrosis factor (TNF-α) play certain roles in malaria pathogenesis, by increasing the surface expression of adhesion molecules on cerebral endothelial cells which enhance parasite attachment leading to cerebral malaria (Hommel 1990; Grau and De Kossodo, 1994; Angulo and Fresno, 2002). Excessive production of TNF−α in patients with cerebral malaria might be as a result of genetic variation in the host's ability to produce these cytokines (McGuire, 1994, 1999).

For Severe Malaria cases, it has been identified that chemokine interferon inducible protein (CXCL10 and CXCL4), soluble tumor necrosis factor receptor (sTNF-R), interleukin 8(IL-8), interleukin 1 receptor antagonist (IL-1ra), IL-1β, TNF-α , soluble Fas ligand (sFas), angiopoietin

I (ANG I), ANG II, intracellular adhesion molecule I (ICAM-I), vascular cell adhesion molecule I (VCAM-I) could be related to the severity of cerebral malaria cases (Mshana *et al.,* 1991; Friedland *et al.,* 1993; Armah *et al.,* 2007; John *et al.,* 2008; Conroy *et al.,* 2009b; Lovegrove *et al.,* 2009; Olszewski and Llinas, 2011; Jain *et al.,* 2014). Also, the decreased levels of RANTES and vascular endothelial growth factor (VEGF) has been related to severe malaria cases (John *et al.,* 2006).

Also, severe malaria patients have been reported with increased plasma levels of TNF−α, IFN−γ, interleukin IL-1β, and IL-6 and reduced production of IL-4 and transforming growth factor (TGF−β), (De Kossodo and Grau, 1993; Peyron, *et al.,* 1994); however a reversed relationship of IFN−γ and TNF−α are involved in conferring protection against *Plasmodium* infection (Taylor-Robinson *et al*., 1993; Sedegah *et al*., 1994; Winkler *et al*., 1999). The anti-inflammatory cytokines IL-10 has being associated both the progression of disease to severe malaria or with protection against progression to severe malaria in humans (Peyron *et al*., 1994; Ho *et al.,* 1998; Kurtis *et al.,* 1999; Nussenblatt *et al.,* 2001).

A report by Othoro *et al.,* (1999) has shown that the balance between IL-10 and TNF−α concentrations determines the severity of anemia in infected children (Othoro *et al.,* 1999). All of these activities implies that the outcome of *P. falciparum* infection may depend on a balance between appropriate concentrations of these immune regulatory factors.

Prakash *et al.,* (2006) was able to propose a model for cytokine activity which suggests that there is an increase in the levels of IL-5, IL-1β, IL-10, and IL-2 with an increase in infection; also, the levels of IL-12, IL-5, and IL-6 can be used to discriminate between severe malaria and mild malaria; furthermore the levels of IL-1β, IL-12, and IFN−γ are indicators that helps to

discriminate cases of cerebral malaria and severe malaria where high level of IL-1β is associated with cerebral malaria while high levels of IL-12 and IFN–γ levels are associated with severe malaria (Table 2).

In patients with cerebral malaria, there could be cases of immunologic dysfunction which results from lesions that gives rise to an alteration in the blood-brain barrieras a result of the impairment of the urokinase-type Plasminogen Activator receptor (UPAR/CD87); the uPAR (CD87) degrades the extracellular matrix through proteolysis, it is usually elevated when the immune system is activated and there is inflammation as seen with malaria (Fauser *et al.,* 2000; Hawkes *et al*., 2013).

Table 2: Malaria Related Markers and their Functions

Biomarker	Location	Function	Reference
HRP2	Secreted in serum ofinfected patient		Brooks *et al.,* 2010
PLDH	Inside infected RBCs	Parasite Metabolism to release ATP	Brooks *et al.,* 2010
Aldolase	Inside infected RBCs		Brooks *et al.,* 2010
Hemozoin	Inside digestive vacuole of parasite	A product of the digestion of blood by the parasite. Prevents parasites' toxicity be heme	Brooks *et al.,* 2010
Glutamate Dehydrogenase	Inside infected RBCs	GDH are ubiquitous enzymes responsible for the reversibleoxidative deamination of L-glutamate for production of α-ketoglutarate and ammonia utilizing NAD (H) or NADP (H) as co-factors	Storm *et al.,* 2011
TNF	Produced by the T-Lymphocytes	Pro-inflammatory Th1 Cytokine, Plays role in protection against *Plasmodium* infection	Taylor-Robinson*et al*., 1993; Sedegah*et al*., 1994; Winkler *et al*., 1999
TGF-β	Produced by the T-Lymphocytes	Anti-inflammatory	

		CytokinePrognostic biomarker that can identify patients at risk for Cerebral Malaria associated Mortality	
IL-1β	Produced by the T-Lymphocytes	Prognostic biomarker that can identify patients at risk for Cerebral Malaria associated Mortality	Prakash *et al.*, 2006
IL-2	Produced by the T-Lymphocytes	Indication of Mild Malaria	Prakash *et al.*, 2006
IL-4/ CXCL-4	Produced by the T	Prognostic biomarker that can identify patients at risk for Cerebral Malaria associated Mortality	Prakash *et al.*, 2006
IL-5	Produced by the T cells	Indication of Mild Malaria	Prakash *et al.*, 2006
IL-6	Produced by the T cells	Indication of Mild Malaria	Prakash *et al.*, 2006
IL-8	Produced by the T cells	Indication of Cerebral Malaria	John*et al.*, 2008
CXCL-9	Produced by the T cells	Predicts Cerebral Malaria	Lucchi *et al.*, 2011
IL-10/ CXCL-10	Produced by the T	Anti inflammarory Cytokine. Prognostic biomarker that can identify patients at risk for Cerebral Malaria associated	Prakash *et al.*, 2006

		Mortality and also as a diagnostic marker with increased level	
IL-12	Produced by the T-Lymphocytes	Indication of Mild Malaria; It down regulates in infection with severe malaria	Prakash *et al.*, 2006
IFN-γ	Produced by the T-Lymphocytes	Indication of Mild Malaria	Prakash *et al.*, 2006
ICAM	Produced by the T-Lymphocytes		
VCAM	Produced by the T-Lymphocytes		
ANG I	Produced by the T-Lymphocytes	Diagnostic biomarker that can identify CM patients when level is decreased; act as endothelial regulator	Lucchi*et al.*, 2011
ANG II	Produced by the T-Lymphocytes	Diagnostic biomarker that can identify CM patients when level is increased act as endothelial regulator	Luchi*et al.*, 2011

2.23 Prevention and Control of Malaria

The control of malaria is rather dynamic and not static. This process will help the infection of the malaria parasite into the host environment. However, the following process could be useful for the prevention of malaria and they include; the use of insecticide treated net to prevent mosquitoes from feeding on humans, prevent or reduce breeding of mosquitoes, larvae destruction, and elimination of malaria parasite in human host and to destroy adult mosquitoes (WHO, 2013a,b). These processes could be achieved if there are established control programme with trained professional available to run the program and also the availability of health facilities in rural and urban areas to help in the control and prevention of malaria to a good level, as good treatment and case management can be obtained from good health care centers. These in turn will create a suitable condition of early detection of malaria parasite and treatment of the infection in individuals. It is also important to note that for an effective malaria control program to be achieved; multiple strategies are needed to control.

Non usage of mosquito nets either the non-treated or the insecticide treated nets has been attributed to the increase rate of malaria especially (CDC, 2010), therefore the usage of treated net is advice since this method serves as a form of vector prevention when the mosquitoes come in contact with the nets and die. Recently the Nigerian government has promoted the use of insecticide treated nets which good and less costly method of malaria prevention. This method will also prevent asymptomatic carriers of *Plasmodium* parasite from being bitten by mosquitoes (CDC, 2010).

The intermittent administration is a full treatment courses of an antimalarial medicine to children especially under 5 during the malaria season in areas of highly seasonal transmission; this is another way by which malaria transmission can be prevented (WHO,

2013a) . This Seasonal malaria chemoprevention (SMC) is to prevent malarial illness by maintaining therapeutic antimalarial drug concentrations in the blood; where most malarial disease and deaths occur during the rainy season within a shortest period of time usually 3-4 months (WHO, 2013a). This method therefore helps in the administration of effective antimalarial treatment usually the use of sulfadoxine-pyrimethamine and amodiaquine at monthly intervals in areas with highly seasonal malaria transmission (WHO, 2013a; WHO, 2015a). This has given a positive result of about 75% protective against uncomplicated and severe malaria in children under 5 years of age.

Also, current available drugs that can reduce transmission are those of the 8- aminoauinoine drug family such as primaquine (Domingo *et al.,* 2013). This antimalarial drug does not cure malaria illness, but is known to kill the gametocyte stage of the malaria parasite in human in other to reduce the rate of being transmitted to the vector (WHO, 2013a). Although, high intake of this drug is known to cause haemolysis in patient who are with glucose 6 sulphate dehydrogenase (G6PD) deficient; therefore in 2013, the WHO amended their guideline, reducing the dosage of this drug from 0.75 mg/kg to 0.25 mg/kg to reduce the risk of haemolysis.

Other means of malaria control in areas of high endemicity is the use of insecticide. This useful tool for the elimination of mosquito is likely to be implemented by all individuals; as this strategy will help kill the mosquito's vectors that carry these parasites and thus reduce or eradicate the malaria parasite (WHO, 2016). Some mosquitoes have become resistant to some insecticides, therefore alternative insecticides should be made available when this control measure is being used. Also, use of Laviparous fishes such as *Nothobranchius guentheri* and *Cynolebius whitec* especially in ecological zones where pond, marches, swamp are available

will help reduce the development of the vector parasite (WHO 2013b). These fishes eat up the anopheles larva and thus prevent the development of the larvae to adult mosquitoes that are vectors of malaria parasite. There are other biological agents that have been successfully used to control mosquitoes includes fungi. These agents produce toxins harmless to humans and animals but potent to mosquitoes (WHO 2013b).

The human immune system is well adapted to protect the body from non- self via the innate and adaptive immune system. The ability of the malaria parasite to adapt to the parasitic way of life in their host depends on the ability to invade and modify the host cell. Thus identifying protein regions that are directly involved in mechanism used by the parasite for infecting cells as well in developing into new invasive forms is essential for survival in the host. In the host environment, most parasitic association develops sophisticated adaptation to exploit their host and organs. Parasitism therefore could be described as the regular way of life on earth and most cases (Brooks *et al.*, 2010); it is inevitable as the parasite typically produce long- lasting chronic infection in order to maximize their opportunities for successful transmission in the intermediate host.

For *Plasmodium falciparum,* which is the most common and also attributed to a cause a very disease state achieve their mechanism either through their ability to alter the surface coat, short incubation period, high reproductory rate in which close to 40,000 merozoites are released into the blood stream also the unique nature to sequester in deep tissues which are only seen in them (WHO, 2010b). But for *Plasmodium malariae,* Information about the prepatent period, or the period of time between the infection of the parasite and demonstration of that parasite within the body, associated malaria is limited, but some studies have suggested that there is great variation, often the length of time depending on the strain

of *P. malariae* parasite; usually, the prepatent period ranges from 16 to 59 days (Collins and Jeffery, 2007)**.**

Plasmodium malariae are known to causes chronic infection that in some cases can last a lifetime. The parasite has several differences between it and the other *Plasmodium* parasites, one being that maximum parasite counts are usually low compared to those in patients infected with *P. falciparum* (Collins and Jeffery, 2007)**.** The reason for this can be accounted for by the lower number of merozoites produced per erythrocytic cycle, the longer 72-hour developmental cycle compared to the 48-hour cycle of *P. falciparum*, the preference for development in older erythrocytes and the resulting earlier development of immunity by the human host (Collins and Jeffery, 2007*).* Another defining feature of *P. malariae* is that the fever manifestations of the parasite are more moderate relative to those of *P. falciparum* and *P. vivax* and fevers show quartan periodicity (Bruce *et al*, 2006).

Despite all the strategies put together by the immune system of the host to avoid a disease state by the parasite, the parasite still finds a way to evade them to or strike a balance in which there is a co-existence between the two different organisms without inflicting any harm. The human *Plasmodium* parasites are potential human malaria parasite that could result into complicated and uncomplicated malaria infection in man due to the different mechanism that makes up their survival in the host (Brooks *et al.,* 2010). Therefore, beyond reducing immunopathology, new implementation techniques such as vaccine would potentially establish robust, long-lasting protection without parasite persistence.

2.24 Management of Malaria

In respect to the fact that pregnant women are prone to malaria infection due to their compromised immune system and that they have a tendency to transfer malaria to a new born. It is paramount that they should be properly treated in the advent of malaria during pregnancy to avert the possibility of infecting the newborn. Malaria can be managed during pregnancy with Intermittent Preventive Treatment (IPTp) with sulphadoxin-primethamine (Oyibo *et al.,* 2009; Menendez *et al.,* 2011). Other forms of management are included supervised oral quinine plus clindamycine, Atemisin combination therapy (ACT). Also supervised Artesunate-Atovaquone-proquanil (APP) can be used in cases of recrudescence.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area and participant recruitment

This study was carried out in Anambra and Lagos state. The selection of study area was random however, with the aim of including rural areas as well as urban areas both in Lagos state and Anambra state; therefore, the researcher included rural and urban dwellings in the study area. Participants in Anambra state were recruited from Iyenu mission Hospital, Ogidi (Rural), Nnamdi Azikiwe University Teaching Hospital (Urban) and Aguleri community (Fig 5). Participants in Lagos was recruited from Agura Health Care Centre, Ikorodu (Rural) and Regina Mundi Catholic Hospital, Mushin (Urban) (Fig 6). The study included a total of 544 participants a total of 350 were consented, from health facilities of which 172 participants were from Lagos state and 178 from Anambra state. In Aguleri community, 194 individuals were consented. Consented participants included: NAUTH 43 participants, Iyienu Mission Hospital 135 participants, Regina Mundi Catholic Hospital 41 participants and Agura Health Centre 131 participants.

Sample collection in Anambra state started on the 11th of July 2016 and ended in Early August of the same year. Sample collection in Lagos stated started in 2nd June 2016 and stopped in Early August of the same year. Asymptomatic carriers and healthy individuals were recruited from Aguleri Community in Anambra State on the 20th of January 2017. In the hospitals and health care centers, participants with history of fever presenting with uncomplicated malaria that are directed to do a laboratory test for malaria parasite detection were approached and consented and patient signs the consent forms. Venous blood sample was collected and assayed for the presence of malaria parasite and immunological profile.

Fig 5: Map of some LGA in Anambra State with the red spots indicating the study area (Source: Google Map)

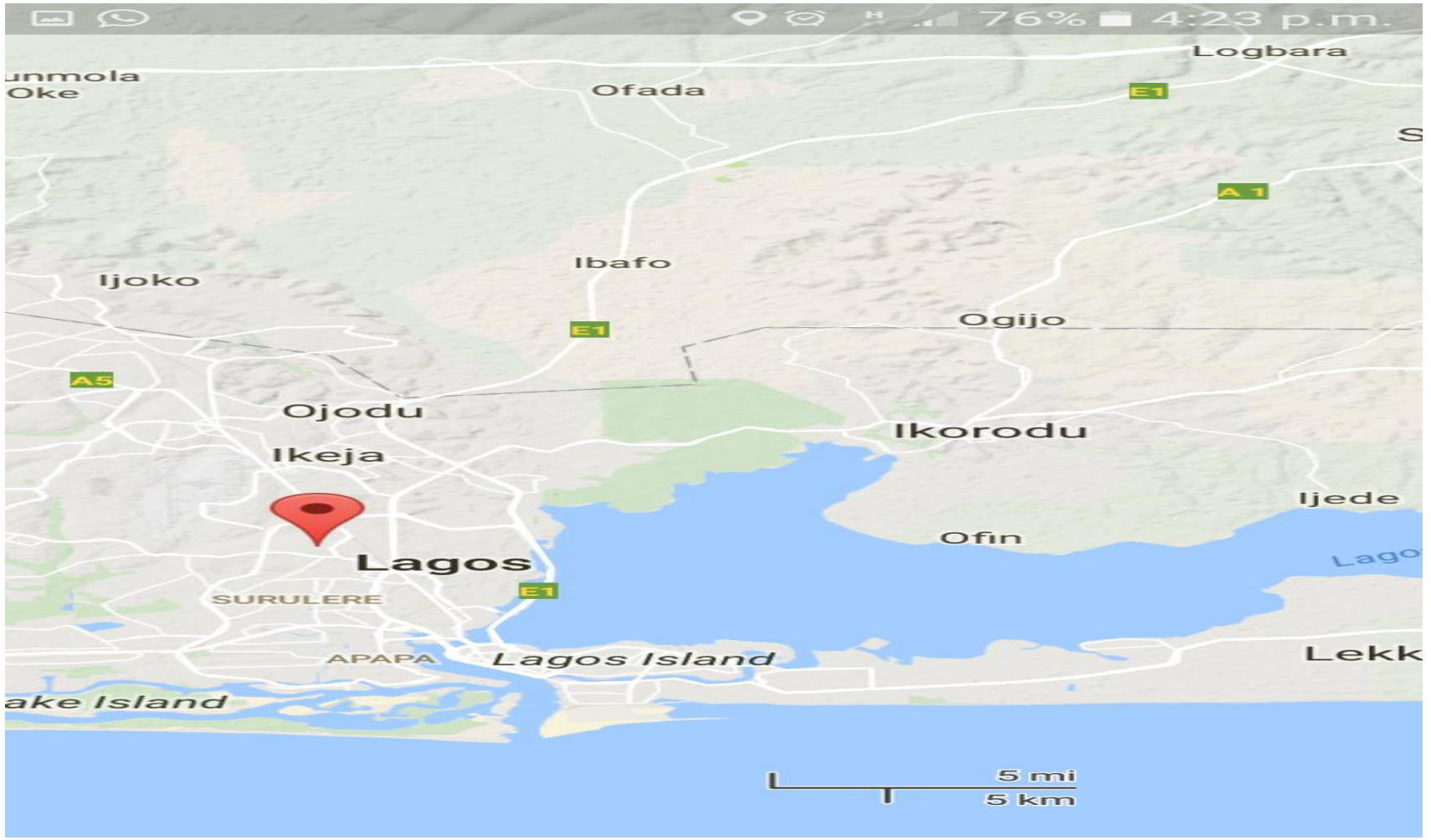

Fig 6: Map of some LGA in Lagos State with the red spots indicating the study area (Source: Google Map)

The sample required will include symptomatic positive malaria patients, asymptotic positive individuals and negative controls; this will help tell the different immune status at different heath conditions. For the asymptotic carriers and healthy individuals, samples were collected from the community after consent has been given and duly signed.

3.2 Study Design

This is a cross sectional study which included 544 participants from Anambra and Lagos state cutting across seven health centers and one community, consisting of individuals suspected with malaria and individuals from the community without clinical episodes of malaria. Consent forms were used to obtain approval from the study participants. The sampling procedure was random.

3.3 Ethical Approval

Ethical Approvals were obtained from Nnamdi Azikiwe University Teaching Hospital and the Anambra state ministry of Health (Appendix III and IV).

3.4 Inclusion Criteria

Individuals presenting with fever at selected health facilities within the last 48hours and agree to the terms in the consent forms and sign them was included in this study

Individuals from the community without clinical episode of malaria and sign the consent with agreement with the protocol was included in this study

3.5 Exclusion Criteria

An individual that do not give consent, and as such do not sign the consent forms was not included in this study.

3.6 Sample Size

Sample size for this study was calculated using the formular: by smith (2013):

$$N = \frac{(Z)^2\delta^2}{\text{Margin of Error}^2}$$

Where:

N= Number of samples or simple size

Z=Z-value (constant and it is dependent on or one tailed test)

δ =standard Deviation

Z=1.96 at 95% confidence level

δ=0.5 Confidence interval

Margin of Error=5%=0.05

$$N = \frac{(1.96)^2 \times (0.5)^2}{(0.05)^2}$$

N= 384.16

$$N \cong 384$$

Although the sample size calculated was 384, total number of participants recruited was 544 so as to have a prevalence determination of high power.

3.7 Sample Collection

Questioners were filled and venous blood collected in EDTA bottles after administration of informed consent (Appendix I). At enrolment, clinical information was taken and entered into standardized forms (Appendix II); 2ml of blood samples were collected into EDTA containers. Rapid Diagnostic test (RDT) and malaria microscopy was done on the samples collected. The RDT will help as a guide for treatment at the facility and community while microscopy is the gold standard for the study. If the malaria test is negative using RDT, the patient will be referred for care for non-malaria illness. However, the collected samples, all positives and randomly selected negatives were used for the determination of the serum

levels of immunoglobulin G, pro-inflammatory cytokine (TNF-α) and IL-8 in individuals with symptomatic and asymptomatic malaria.

3.8 Parasite Density Determination among symptomatic group

3.8.1 Blood Film Preparation and Staining

Thick and thin blood films were made on the same slide and slides were stained following the WHO standard procedure for the preparation of blood films and staining in research settings (WHO, 2015b).

3.8.2 White blood Cell (WBC) Count Determination

A full blood count was done using a heamatology analyzer from which the absolute WBC was gotten.

3.8.3 Malaria Microscopy for Symptomatic group

The Malaria microscopy was carried out following the WHO standard and there were be two blinded independent reading of the slides to minimize errors (WHO, 2015b). The patient's absolute white blood cell count was used to determine the patient's parasite load per micro-litre of blood

3.9 Parasite Density Determination among asymptomatic group

3.9.1 Blood Film Preparation and Staining

Thick and thin blood films were made on the same slide and slides were stained following the WHO standard procedure for the preparation of blood films and staining in research settings (WHO, 2015b).

3.9.2 Malaria Microscopy for Asymptomatic Group

The Malaria microscopy was carried out following the WHO standard and there were be two blinded independent reading of the slides to minimize errors (WHO, 2015b). Malaria parasite density was computed using standard WBC of 8000cells/mL; this was because of the sample collection finished late and taking the samples to the laboratory was not possible on the same day.

3.10 Discrepancy Determination Using Automated WBC Count, Assumed Value of 8,000 cells/mL and 6,000 cells/mL

The discrepancy significance between the parasite density values obtained with the automated WBC count, assumed value of 8,000 cells/mL and 6,000 cells/mL was determined by first determining the discrepancy between the parasite density values obtained with the automated WBC count against that obtained with assumed value of 8,000 cells/mL and 6,000 cells/mL using the formula:

Percentage Discrepancy= Difference X 100

Mean

The statistical significance of the discrepancies was determined using the Mann Whitney Test

3.11 Immunological Assay

The blood samples collected in the EDTA bottles was spurned at 4000 revolution per minute for ten minutes. The plasma was separated from the EDTA container into cryogenic vials and stored frozen at -70°C until required for cytokine assays. The separated plasma was used for the ELISA to determine the serum levels of Immunoglobulin G, proinflammatory Cytokine (TNF-α) and IL-8 in Individuals with Symptomatic and Asymptomatic Malaria Infection. All

the plasma samples were assayed in a single day to eliminate freeze-thaw cycles. **MABTEC ELISA** product was used for this assay.

3.11.1 Immunoglobulin G Assay

Following the manufacturer's instructions the following steps was carried out:

Day 1

A high protein binding ELISA plate was coated with MT145 antibody, diluted to 2 µg/ml in PBS at pH of 7.4, by adding 100 µl/well. This platewas Incubated overnight at 4-8°C.

Day 2

1. The pre coated plate was washed twice with PBS 200µl per well

2. Plate was blocked by adding 200µl/well of PBS with 0.05% Tween 20 containing 0.1% BSA (incubation buffer). Tkhe plate was then Incubated for 1 hour at room temperature

3. After one hour incubation, the plate was washed five times with PBS-Tween

4. The IgG standard was prepared by reconstituting contents of vial 3 in 500µl PBS to a concentration of 50µg/ml.

5. Dilutions of the stock using the standard range of 0.2-100ng/ml was prepared

6. 100µl was added per well of samples or standards diluted in incubation buffer and incubated for 2 hours at room temperature.

7. The plate was washed five times with PBS containing 0.05% Tween 20

8. 100µl of mAb MT8F19-biotin at 1 µg/ml in incubation buffer or Assay was added per well and plate was incubated for 1 hour at room temperature.

9. After the 1hour incubation plate was washed five times with PBS-Tween

10. 100µl of MT78-ALP diluted 1:1000 in incubation buffer was then added per well and incubated for 1 hour at room temperature

11. Plate was again washed five times with PBS-Tween

12. 100µl/well of p-nitrophenylphosphate (pNPP) substrate solution was then added

13. After 15mintes of incubation at room temperature 100µl of stop solution was added per well and the optical density was measured at 405ng/dl

3.11.2 Human IL-8 (CXCL8) ELISA Assay

Following the manufacturer's instructions the following steps was carried out:

Day 1:

A high protein binding ELISA plate was coated with mAb MT8H6, diluted to 2 µg/ml in PBS at pH of 7.4, by adding 100 µl/well. This plate was Incubated overnight at 4-8°C.

Day 2

1. The pre coated plate was washed twice with PBS 200µl per well

2. Plate was blocked by adding 200µl/well of PBS with 0.05% Tween 20 containing 0.1% BSA (incubation buffer). The plate was then Incubated for 1 hour at room temperature

3. After one hour incubation, the plate was washed five times with PBS containing 0.05% Tween20

4. The IL-8 standard was prepared by reconstituting contents of vial 4 in 1 ml PBS to a concentration of 0.6 µg/ml, it was left at room temperature for 15 minutes and then the constituent was mixed using a vortex.

5. Dilutions of the stock using the standard range of 4-400pg/ml was prepared

6. 100µl was added per well of samples or standards diluted in incubation buffer and incubated for 2 hours at room temperature.

7. The plate was washed five times with PBS containing 0.05% Tween 20

8. 100µl of mAb MT8F19-biotin at 1 µg/ml in incubation buffer or Assay was added per well and plate was incubated for 1 hour at room temperature.

9. After the 1hour incubation plate was washed five times with PBS containing 0.05% Tween 20

10. 100µl of Streptavidin-HRP diluted 1:1000 in incubation buffer was then added per well and incubated for 1 hour at room temperature

11. Plate was again washed five times with PBS containing 0.05% Tween 20

12. 100µl/well of TMB substrate solution was then added

13. After 15mintes of incubation at room 7temperature 100µl of stop solution was added per well and the optical density was measured at 450ng/dl

3.11.3 TNF-α ELISA Assay

Following the manufacturer's instructions the following steps was carried out:

Day 1:

A high protein binding ELISA plate was coated with mAbTNF3/4, diluted to 2 µg/ml in PBS at pH of 7.4, by adding 100 µl/well. This plate was Incubated overnight at 4-8°C.

Day 2

1. The pre coated plate was washed twice with PBS 200µl per well

2. Plate was blocked by adding 200µl/well of PBS with 0.05% Tween 20 containing 0.1% BSA (incubation buffer). The plate was then Incubated for 1 hour at room temperature

3. After one hour incubation, the plate was washed five times with PBS containing 0.05% Tween20

4. The TNF-α standard was prepared by reconstituting contents of vial 4 in 1 ml PBS to a concentration of 1µg/ml, it was left at room temperature for 15 minutes and then the constituent was mixed using a vortex.

5. Dilutions of the stock using the standard range of 29-2900pg/ml was prepared

6. 100µl was added per well of samples or standards diluted in incubation buffer and incubated for 2 hours at room temperature.

7. The plate was washed five times with PBS containing 0.05% Tween 20

8. 100µl of mAbTNF5-biotin at 1 µg/ml in incubation buffer or Assay was added per well and plate was incubated for 1 hour at room temperature.

9. After the 1hour incubation plate was washed five times with PBS containing 0.05% Tween 20

10. 100µl of Streptavidin-HRP diluted 1:1000 in incubation buffer was then added per well and incubated for 1 hour at room temperature

11. Plate was again washed five times with PBS containing 0.05% Tween 20

12. 100µl/well of TMB substrate solution was then added

13. After 15mintes of incubatikon at room temperature 100µl of stop solution was added per well and the optical density was measured at 450ng/dl

3.12 Disease pathogenesis in Individuals with symptomatic and asymptomatic malaria infection in relation to age and sex

.The disease pathogenesis in relation with the serum profile of Immunoglobulin G and pro-inflammatory cytokine (IL-8 and TNF-α) in Individuals with symptomatic and asymptomatic malaria infection in relation to age and sex was determined by using the information of symptoms on the case report forms (CRF) (Appendix II) and values obtained from 3.7, 3.8, 3.9 and 3.10.

3.13 Data Analysis

The discrepancy significance between the parasite density values obtained with the automated WBC count, assumed value of 8000 cells/mL and 6,000 cells/mL was determined using the Mann Whitney Test because the values were not nominal. The specific serum levels of Immunoglobulin G, proinflammatory cytokine TNF−α and IL-8, will be determined by

recording and grouping the specific measured concentrations of these markers in the sera analyzed into the symptomatic and asymptomatic to determine the level ranges of the different markers found in this two groups. Furthermore, the Spearman's and Pearson correlation coefficient was used to determine the relationship between the serum levels of Immunoglobulin G, TNF−α and IL-8, association of markers with symptom was done using ANOVA in Individuals with symptomatic and asymptomatic malaria infection; this will help to determine the specific exact combined concentration range at different clinical presentations. The SPSS statistical software was used in data analysis.

CHAPTER FOUR

RESULTS

Of the 350 symptomatic individuals from health facility seventy seven (77) were positive malaria positive cases. The study population for the facility includes: 88(49.4%) males and 90 (50.6%) females from health facilities in Anambra State, 54(31.4%) males and 118 (68.6%) females from health facilities in Lagos. From Aguleri community 16 of the 178 individuals in Anambra state was positive.

Samples selected from facilities both in Anambra and Lagos state for the immunology profile included 58(45.3%) male and 70 (54.7%) female with a mean temperature of 37.3±1.0 and mean age of 23.4±19.8. The symptoms which include fever, chills, body pains and headache were mostly common in participants from the facilities when compared to the community (Table 3).

It was also observed that some of the participants from the symptomatic group had taken medications before coming to the clinic; 3 (2.3%) had taken chloroquine, 8(6.3%) had taken Sulphadoxine-Pyremetamine and 34(26.8%) had taken Artemisinin Combination Therapy (ACT) from the health facilities visited. From the community only one participant had taken ACT (Table 3). The age comparism for this study was done by grouping participants into 4 age groups (Table 3). This was done to determine how the result will vary among the different age groups.

Table 3: Baseline characteristics of the study population assayed for immune markers

Character	Symptomatic (n=128)	Asymptomatic (n=46)	P
Sex			
Male	58(45.3%)	26 (56.5%)	0.23
Female	70 (54.7%)	20 (43.5%)	
Temperature (^{o}C)			
Mean±SD	37.3±1.0	36.3±0.7	<0.001
≥ 37.5	53 (41.4%)	1 (2.2%)	
<37.5	75 (58.6%)	45 (97.8%)	<0.001
Age (years)			
Mean±SD	23.4±19.8	15.9±12.3	0.017
Age group1 (years)			
1-10	47 (36.7%)	15(32.6%)	0.005
11-20	26 (20.3%)	22(47.8%)	
21-30	14 (10.0%)	3 (6.5%)	
31-40	16 (12.5%)	3 (6.5%)	
>40	25 (19.5%)	3 (6.5%)	
Age group2 (years)			0.002
1-5	21(16.4%)	4 (8.7%)	
6-15	46 (35.9%)	31(67.4%)	
16-25	13 (10.2%)	4(8.7%)	
>25	48 (37.5%)	7(15.2%)	
Age group3			
1-5	21 (16.4%)	4(8.7%)	0.23
>5	107 (83.6%)	42 (91.3%)	
Age group4			0.005
1-15	67 (52.3%)	35 (76.1%)	
>15	61 (47.7%)	11 (23.9%)	
Reported fever duration			
Mean±SD	43.3±24.9	37.7±12.8	0.557
N	113	7	
Symptoms			
Fever	113 (88.3%)	10 (21.7%)	<0.001
Chills	71 (55.5%)	9 (19.6%)	<0.001
Body pains	52 (40.6%)	4 (8.7%)	<0.001
Headache	96(75%)	10 (21.7%)	<0.001
Medications taken			
Chloroquine	3 (2.3%)	0 (0%)	0.567
Sulfadoxine-pyrimethamine	8 (6.3%)	0 (0%)	0.112
ACT	34 (26.8%)	1 (2.2%)	<0.001

4.1 Malaria prevalence in Lagos and Anambra State among symptomatic group

544 individuals were consented for this study; a total of 350 were consented, from health facilities of which 172 participants were from Lagos state of which 61 were positive for malaria and 178 from Aguleri community in Anambra state of which 16 was positive for malaria. The symptomatic malaria prevalence for the study was 35.5% in Lagos, 9% in Anambra (Fig 7). The overall study prevalence among symptomatic was 22% (Fig 7). Infected individuals were all single specie infection of *Plasmodium falciparum* alone with parasitaemia ranging from 15-451,440 with a Geometric mean parasite density (GMPD) of 8,009 in symptomatic individuals (Table 4).

4.2 Malaria prevalence in Anambra State among Asymptomatic Group

A total of 194 individuals were consented from the community, 19 (9.8%) was positive for malaria (Figure 8). A total of 105(54.1%) males, 89 (45.9%) females from community who consented to the study were screened for malaria parasite. Infected individuals were all single specie infection of *Plasmodium falciparum* alone with parasitaemia ranging from 63 – 13,084 with a GMPD of 953 in asymptomatic individuals (Table 4, Fig 9). From the community samples used for immune profiling included 26 (56.5%) male and 20 (43.5%) female with a mean temperature of 36.3±0.7 and mean age of 15.9±12.3 (Table 3)

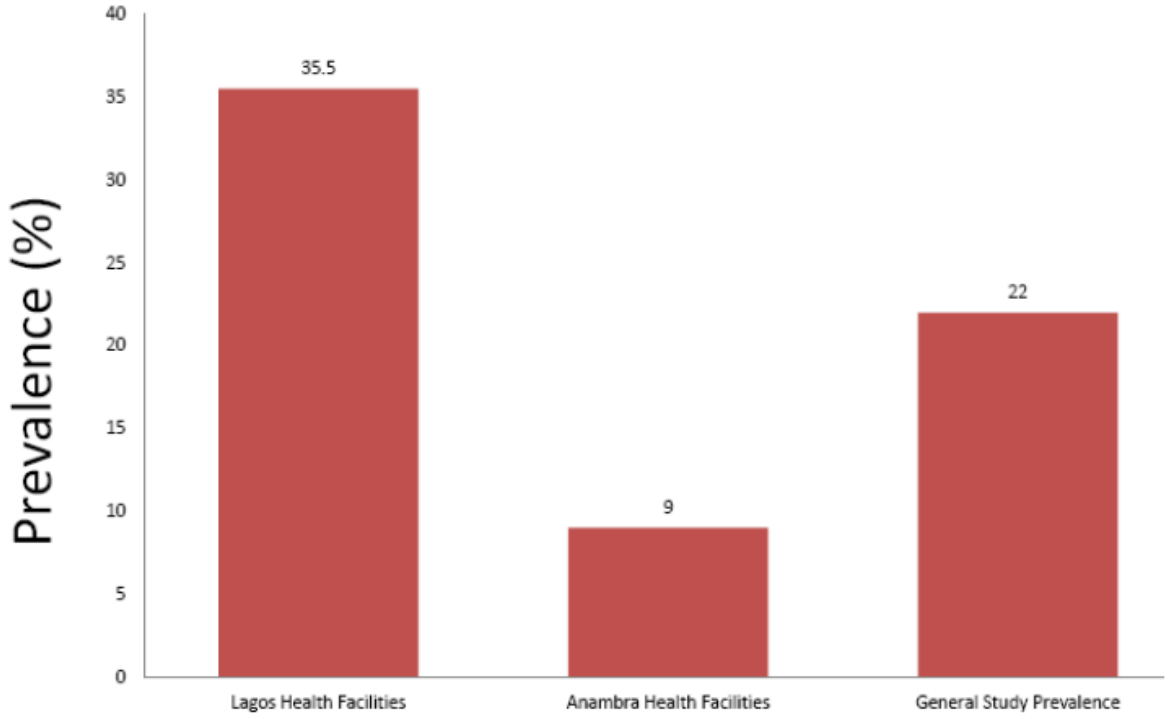

Fig 7: Symptomatic study Prevalence in Lagos and Anambra State

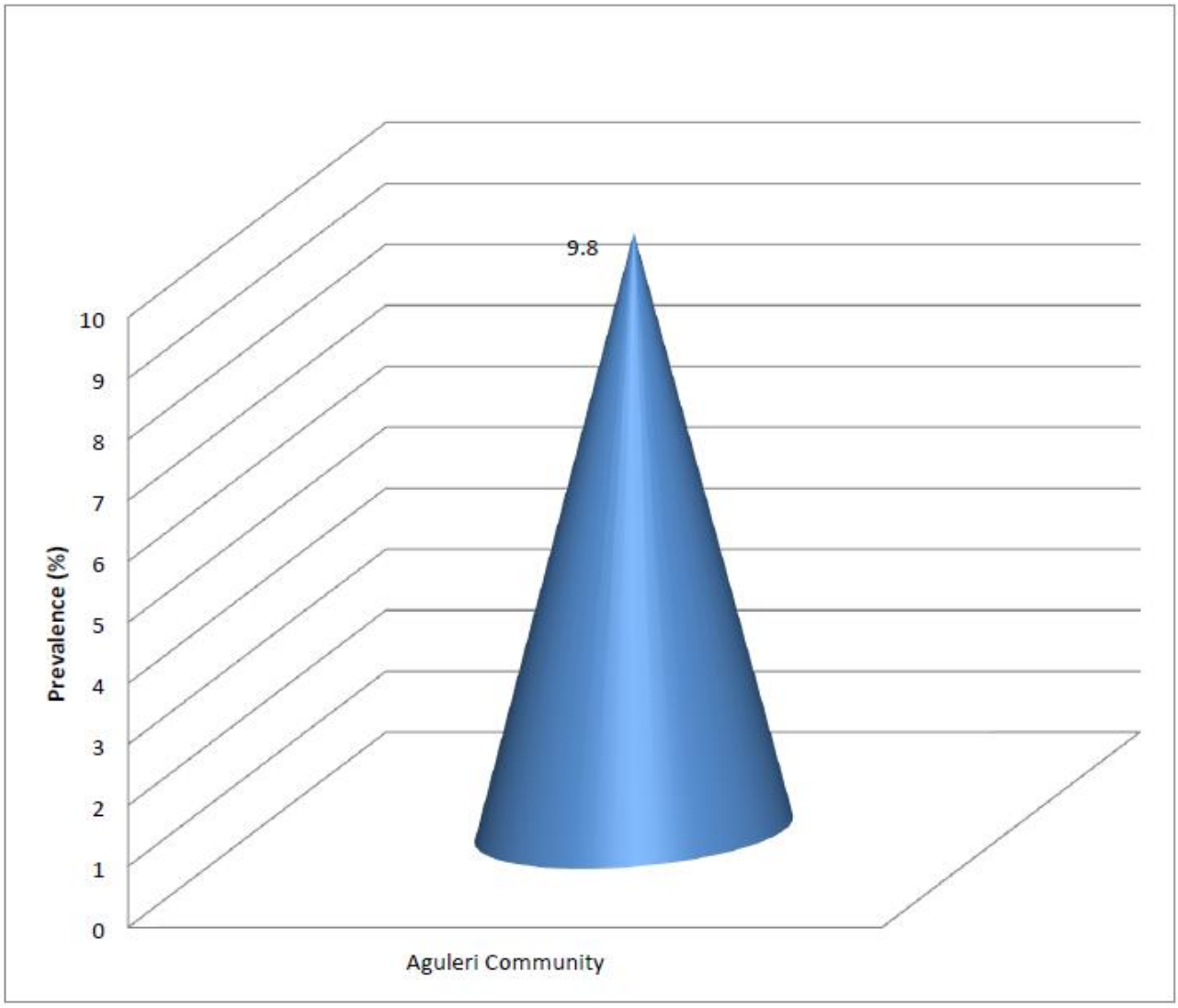

Fig 8: Asymptomatic Study Prevalence in Aguleri Community, Anambra State

Table 4: Malaria infection rate of the study population assayed for immune markers

Character	**Symptomatic**	**Asymptomatic**	
Microscopy	77 (60.2%)	19 (41.3%)	<0.001
RDT	81 (63.3%)	14 (30.4%)	<0.001
Asexual Parasite density (parasites/µL)			
Geometric mean parasite density(GMPD)	8,009	953	0.001
kRange	15-451,440	63 – 13,084	
n	77	16	
Parasite density group			
1-1,000	11 (14.3%)	8 (50.0%)	<0.001
1001-10,000	27 (35.1%)	7 (43.8%)	
k>10,000	39 (50.6%)	1 (6.2%)	
Sexual Parasite density (parasites/µL)			
GMPD	48	93	0.788
Range	48- 537	16 – 2,544	
N	7	4	
Plasmodium falciparum stages			
Trophozoites only	69 (89.6%)	16 (84.2%)	
Gametocytes only	0	3 (15.8%)	
Trophozoites and gametocytes	5 (6.5%)	0	
Troph, Gam and schizonts	1 (1.3%)	0	
Troph and schizont	2 (2.6%)	0	

P≤0.001

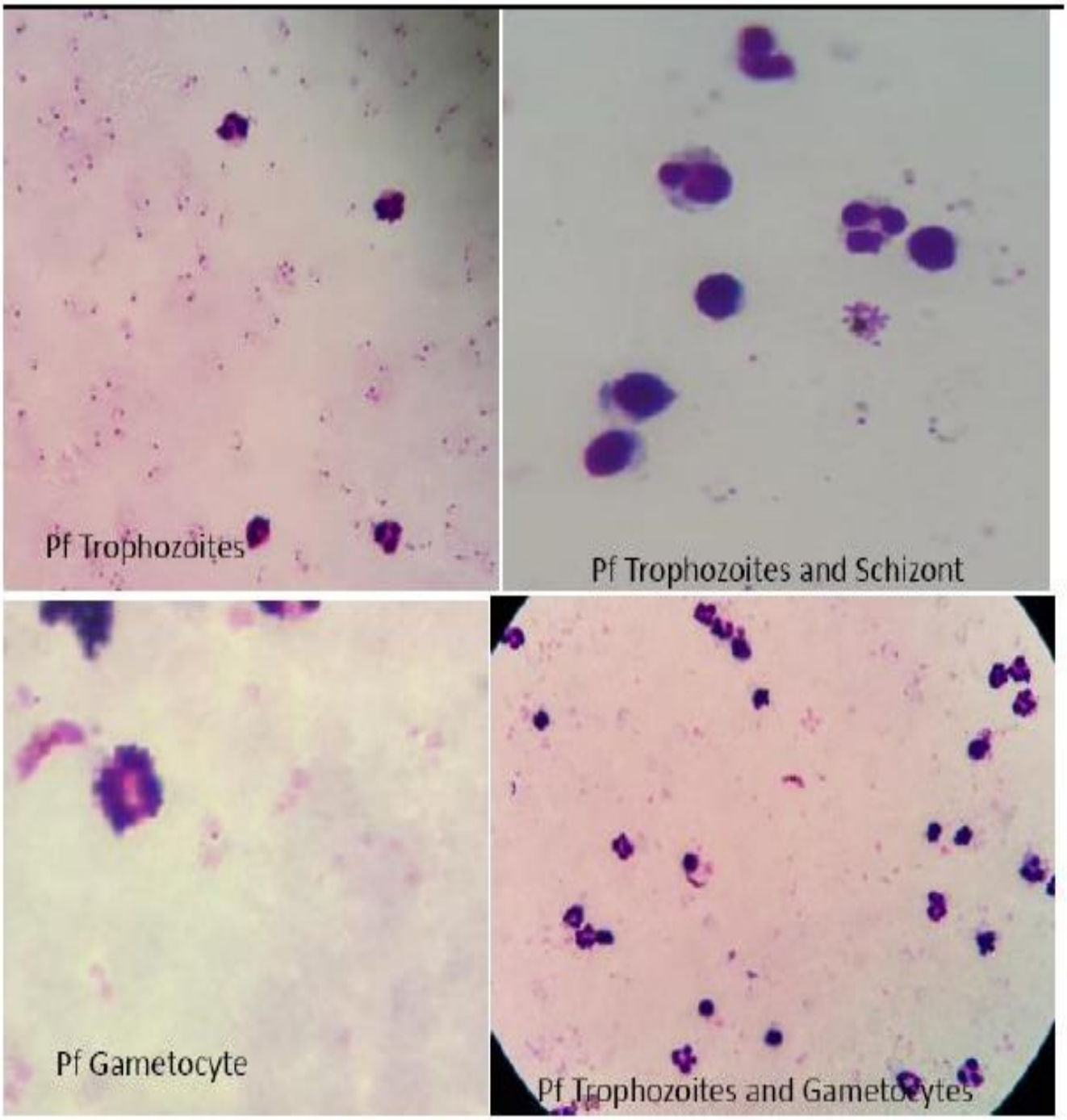

Fig 9: Image of Parasite Stages found under the microscope

4.3 Discrepancy significance between the parasite density values obtained with the automated WBC count, assumed value of 8,000 cells/mL and 6,000 cells/mL

To determine the statistical significance and discrepancy between the parasite density values obtained with the automated WBC count, assumed value of 8,000 cells/mL and 6,000 cells/mL, parasite density of 68 malaria Positive samples from Lagos state and Anambra state were calculated using actual patient's WBC which was determined using a haematology analyzer and using a fixed WBC count of 8,000 cells/mL and 6,000 cells/mL respectively. Percentage discrepancy was also calculated.

Using the two tailed Mann Whitney test, there was no statistical significance between the parasitaemia obtained with actual WBC and assumed parasitaemia with WBC count of 8,000 cells/mL ($p=0.2892$) and 6,000 cells/mL ($p=0.8858$). This implies that assumed parasitaemia calculated using a fixed WBC count of 8,000 cells/mL and 6,000 cells/mL can be used in place of parasitaemia calculated using actual patient's WBC where the actual patient's WBC is not available

The parasitaemia discrepancy between the actual parasitaemia and assumed parasitamia with WBC count of 8,000 cells/mL is 1.5%, 4.4%, 13.2%, 5.9%, 9.0% and 66.2% at 0-5%, >5-10%>10-15%, >15-20% >20-25% and>25 respectively; while parasitaemia discrepancy between the actual parasitaemia and assumed parasitamia with WBC count of 6,000 cells/mL is 5.9%, 10.3%, 11.8%, 16.2%, 5.9% and 54.4% at 0-5%, >5-10%>10-15%, >15-20% >20-25% and>25 respectively (Table 5).

Table 5: Parasite Density discrepancy with White blood cell count of 8000 Cells/mL and 6,000 cells/mL

Parasitaemia Discrepancy Grouping	N (8000 cells/mL)	N (6000 cells/mL)	% (8000 cells/mL)	% (6000 cells/mL)
0-5	1	4	1.5	5.9
>5-10	3	7	4.4	10.3
>10-15	9	8	13.2	11.8
>15-20	4	11	5.9	16.2
>20-25	6	4	8.8	5.9
>25	45	37	66.2	54.4

($P \leq 0.001$)

4.4 Immunoglobulin G levels in individuals with symptomatic and asymptotic malaria infection

The Immune Profile of IgG, IL-8 and TNF−α in individuals with symptomatic and asymptotic malaria infection was assayed and the concentration of theses markers in selected study population was determined. Immunoglobulin G was seen to range from 69-202ng/ml with a mean of 97.3 ±24.5 SD in Symptomatic individuals while asymptomatic concentration range was from 92.0-267.7ng/ml with a mean of 127.4±42.2 SD with a p-value of <0.001which is significant (Table 6, Figure 10).

4.5 Pro-inflammatory cytokine (IL-8 and TNF−α) levels in individuals with symptomatic and asymptotic malaria infection

The Immune Profile of TNF−α in individuals with symptomatic and asymptotic malaria infection was seen to range from 32.3 - 210.3pg/ml with a geometric mean of 50.6pg/ml in Symptomatic individuals while asymanptomatic ranged from 39.2 – 62.3pg/mlwith a geometric mean of 44.8pg/ml with a p-value of <0.001busing the b –Mann Whitney Test (Table 6, Figure 11). This indicates that there is significant difference in the profile of TNF−α in the symptomatic and asymptomatic patients with the asymptomatic patients having the higher concentration range.

The Immune Profile of IL-8 in individuals with symptomatic and asymptotic malaria infection ranges from 43.4 – 3,096.1pg/ml with a geometric mean of 126.9pg/ml in Symptomatic individuals while asymptomatic range was from 54.5 – 1,468.2pg/ml with ageometric mean of 111.7pg/ml and a p-value of 0.671b using the b –Mann Whitney Test (Table 6, Figure 12).

Table 6: Immune Profile of IGG, IL-8 and TNF-α in individuals with symptomatic and asymptotic malaria infection

Immune Marker	Symptomatic (n=128)	Asymptomatic (n=48)	P
IGG			
Mean ± SD	97.3 ±24.5	127.4±42.2	<0.001
Range	69.0-202.0	92.0-267.7	
TNF-α			
Geometric mean	50.6	44.8	<0.001[b]
Range	32.3 - 210.3	39.2 – 62.3	
IL-8			0.671[b]
Geometric mean	126.9	111.7	
Range	43.4 – 3,096.1	54.5 – 1,468.2	

(P≤0.001)

b –Mann Whitney Test

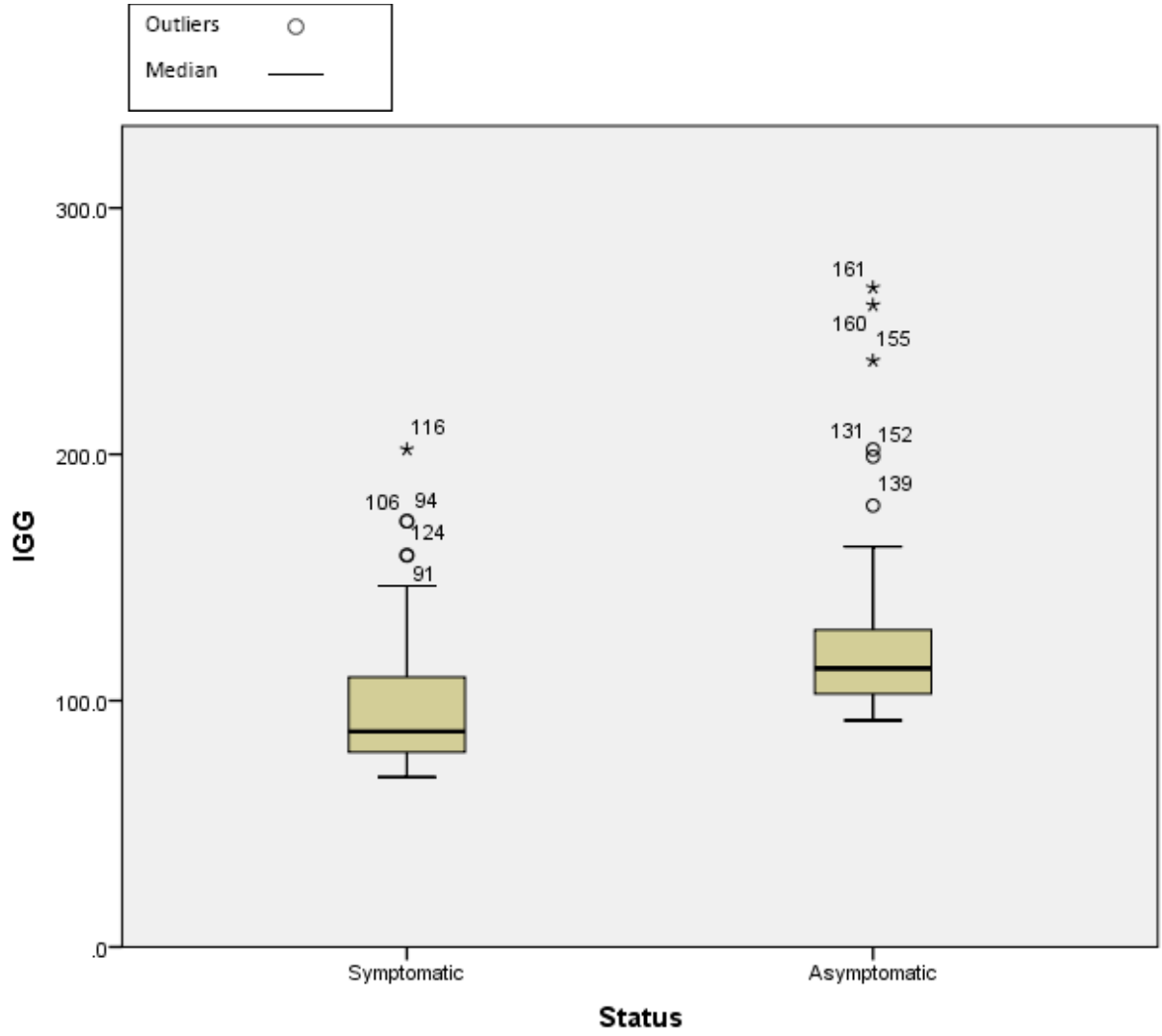

Fig 10: Logarithmic transformed distribution of IgG(ng/ml) among symptomatic and asymptomatic individuals. The boxes illustrate the total observations equivalent to the first quartile and the third quartile. The median is represented by the horizontal line. The outlier is shown as a circle point.

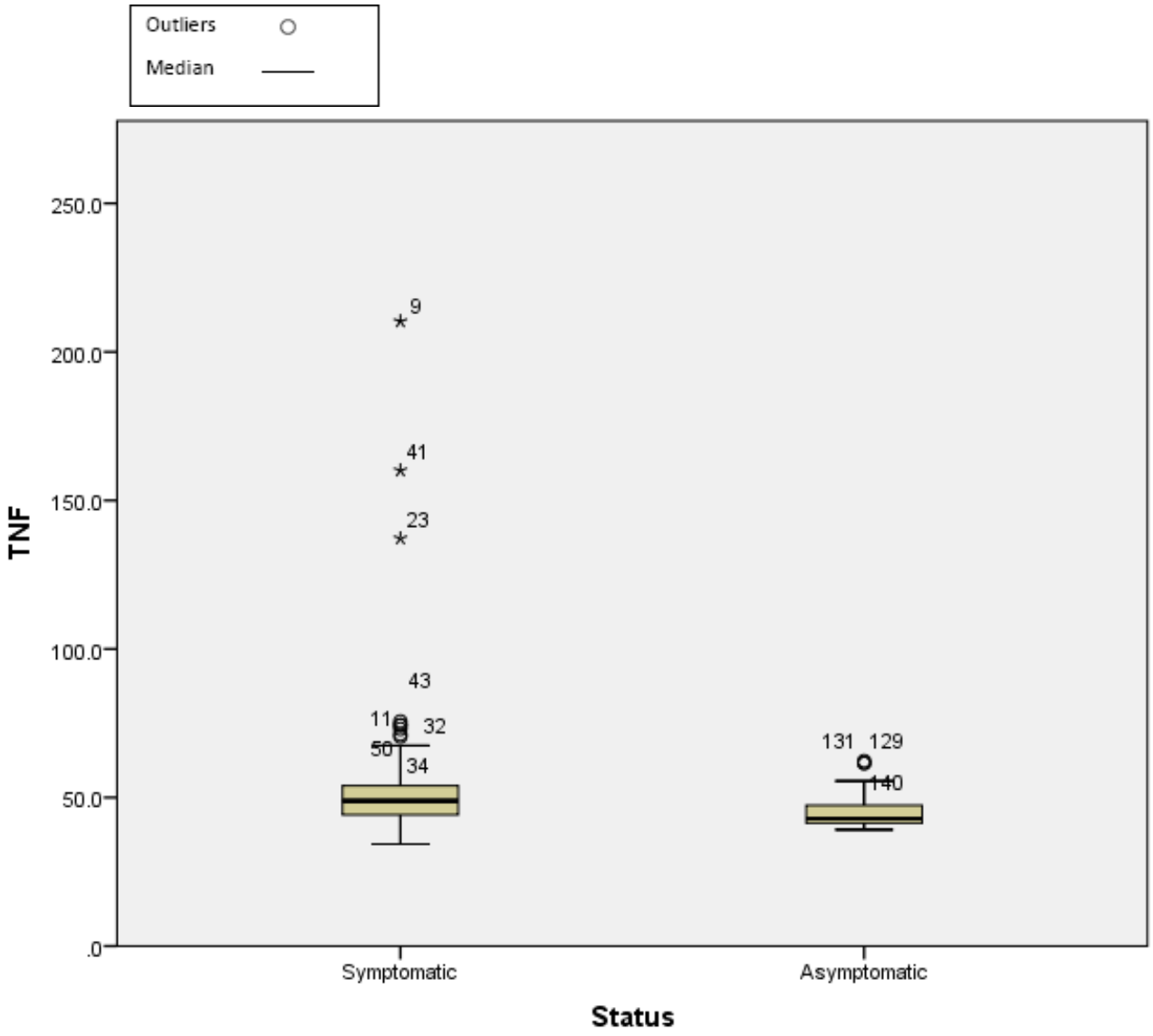

Fig 11. Logarithmic transformed concentration of TNF-α (pg/ml) among symptomatic and asymptomatic individuals.

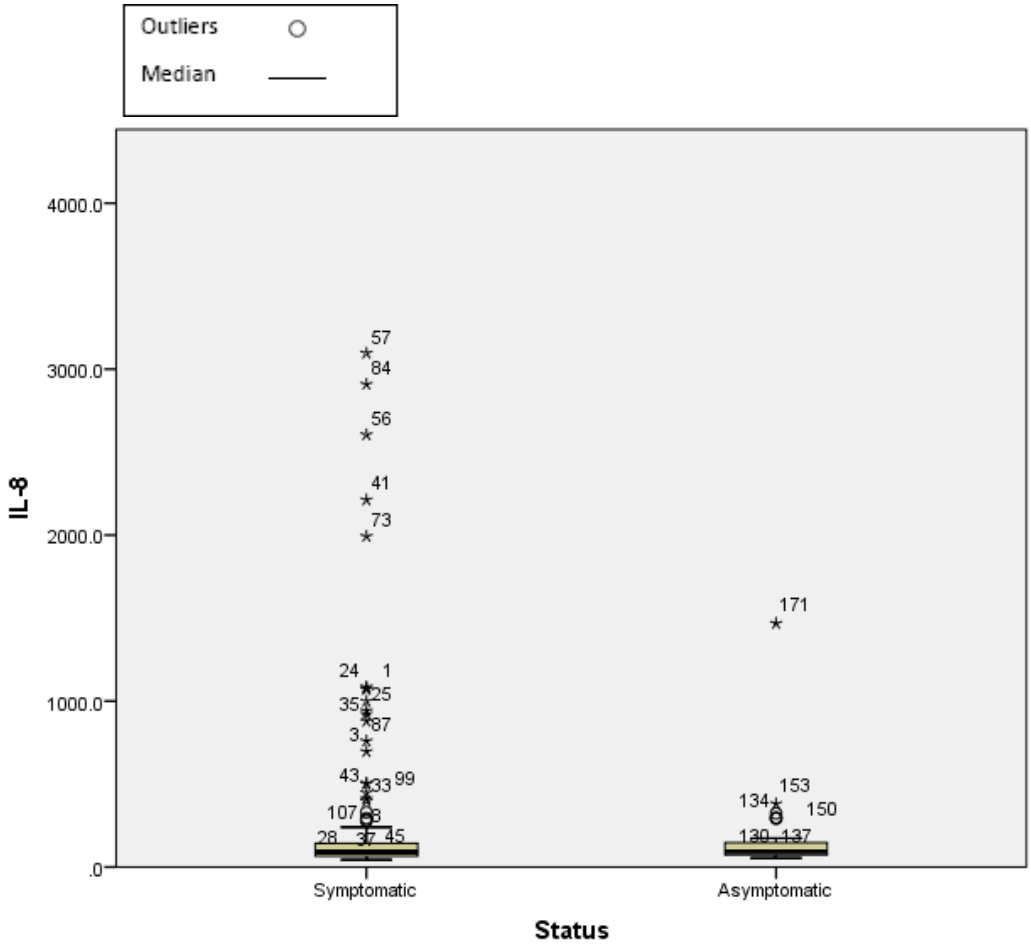

Fig 12: Logarithmic transformed concentration of IL-8(pg/ml) among symptomatic and asymptomatic individuals.

1.6 Disease pathogenesis in relation with the serum profile of Immunoglobulin G and pro-inflammatory cytokine (IL-8 and TNF–α) in Individuals with symptomatic and asymptomatic malaria infection in relation to age and sex.

4.6:1 Correlation of Immune Profile of IgG, IL-8 and TNF–α

The Correlation of Immune Profile of IgG, IL-8 and TNF–α in individuals with symptomatic and asymptotic malaria infection was assayed. ForIgG the Pearson correlation was -0.175 for symptomatic and -0.074 for asymptomatic, IL-8 Spearman's correlation was determined as 0.147 and 0.135 for symptomatic and asymptomatic respectively, while TNF–α Spearman's correlation was -0.005 and -0.332 for symptomatic and asymptomatic respectively (Table 7). Also the mean of the assayed immune markers concentrations in the study population was compared alongside the presentation of fever which is a symptom associated with malaria and the malaria mkicroscopy status of the participantse. The mean for IgG and IL-8 were significant for both parameters while those of TNF–α were shown to be significant for malaria microscopy (Table 8).

Furthermore, the parasitaemia was groupedinto 1-1000, 1001-10,000 and >10,000 which was compared with the mean concentrations of the assayed immune markers to determine if there is any relationship between the increase in parasitaemia and an increase in the concentration of these markers; the resultshowed that there was no statistical difference in the mean of the different grouped parasitaemia (Table 9, Figure 13, 14 and 15). However, the range of the concentration of the markers clearly shows that an increase in parasitaemia could also mean a possible increase in the concentration of the markers for TNF–α and IL-8.

Table 7: Correlation of Immune Profile of IGG, IL-8 and TNF-α in individuals with symptomatic and asymptotic malaria infection

Cytokines		Asexual parasite density	
		Symptomatic	Asymptomatic
IgG			
	Pearson correlation	-0.175	-0.074
	n	77	16
	P	0.127	0.786
TNF			
	Spearman's correlation	-0.005	-0.332
	n	77	16
	P	0.964	0.209
IL8			
	Spearman's correlation	0.147	0.135
	n	77	16
	P	0.202	0.617

Table 8: Comparison of means for IgG, TNF-α and IL-8 in relation to documented fever and Malaria Parasite Detection.

Cytokines		Body temperature		Microscopy	
		≥37.5	<37.5	Pos	Neg
IgG					
	Mean±SD	88.3±17.3	103.7±26.8	85.2±13.1	115.6±26.3
	n	53	75	77	51
	P	<0.001		<0.001	
TNF					
	Geometric mean	52.2	49.6	53.2	47.0
	Range	39.4-160.1	34.3 – 210.3	34.3-210.3	39.2-137.2
	N	53	75	77	51
	P	0.009		<0.001	
IL8					
	Geometric mean	185.5	97.0	158.3	90.8
	Range	52.9-3096.1	43.4-2604.8	52.9-3096.1	43.4-2908.8
	N	53	75	77	51
	P	<0.001		<0.001	

Table 9: Comparison of grouped parasitaemia with immune markers

Cytokine		Asexual Parasite density (parasites/μL)		
		1-1000	1001-10,000	>10,000
IgG				
	Mean±SD	85.4±12.1	87.6±18.3	83.5±8.4
	n	11	27	39
	P		0.465	
TNF-α				
	Geometric mean	53.8	53.6	52.7
	Range	45.7-73.1	39.4-160.1	34.3-210.3
	N	11	27	39
	P		0.509	
IL8				
	Geometric mean	119.8	160.8	169.4
	Range	68.0-2604.8	57.7-2211.9	52.9-3096.1
	N	11	27	39
	P		0.900	

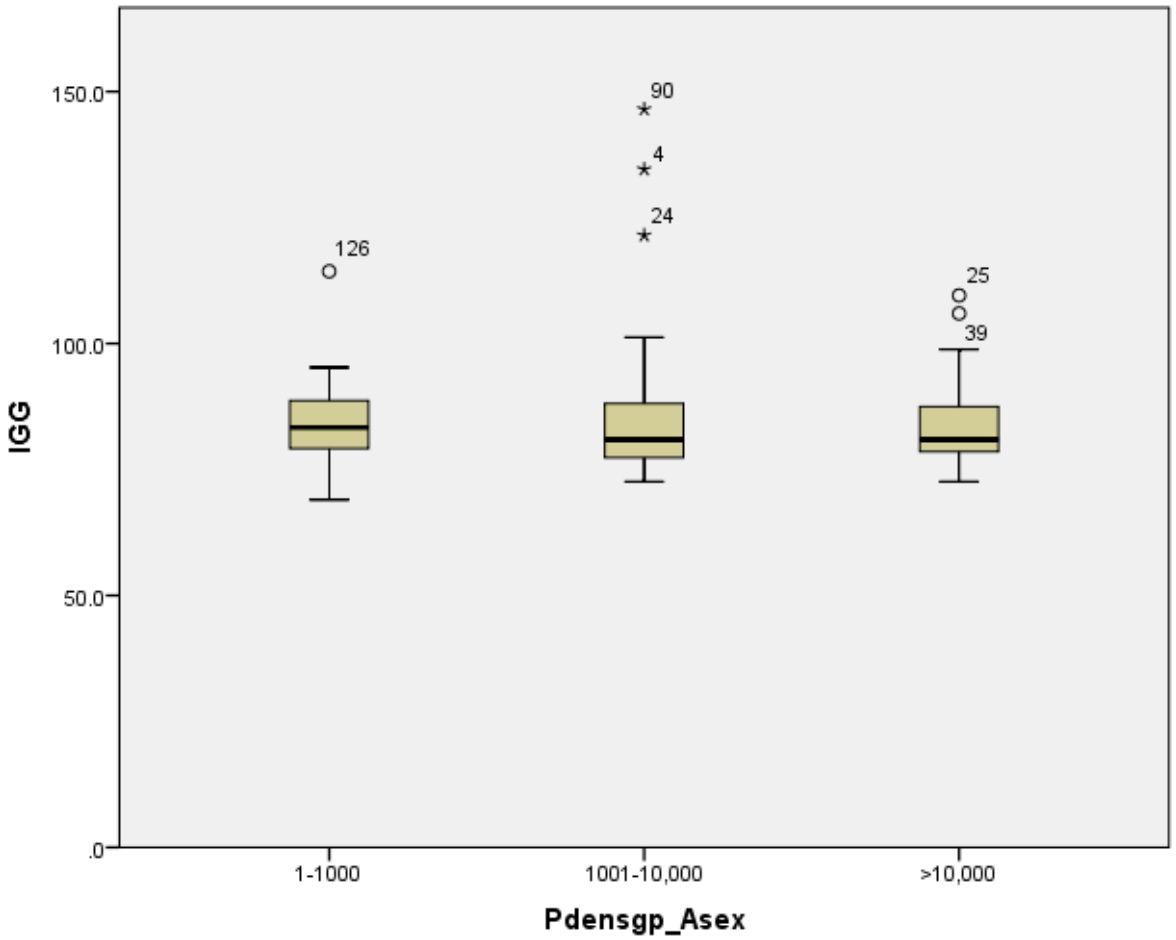

Fig 13:Grouped asexual parasitaemia level with IgG (ng/mL).

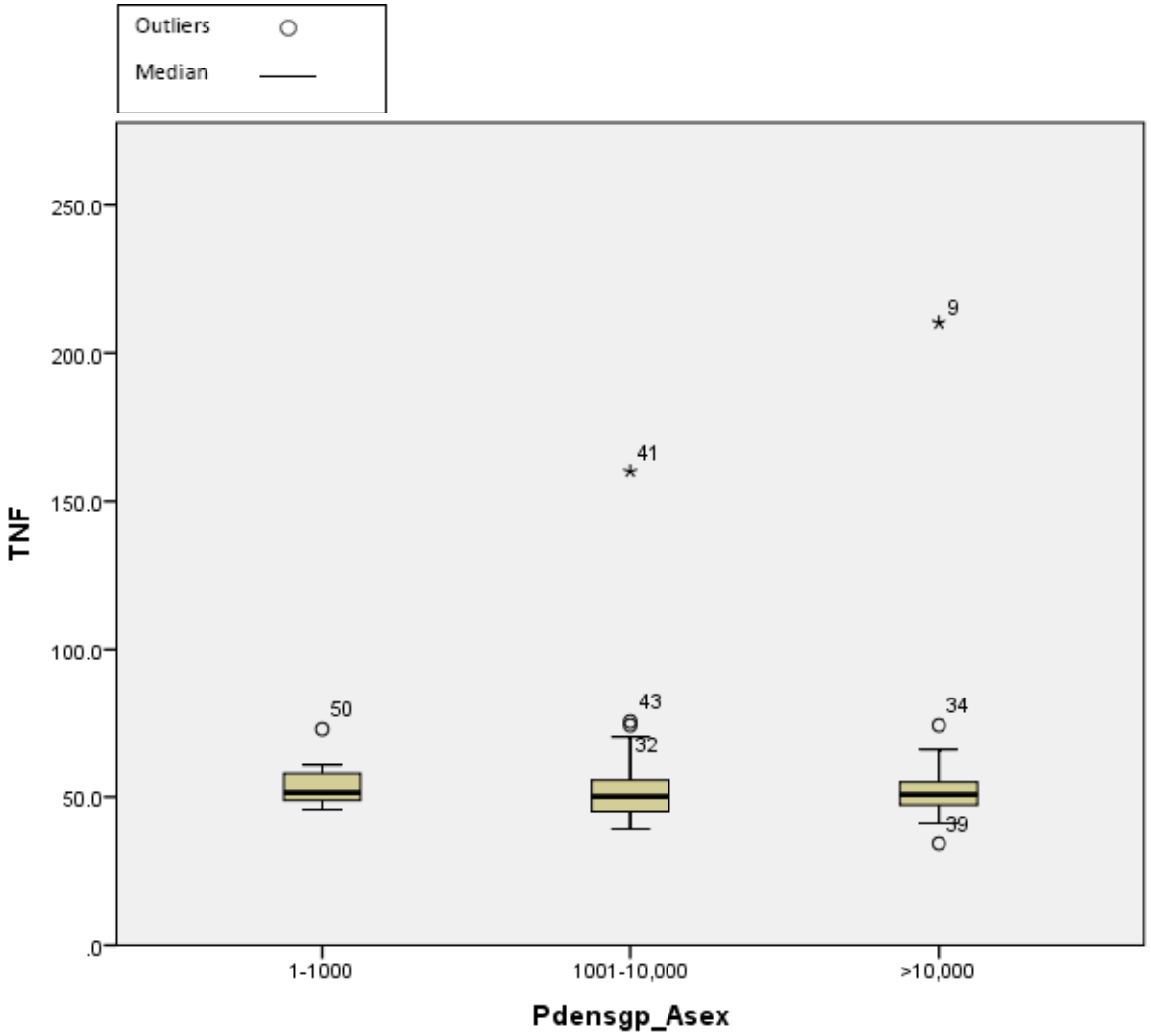

Fig 14:.Grouped asexual parasitaemia level with TNF-α (pg/mL).

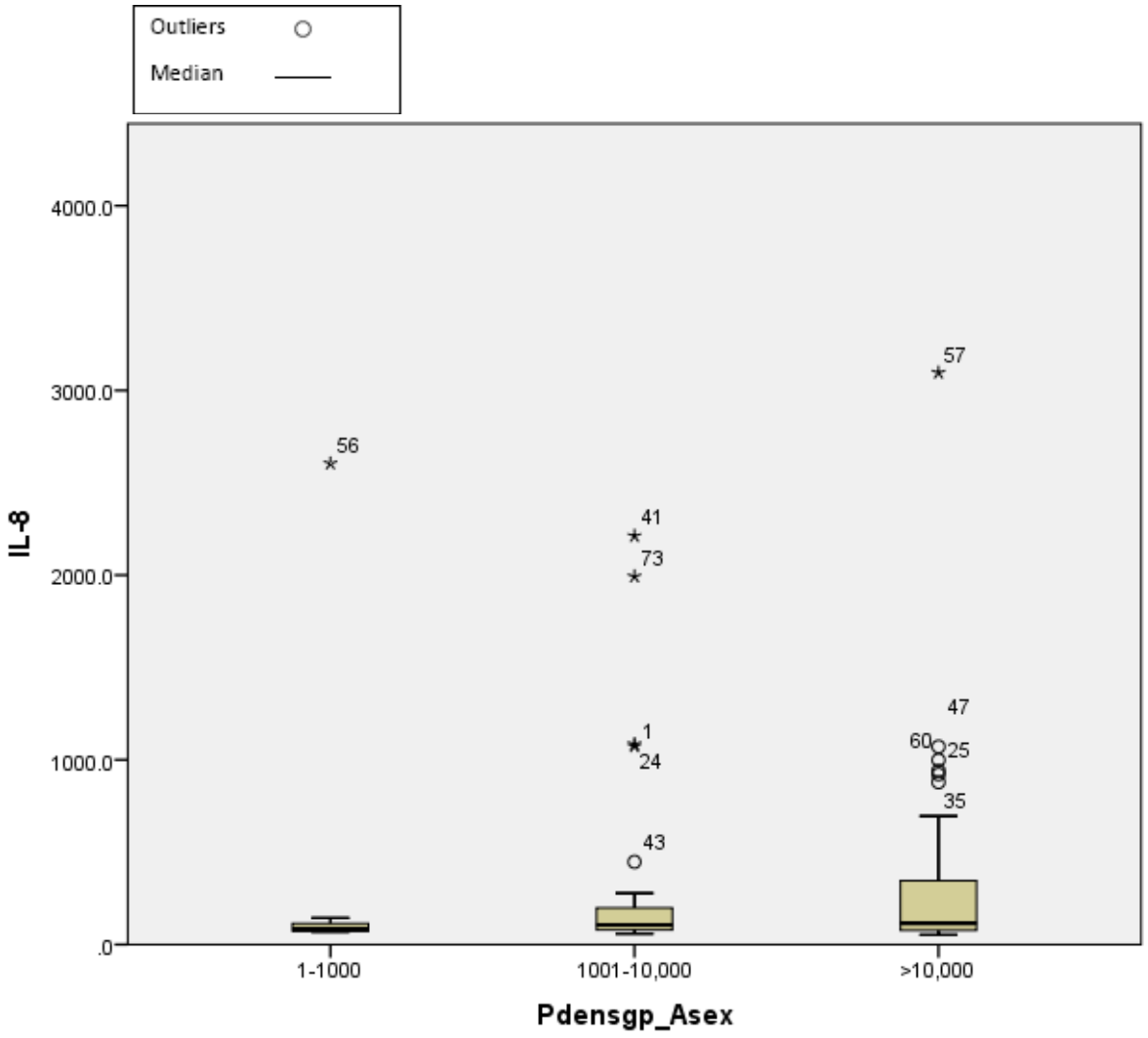

Fig 15:Grouped asexual parasitaemia level with IL-8 (mg/L).

4.6.2 Relationship of Immune Markers with Symptoms, age and sex

The relationship between the assayed markers with symptoms, age and sex was analyzed; the association was determined using eta while the test analysis of variance (ANOVA) was used to determine the statistical significance of the test. The study showed some strong association which was significant between Immunoglobulin G with the presentation of fever, both documented and reported ($p<0.001$: $\phi = 0.310$; $p=0.003$: $\phi = 0.259$ respectively) also there was a recorded strong association between the means of IgG and the microscopy readings which was significant ($p<0.001$: $\phi=0.610$) among the symptomatic group (Table 10). There were also other parameters that showed some level of association which was not significant (Table 10).

The association between the assayed markers with different age groups was assayed for the symptomatic group and they all showed some level of association some strong, others weak, these relationships are not significant however, for age group 2 and 3, IL-8 association was seen to be significant($p=0.001$: $\phi = 0.355$; $p = 0.001$: $\phi = 0.282$ respectively) (Table 11).

There were also some recorded association which was not statistically significant among the asymptomatic group however there was an association between the presentation of fever (reported) and TNF−α which was statistically significant ($p = 0.003$: $\phi = 0.428$) (Table 12, 13).

Table 10: Correlation of Immune Markers with Symptoms and sex among the symptomatic group

	Sex		Reported Fever		Documented Fever		Body pain		Headache		Microscopy	
IgG	Male	Female	Yes	No	>=37.5	<37.5	Yes	No	Yes	No	Positive	NMPS
Mean	101.397	93.944	95.024	114.627	88.329	103.676	103.335	93.207	99.459	90.908	85.206	115.613
N	58	70	113	15	53	75	52	76	96	32	77	51
Std. Deviation	27.4318	21.3593	23.4738	25.8172	17.3066	26.8237	26.7613	22.0430	25.3707	20.6585	13.1266	26.3339
Geometric Mean	98.310	91.923	92.667	112.162	86.973	100.687	100.448	91.062	96.742	89.070	84.386	112.901
P	0.086		0.003		<0.001		0.021		0.087		<0.001	
Association	0.152		0.259		0.310		0.204		0.152		0.610	
TNF												
Mean	52.470	52.689	53.094	48.792	53.664	51.831	51.396	53.406	52.566	52.661	55.511	48.180
N	58	70	113	15	53	75	52	76	96	32	77	51
Std. Deviation	19.9723	20.8053	21.4275	8.1826	16.8867	22.5632	23.3346	18.1517	20.2269	21.0518	23.1383	14.3306
Geometric Mean	50.392	50.834	50.962	48.227	52.171	49.574	49.288	51.575	50.683	50.486	53.194	46.999
P	0.952		0.444		0.618		0.585		0.982		0.046	
Association	0.005		0.068		0.045		0.049		0.002		0.177	
IL-8												
Mean	339.575	208.247	283.678	147.802	407.757	168.820	132.725	360.144	214.706	426.901	335.795	165.028
N	58	70	113	15	53	75	52	76	96	32	77	51
Std. Deviation	631.1023	412.2539	552.0760	206.2642	662.6271	374.3740	161.0994	653.7677	411.8603	757.3506	581.1177	410.2842
Geometric Mean	143.301	114.707	132.917	89.388	185.547	96.994	94.700	154.993	116.012	165.978	158.312	90.836
P	0.160		0.348		0.011		0.015		0.047		0.071	
Association	0.125		0.084		0.225		0.214		0.176		0.160	

Table 11: Correlation of Immune Markers within different age groups among the symptomatic group

	Age Group 1					Age Group 2				Age Group 3		Age Group 4	
	1-10	11-20	21-30	31-40	>40	1-5	6-15	16-25	>25	1-5	>5	1-15	>15
IgG													
Mean	89.459	101.165	88.733	104.387	108.391	88.773	95.557	90.595	104.573	88.773	98.999	93.431	101.594
N	47	26	14	16	25	21	46	13	48	21	107	67	61
Std. Deviation	17.4324	32.3085	11.5172	26.4292	25.5545	18.0790	26.5820	19.4812	24.6055	18.0790	25.2835	24.3079	24.1552
Geometric Mean	88.042	97.157	88.096	101.575	105.661	87.340	92.704	89.042	102.005	87.340	96.294	90.988	99.093
P			0.007				0.044			0.080		0.059	
Association			0.328				0.254			0.155		0.167	
TNF													
Mean	59.746	50.470	48.394	49.209	45.854	51.524	59.585	49.583	47.167	51.524	52.799	57.059	47.681
N	47	26	14	16	25	21	46	13	48	21	107	67	61
Std. Deviation	31.2411	8.2501	5.4311	7.4847	5.3867	8.9409	31.6373	5.5203	6.1812	8.9409	21.9301	26.8489	6.0843
Geometric Mean	55.532	49.898	48.125	48.717	45.575	50.800	55.293	49.310	46.804	50.800	50.601	53.844	47.327
P			0.040				0.025			0.794		0.009	
Association			0.279				0.269			0.023		0.231	
IL-8													
Mean	404.637	270.562	349.897	73.114	86.070	600.078	233.379	485.026	96.463	600.078	202.533	348.315	179.271
N	47	26	14	16	25	21	46	13	48	21	107	67	61
Std. Deviation	652.5777	416.7100	810.7670	23.3402	59.6522	817.2828	365.3250	942.8470	108.0171	817.2828	421.6203	568.1386	461.1646
Geometric Mean	186.373	150.676	127.244	70.081	75.177	265.359	138.868	164.236	78.571	265.359	109.774	170.118	91.940
P			0.065				0.001			0.001		0.069	
Association			0.262				0.355			0.282		0.162	

Table 12: Correlation of Immune Markers with Symptoms and sex among the asymptomatic group

	Sex		Reported Fever		Documented Fever		Body pain		Headache		Microscopy	
IgG	Male	Female	Yes	No	>=37.5	<37.5	Yes	No	Yes	No	Positive	NMPS
Mean	121.220	135.530	139.230	124.167	162.560	126.662	157.046	124.622	143.176	123.071	122.409	130.984
N	26	20	10	36	1	45	4	42	10	36	19	27
Std. Deviation	30.9231	53.2887	66.7372	33.0861		42.3497	74.7154	38.1230	65.2642	33.2422	27.1348	50.4218
Geometric Mean	118.369	127.954	128.594	120.790	162.560	121.676	146.286	120.388	132.969	119.672	120.041	124.165
P	0.259		0.324		0.406		0.144		0.186		0.504	
Association	0.170		0.149		0.125		0.219		0.199		0.101	
TNF												
Mean	44.543	45.882	43.384	45.608	61.573	44.759	42.565	45.369	41.968	46.002	44.350	45.670
N	26	20	10	36	1	45	4	42	10	36	19	27
Std. Deviation	5.3740	6.3549	3.6212	6.2171		5.2937	.9623	6.0042	1.3632	6.2462	5.6818	5.9129
Geometric Mean	44.264	45.513	43.258	45.242	61.573	44.487	42.556	45.023	41.948	45.630	44.046	45.343
P	0.443		0.288		0.003		0.361		0.050		0.453	
Association	0.116		0.160		0.428		0.138		0.290		0.114	
IL-8												
Mean	172.351	123.010	107.215	163.033	75.941	152.564	157.896	150.232	128.567	157.102	179.546	130.739
N	26	20	10	36	1	45	4	42	10	36	19	27
Std. Deviation	274.2842	83.3937	34.2635	239.3743		215.0427	149.7264	219.3662	95.1422	236.1967	320.1006	80.7483
Geometric Mean	117.357	104.798	102.676	114.372	75.941	112.684	119.998	110.963	109.564	112.328	107.665	114.667
P	0.442		0.470		0.726		0.946		0.712		0.450	
Association	0.116		0.109		0.053		0.010		0.056		0.114	

Table 13: Correlation of Immune Markers within different age groups among the asymptomatic group

	Age Group 1					Age Group 2				Age Group 3		Age Group 4	
	1-10	11-20	21-30	31-40	>40	1-5	6-15	16-25	>25	1-5	>5	1-15	>15
IgG													
Mean	128.889	129.945	102.357	134.133	120.246	126.353	121.240	179.714	125.661	126.353	127.546	121.824	145.317
N	15	22	3	3	3	4	31	4	7	4	42	35	11
Std. Deviation	30.2483	52.4709	12.6778	56.8365	14.4779	25.6494	31.7429	97.5640	34.8028	25.6494	43.6716	30.8195	65.7729
Geometric Mean	126.004	122.911	101.849	126.911	119.688	124.547	118.206	158.595	122.258	124.547	122.247	118.914	134.392
P			0.870				0.072			0.958		0.108	
Association			0.171				0.389			0.008		0.240	
TNF													
Mean	46.018	44.665	48.652	42.440	43.186	47.037	45.149	47.596	42.511	47.037	44.943	45.365	44.360
N	15	22	3	3	3	4	31	4	7	4	42	35	11
Std. Deviation	7.3137	4.4391	11.2883	.4304	1.5517	9.9357	5.3389	9.3375	1.2808	9.9357	5.4041	5.8508	5.8071
Geometric Mean	45.535	44.467	47.837	42.439	43.167	46.325	44.870	46.983	42.495	46.325	44.660	45.034	44.075
P			0.647k				0.472			0.496		0.621	
Association			0.240				0.240			0.103		0.075	
IL-8													
Mean	111.872	181.834	74.354	165.105	181.510	151.943	157.236	78.918	163.367	151.943	150.799	156.631	132.658
N	15	22	3	3	3	4	31	4	7	4	42	35	11
Std. Deviation	79.8193	291.7034	25.9520	138.7361	172.6411	109.9319	250.1128	24.3735	130.8048	109.9319	221.0944	237.2047	110.7225
Geometric Mean	95.098	126.156	71.566	131.097	136.441	123.686	112.159	76.145	128.973	123.686	110.644	113.421	106.482
P			0.852				0.923			0.992		0.749	
Association			0.178				0.106			0.102		0.049	

CHAPTER FIVE

DISCUSSION

5.1 Malaria Prevalence in Lagos and Anambra and Lagos State among Symptomatic and Asymptomatic Individuals

Human Malaria caused by *Plasmodium species* is a disease of public health importance in Nigeria including Anambra and Lagos state (Agboola *et al.,* 2010; Onyido *et al.,* 2014) due to its prevalence, incidence, morbidity and mortality (Yilgwan, 2011; WHO, 2017). Knowledge on the burden of malaria is an essential aspect of planning in the control and implementation of intervention strategies for Malaria in Nigeria. One of the objectives of this study was to determine the prevalence of malaria in Lagos and Anambra state using selected health facility as reference samples. The reference sample were to represent a rural and urban area. The malaria prevalence for the study was 35.5% in selected facilities in Lagos state, 9% in selected facilities in Anambra state and 9.8% from the community which is also in Anambra state.

The prevalence of malaria in Lagos state was relatively higher than the prevalence in Anambra state. Although a retrospective study on malaria infection in Anambra from 2005 to 2010 showed that seasonal prevalence occurred with 52.3% malaria parasite infection during rainy season and 47.7% during the dry season (Okeke *et al.,* 2015). The 2015 national malaria indicator survey recorded malaria prevalence of 10.2% (NMEP *et al.,* 2016). This study recorded a prevalence of 9% in facilities visited in Anambra state.

Another study in Aguleri community of Anambra state reported that malaria transmission was high due to lots of breeding site of the mosquitoes vector which is as a result of plants grown around houses as majority of the population of the areas are farmers, also there were

accumulation of water bodies seen in the town and these could also serve as a best sites for mosquitoes to breed, 100% of the individual sampled for this study have the knowledge of the usefulness of insecticide treated net but only 26.9% of the individual make use of it (Egbuche *et al*., 2013).

However, this study recorded a reduced prevalence of 9.8% in Aguleri community irrespective of the environmental condition of the community, the community prevalence reported in this study is close to what was recorded by the National Malaria Indicator survey 2015 (NMEP *et al.,* 2016). The reduction in prevalence in both selected health facilities and Aguleri community in Anambra state could be attributed to the integrated control measures effectively been executed in the state and thus could stand as evidence to buttress the achievement in the malaria control programme of the state thus far.

On the other hand the study recorded a high prevalence in Agura health care centre in Ikorodu, Lagos however, in Regina Mundi catholic Hospital Lagos, all the samples collected were negative. This basically indicates that malaria incidence in Lagos is more in the rural/ sub-urban area of the state. This is supported by a study done in Ibeshe community in Ikorodu Lagos state during the dry season which showed a prevalence of 14.7% (Aina *et al.*, 2013). This study was carried out during the rainy season in Regina Mundi catholic hospital and Agura health centre with a prevalence of 35.5%; all positives seen were from Agura health centre. The high prevalence could be because of a high rate of transmission during the rainy season. This implies that intensive control measures should be implemented in areas such as this which are at the outskirt of Lagos metropolis.

Of all the human malaria species, *Plasmodium falciparum* has been identified as the most prevalent species (NMEP *et al.,* 2016; WHO, 2017). This fact is further reaffirmed with the

findings from this study which showed that the 100% of human malaria cases encountered in this study were as a result of *Plasmodium falciparum.*

5.2 Discrepancy Significance between Parasite Density with automated WBC, WBC of 8,000cells/ml and 6,000cells/ml

There was also a need to look at the method of parasite density determination with assumed WBC count since some facilities might have to estimate WBC to determine parasite density where actual WBC is not available. Diagnostic quality is a core part of malaria control and possible elimination in Nigeria. The parasite density discrepancy between the parasitaemia obtained with actual WBC and assumed parasitaemia with WBC count of 8,000cells/mL and 6,000cells/mL has been a contending issue (Jeremiah and Uko, 2007; Omalu *et al.,* 2008; Alves-Junior *et al.,* 2014). This study gives further insight on the deviation of these assumed parasite densities from the actual parasite density and how it could potentially affect the treatment and management of malaria cases.

Some studies suggest that assumption of WBC Count of 8,000 Cells/mL overestimates malaria parasite density (Jeremiah and Uko, 2007; Omalu *et al.,* 2008; Alves-Junior *et al.,* 2014) and a recommendation that 6,000 cells/mL be used in Nigeria which has been applied in some malaria studies (Udomah *et al.,* 2016). This study however did not find any significant statistical difference in the Assumed parasitaemia calculated using a fixed WBC count of 8,000 cells/mL and 6,000 cells/mL, thus these computations can be used in place of parasitaemia calculated using actual patient's WBC where the actual patient's WBC is not available.

5.3 Immune Markers

Cytokines are a relevant factor in patients with clinical malaria, both in severe and mild cases (Prakash *et al*., 2006). Cytokines have been identified in various studies to play specific roles in disease pathogenesis or protection against severe forms of the disease (Aucan *et al.,* 2000; Choudhury, *et al*., 2000; Kinyanjui *et al.,* 2004; Iriemenam *et al.,* 2009a; Medina *et al.,* 2011; Bostrom *et al.,* 2012; Tran *et al.,* 2012; Rovira-Vallbona *et al.,* 2012).Malaria infection has been identified to result in the characteristic changes in cytokine production which are as a result of the host immune activity in response to the infection (Lyke *et al.,* 2004).

The balance between pro- and anti-inflammatory cytokines are known to play vital role in immune responses and disease pathogenesis of malaria infection, although, the role of these markers in disease pathogenesis and relationship to host protection is still not well established (Prakash *et al*., 2006). This study profiled IgG which is known to confer protection against malaria and pro inflammatory cytokines IL-8, and TNF−α which plays different roles in the disease pathogenesis correlating and associating them with the disease presentation, age and sex of study participants.

5.3.1 IgG in Individuals with Symtomatic and Asymptomatic Malaria Infection

The Immune Profile of IgG, was seen to range from 69-202ng/ml with a mean of 97.3 ±24.5 SD in Symptomatic individuals while asymptomatic concentration range was from 92.0-267.7ng/ml with a mean of 127.4±42.2 SD with a p-value of <0.001 which is significant. Though the mean of the symptomatic is seen to be relatively lower than that of the asymptomatic, however the parasite range shows that the concentrations are quite similar, this which agrees with some earlier study by Perlman and Troye-Blomberg, (2002). This is

possibly the case because Nigeria is a country that is endemic for malaria and most of the population would have some form of immunity to the disease.

Furthermore, mean IgG plasma level was seen to be higher among the parasitemic asymptomatic group (122.409ng/ml) than the parasitemic symptomatic group (85.206ng/ml), this could possibly be the reason why individuals from that group are without symptoms even with the presence of the malaria parasite which is supported by a study in Kenya that identified that asymptomatic infection and anti-VSA IgG antibodies are associated with protection against clinical malaria (Kinyanjui *et al.,* 2004).

This study showed some strong significant association between Immunoglobulin G with the presentation of fever, both documented and reported and microscopy readings which was significant among the symptomatic group (Table 8). This could indicate that the increase in the concentration of IgG in this group could be an indication of its protective role since the antibody has recognized the presence of the disease organism and is set to clear the infection. Other malaria associated symptoms which were assayed did not show any significant relationship with IgG (Table 8).

Studies have shown that adults have the highest concentration of IgG1 (5-12 mg/ml), followed by IgG2 (2 - 6 mg/ml), IgA1 (0.5 - 2 mg/ml), IgM (0.5 - 1.5mg/ml), IgG3 (0.5 - 1.0 mg/ml), IgG4 (0.2 - 1.0 mg/ml), IgA2 (0 - 0.2 mg/ml), IgD (0 - 0.4 mg/ml) and IgE (0 - 0.002 mg/ml) (Shakib and Stanworth, 1980; French, 1986). It has been noticed that individuals who have malaria parasites at the time when their antibodies are being measured usually have high levels of antibodies compared to those that do not have the parasite (Kinyanjui *et al.*, 2007).

Among people living in endemic areas, levels of antibodies to malaria antigens are seen to vary with the level of malaria transmission that is, they are highest during the period of high transmission (usually rainy season) and lowest or undetectable during the periods of low transmission (Kinyanjui *et al,* 2007, Cavanagh *et al.,* 1998). It is evident that Immunoglobulin G has a role to play in the boosting of the immune system though it has been implicated in to be associated with susceptibility to malaria (Riley *et al.,* 2000). The major setback in the role played by immunoglobulin G in malaria is the fact that it has a short half-life (Kinyanjui *et al.,* 2007).

5.3.2: IL-8 and TNF-α in Individuals with Symptomatic and Asymptomatic Malaria Infection

TNF−α and IL-8 in study population was seen to be higher in symptomatic participants than asymptomatic participants, however, there was a patient from the community who presented with documented fever at the point of sample collection, this participant TNF−α plasma concentration was seen to be significantly higher than others in the same group; this indicates that TNF−α is a marker of inflammation which is seen to increase with the presentation of inflammatory symptoms such as fever. This is supported by a study on Ghanaian children which showed that only patients with uncomplicated malaria had a positive correlation with levels of TNF−α and soluble TNF− α R1 and TNF− α R2 in serum (Akanmori *et al.,* 2000).

Also, TNF−α was seen to have higher concentrations in individuals with parasitaemia than those negative for malaria parasite, thus identifying it as a marker associated with malaria infection which is supported in a study carried out by Angulo and Fresno (2002); this therefore indicates that tumor necrosis factor- α (TNF-α), among others are crucial for the fast clearance of *Plasmodium falciparum* and the disease pathology (Sam *et al* 1999;

D'Ombrain, *et al.,* 2008; Nmorsi *et al.,* 2010). A study on Ghanaian Children identified that children with cerebral malaria had high levels of TNF−α (Akanmori *et al.,* 2000), another study on mice infected with malaria showed that TNF-α was initially beneficial by reducing the parasite load and later detrimental by decreasing the humoral response (Mugweru *et al.* 2013). This could imply that the more quantity of TNF−α produce could tip the balance of protection thus heading towards a more complicated phase of the disease which could be life threatening. This therefore identifies TNF−α as a possible prognostic marker that can be used to identify the disease progression to complicated cases.

The association between the assayed markers with different age groups and sex was assayed an there was no significant relationship though they all showed some level of association however, for age group 2 and 3, IL-8 association was seen to be significant (p=0.001: ϕ =0.355; p=0.001: ϕ =0.282 respectively) (Table 9); within the significant age group, It could be identified that in the age group 1-5 which is the age group that is prone to developing complicated malaria has the highest concentration of IL-8 in their plasma (600.078 pg/ml). This further affirms the role IL-8 plays in complicated malaria cases as reported by a study that the increased levels of IL-8 were found in cerebral malaria patients (John *et al.,* 2008) also another study noted an increase in IL-8 levels that was seen to correlate with disease severity (Lyke *et al.,* 2004).

The malaria disease severity based on symptoms presented in comparison with the asymptomatic cases was also determined and the results were not significant in all cases when compared with parasitaemia, sex, and age. Thus severity of the malaria infection was found to be independent of parasitaemia, sex, and age; which correlates with a study by Prakash and others (2006).

5.4 Conclusion

The National Malaria control programme aims to move from a control phase of malaria to an elimination phase (The malERA Consultative Group on Basic Science and Enabling Technologies, 2011). With this shift in focus, there are the possibilities of the rise in other issues like the increase in susceptibility to the disease with a reduced immunity and *infection* rate which could give rise to severe or complicated cases of malaria hence the need for more grounded preparation during this phase.

Therefore, the development of an effective malaria vaccine and accurate diagnostic procedures are considered as the way towards sustenance of malaria control and possible elimination in Nigeria. Clearance and elimination of malaria infection is anchored on the better understanding of the human-malaria immune response, integrated control measures and the production of an effective life-long malaria vaccine. The integration of these facts is important in Nigeria malaria elimination scheme. Hence, this study addressed the diagnostic concerns in relation to parasite density determination and the roles of some immunological markers while relating them to their possible use as a diagnostic tool.

The study determined that ASP using a fixed WBC of 8,000 cells/mL and 6,000 cells/mL can be used in place of ACP calculated using AcWBC where AcWBC is not available. Fever was identified in elevating plasma level of IgG and TNF−α which could in turn affect parasite clearance and immunity as well as disease pathogenesis. Malaria disease severity based on symptoms was independent of parasitemia, sex, and age.

The transition from asymptomatic malaria cases to mild malaria and then to severe forms of malaria can be sudden and requires immediate intervention, therefore, the use of biomarkers

to stratify severe malaria patients based on risk potential have vital role in patient care and appropriate case management. Also, as Nigeria is moving from the control phase of malaria to the elimination phase where there might be asymptomatic cases with levels of parasitaemia which are undetectable by light microscopy or other conventional method of parasite detection, biomarkers will be crucial for monitoring the possible elimination of malaria.

5.5 Recommendation

Assumed parasitaemia calculated using a fixed WBC count of 8,000 cells/mL and 6,000 cells/mL can be used in place of parasitaemia calculated using actual patient's WBC where the actual patient's WBC is not available. To determine a uniform process of parasite density determination without bias the National malaria control programme and other bodies involved in the fight against malaria should agree on a standard formular that should be used or otherwise use the WHO recommended standard to create uniformity.

With further research and knowledge integration, TNF-α can be used as a possible prognostic marker for the identification of uncomplicated malaria cases that could progress to complicated cases. Also, IL-8 can also be considered as a possible prognostic marker for the identification of the transition from mild malaria to cerebral malaria or complicated malaria

With the proposed shift from prevention to elimination phase of malaria control, there is need for more studies on these identified immune markers which may serve as prognostic markers. Further comprehensive test using some of these immune markers may aid malaria vaccine development.

5.6 Contribution to knowledge

1. The study has produced evidence of reduced malaria transmission in selected health facilities and Aguleri community in Anambra state

2. The study has produced evidence showing that prevalence of malaria in Agura community is relatively high and control measures should be intensified

3. The study also showed that assumed parasitaemia calculated using a fixed WBC count of 8,000 cells/mL and 6,000 cells/mL can be used in place of parasitaemia calculated using actual patient's WBC where the actual patient's WBC is not available.

4. The study showed the association between plasma profile of TNF-α, IgG and parasitaemia in uncomplicated malaria, the immune-regulatory roles of these biomarkers in uncomplicated malaria cases were demonstrated, thus has contributed to the data on the plasma profile of IL-8, TNF-α, IgG, in uncomplicated symptomatic malaria cases and asymptomatic individuals.

5. This study has identified TNF-α as a possible prognostic marker for the identification of uncomplicated malaria cases that could progress to complicated cases.

6. This study identified IL-8 as a possible prognostic marker for the identification of the transition from mild malaria to cerebral malaria or complicated malaria

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APPENDIX I: Informed Consent Form

Immunological Profile of Individuals with Malaria Infection
Informed Consent Form
Name and Address/ Affiliation of Principal Investigator
I am Onwuachusi Ginika Lovelyn, a student of Nnamdi Azikiwe University, Awka
Brief on Nature of Research
If you agree to take part in the research, the following will be done:2ml of venous blood will be required from you. You will be asked questions about the way you feel (symptoms) and treatment you had taken. Rapid Diagnostic Test (RDT) will be done on your sample. If the malaria test is negative, you will be referred for non malaria illness. You will be treated following the procedure at the health facility. The risks involved in this study are minimal. They include the discomfort of a slight delay in the treatment, discomfort in drawing a sample of blood, rare bruising and infection at the site of needle stick. New needles and syringes will be used for each patient so that there will be no risk of transmitting diseases.
Voluntarism
Your participation in this research is entirely voluntary. Whether you choose to participate or not, all the services you receive at this facility will continue and nothing will change.
Confidentiality:
All information that you provide will be considered confidential, and no mention of your name or any other identifying information will appear on the stored samples or in any publication in connection with this study. The information will NOT be stored together with the samples.
Right to Withdraw:
You may change your mind later and stop participating even if you agreed earlier without any repercussion. You will receive the normal services by the clinic. You do not have to explain why you do not wish to participate or withdraw.
Benefits:
There will be no direct benefit from your taking part in this project but it will also not incur any costs if you participate. In the long run, your participation will help to improve the quality of information available on malaria-immune response.
General Statement
The results from this research will help determine the immune profile of anti and pro inflammatory cytokine in relation to immunoglobulin G levels in symptomatic and asymptomatic individuals of different ages. There will be no document stating participants identity, therefore maintaining confidentiality.

Contact information:
If you have any questions, or if any problems arise, you may contact:
Onwuachusi Ginika Lovelyn- *Department of Parasitology and Entomology, NnamdiAzikiwe University, Awka or ANDI Centre of Excellence for Malaria Diagnosis, College of Medicine, University of Lagos, Idiaraba. 08069517287; gonwuachusi@yahoo.com; gokwusi@gmail.com*

Certificate of consent:
I have been invited to participate in a research project and agree to be screened for malaria. I have read the foregoing information (or it has been read to me/my child). I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I give consent/assent voluntarily to participate in this study.

If participant is ≥18 years fill this part:
Name of Participant_____
Signature of Participant_____ Date _____/_____/_____ (*dd/mm/yyy*)

If illiterate (Statement of witness):
I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.
Name of witness AND Thumb print of patient
Signature of witness _____ Date
____/_____/_____(*dd/mm/yyyy*)

If participant is a child<18 years fill this part:
Name of Participant (Child) _____
Signature of Parent_____ Date _____/____/_____(*dd/mm/yyyy*)

If illiterate (Statement of witness):
I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.
Name of witness AND Thumb print of patient
Signature of witness _____ Date
___/___/_____(dd/mm/yyyy)

APPENDIX II: Case Report Form

Immunological Profile of Individuals with Malaria Infection
Case Report Form:

A. Site Information

1. Community ☐ Health Care Centre ☐
2. Site Name and Code _____ Study ID No._____
3. Date ____/___/_____(dd/mm/yyy)

B. Patient Information:

4. Sex: Male ☐ Female ☐
2. Age_____ (Years)

C. Clinical History:

Symptoms	Treatment
Fever Yes ☐ No ☐	What medicine have you taken in the past two weeks?
Duration……(24/28hrs)	Chloroquine Yes ☐ No ☐
Chills Yes ☐ No ☐	SulfadoxinePyrimethamine: Yes ☐ No ☐
Body Pain Yes ☐ No ☐	ACT: Yes ☐ No ☐
Head Ache Yes ☐ No ☐	Others: Yes ☐ No ☐
Others Yes ☐ No ☐	If Others Specify_____ How long ago have you taken the Medicine? _____

Blood Based MRDT Result

RDT Name	Lot No	Expiry Date	Pf (e.g. 2+)	Pan (e.g.2+)	Result (e.g. P.fPos)	Time for test band (mins)

Malaria Microscopy Result

Detection	Species	Parasite Count (Asexual)	Asexual Parasitaemia (P/mL)	Parasite Count (Sexual)	Sexual Parasitaemia (P/mL)

APPENDIX III
Ethical Approval from NnamdiAzikiwe University &Teaching Hospital

NNAMDI AZIKIWE UNIVERSITY TEACHING HOSPITAL

P.M.B. 5025, NNEWI, ANAMBRA STATE, NIGERIA

Chairman
Board of Management

Mrs. Chinyelu Ogoamaka Nwofor
B.Ed, M.Ed, MHP&M, AHA, FCAI
Director of Administration/
Secretary to the Board

Professor Anthony O. Igwegbe
MBBS, FWACS, FICS, FISS
Chief Medical Director/
Chief Executive

Dr. E. A. E. Afiadigwe
B.Sc (Hons) Nig. MBBS (NAU), FWACS, FICS
Chairman
Medical Advisory Committee

E-mail: nauthcmd@yahoo.co.uk
nauthnnewi@hotmail.com
Telegram: TEACHOS NNEWI

Our Ref: NAUTH/CS/66/VOL.8/24

*Your Ref:*_____

Date: 28th January, 2016

Onwuachusi Ginika Lovelyn
Department of Parasitology and Entomology,
Faculty of Biosciences,
Nnamdi Azikiwe University,
Awka.

ETHICS COMMITTEE APPROVAL

RE: PROFILE OF IMMUNOGLOBULIN-G, H, IL-I, INTERFERON-γ, TNF-α, IL-10 AND TGF-β in INDIVIDUALS WITH MALARIA INFECTION

We write to inform you that after due consideration of your research proposal, approval is hereby conveyed for you to commence the study.

The principal investigator is required to send a progress report to the Ethics Committee at the expiration of three (3) months after ethical clearance to enable the Committee carry out her oversight function.

Please note that this approval is subject to revocation if you fail to obtain proper authorization from your study site/unit.

.....
Dr. Joy Ebenebe
Chairman, NAUTH Ethics Committee

.....
Udemezue N.O (Mrs)
Sec., NAUTH Ethics Committee

APPENDIX IV
Ethical Approval from Anambra State Ministry of Health

ANAMBRA STATE OF NIGERIA
MINISTRY OF HEALTH

JEROME UDOJI SECRETARIAT
COMPLEX
P. M. B. 6002
AWKA.

Your Ref :.....

Our Ref : MH/PHD/140/20

22nd January, 2016

PHC/DC Dept.
Ministry of Health
Awka

Onwuachusi Ginika L.
Nike Lake Road
Enugu
11-01-2016

RE: APPLICATION FOR ETHICAL APPROVAL

I am directed to inform you that your *Application for Ethical Approval* has been approved by the Hon. Commissioner for Health.

You can go on with the programme and keep us informed of your activities and final report.

Dr. C.E.N Okafor
Ag. Director PHC/DC

CC: The Honorable Commissioner
Ministry of Health

Appendix V

Protocol for reagent Preparation

1. Phosphate Buffered Saline (PBS), pH7.4: thisrequires the use of 0.086 M disodium hydrogen phosphate (Na2HPO4), 0.020 M monopotassium phosphate (KH2PO4) and 3.08 M sodium chloride

2. Blocking buffer (PBS, 1% BSA): thisrequires the dilution of5g BSA to 500 ml PBS

3. Wash solution (PBS, 0.05% Tween-20): thisrequires the use of 400 ml PBS, 2 ml Tween-20 and fill up with to 4 L with distilled water

4. Dilution buffer (PBS, 0.05% Tween-20, 0.1% BSA): 7this c-onsi7t9e 500 ml PBS, 0.25 ml Tween-20 and 0.5 g BSA (Hycult Biotech 2010).

The Malaria Microscopy and immunological assay (ELISA) were done in ANDi Centre of Excellence for Malaria Diagnosis, College of Medicine, University of Lagos.

Appendix VI: Objectives/ Summary of Findings

S/N	Objectives of the study	Summary of finding
1	To determine malaria prevalence in Lagos and Anambra State among febrile patients	35.5% inLagos (Agura community), 9% in Anambra facility 9.8% from the community which was also in Anambra state
2	To determine malaria prevalence in Anambra state among afebrile participants.	9.8% from the community which was also in Anambra state
3	To determine the discrepancy between the parasite density values obtained with the automated WBC count, assumed value of 8,000 cells/mL and 6,000 cells/mL	The parasitaemia discrepancy between the actual parasitaemia and assumed parasitamia with WBC count of 8,000 cells/mL is 1.5%, 4.4%, 13.2%, 5.9%, 9.0% and 66.2% at 0-5%, >5-10%>10-15%, >15-20% >20-25% and>25 respectively; while parasitaemia discrepancy between the actual parasitaemia and assumed parasitamia with WBC count of 6,000 cells/mL is 5.9%, 10.3%, 11.8%, 16.2%, 5.9% and 54.4% at 0-5%, >5-10%>10-15%, >15-20% >20-25% and>25 respectively
4	To determine Immunoglobulin G levels in individuals with symptomatic and asymptotic malaria infection in relation to age and sex	1. IgG plasma level was seen to be higher among the parasitemic asymptomatic group than the parasitemic symptomatic group which could possibly be the reason why individuals from that group are without symptoms even with the presence of the malaria parasite, thus emphasizing

		the protective role of IgG in malaria cases. 2. This study showed some strong association which was statistically significant between Immunoglobulin G with the presentation of fever, both documented and reported and malaria microscopy among the symptomatic group 3. This study did not identify any statistically significant relationship with Sex and age.
5	To determine pro-inflammatory cytokine (IL-8 and TNF-α) levels in individuals with symptomatic and asymptomatic malaria infection in relation to age and sex	1. TNF-α was seen to have higher concentrations in individuals with parasitaemia than those negative for malaria parasite 2. IL-8 association was seen to be statistically significant in age group 2 and 3 of which the highest concentration is seen in the age group 1-5 which is the age group that is prone to developing complicated malaria. 3. TNF-α and IL-8 concentration in study population was seen to be higher in symptomatic participants than asymptomatic participants
6	To determine the disease pathogenesis in relationship with the plasma profile of Immunoglobulin G and pro-inflammatory cytokine (IL-8 and TNF-α) in Individuals with	1. Fever has been identified to play certain roles in elevating the plasma level of IgG and TNF-α which could in turn have major roles to play in parasite clearance and immunity as well as disease pathogenesis 2. Malaria disease severity based on symptoms presented in comparison with the

	symptomatic and asymptomatic malaria infection in relation to age and sex	asymptomatic cases, wasfound to be independent of parasitemia, sex, and age