CHAPTER ONE

1.1 INTRODUCTION

The term pregnancy refers to a state in which fertilized ovum implants into the maternal uterus and develops into a foetus.The period of development and growth of the foetus is known as gestation. During gestation, normal physiological processes such as changes in the cytokine profile, increased erythropoiesis and thrombocytopenia take place in the maternal subjects. Often, most of these processes become pathological and may complicate or threaten the well-being and survival of the mother, the foetus or both. These conditions include cytokine dysregulation, inflammation and anaemia. Other abnormal conditions may include thrombosis and disseminated intravascular coagulation (DIC) (Hoffbrand and Moss, 2011).It appears to me that of all the haematologic and immunologic conditions, anaemia appears to be the most frequently discussed.

Several factors are known to cause anaemia in pregnancy especially in sub-Saharan Africa. In this region, most factorsinteract to play significant roles in determining the severity of anaemia (Buseri *et al.*,2008*).*The incriminating factors are microbial infections, nutritional deficiencies and inherited or acquired conditions that affect the red blood cell production or destruction (WHO, 2007; Buseri *et al.*,2008). Infections include malaria, human immuno-deficiency virus, tuberculosis and hookworm while nutritional deficiencies include iron, folate, copper, zinc, vitamin B_{12} possibly riboflavin and other micronutrient deficiencies (Guyatt and Snow, 2001).

Malaria and Iron deficiency are the predominant causes of severe anaemia in sub- Saharan Africa and have their respective deleterious impacts on women of reproductive age, the neonates, infants and children(Nyakeriga *et al.*,2005*).* The vulnerability of children and pregnant women to malaria is because children have naive immune system while the immune

systems of pregnant women are potentially compromised. Consequently, the two groups are considered to be the highest risk population for malaria-related death (WHO. 2000; Laishram *et al.*,2012).

Malaria isa known protozoandisease, transmitted to humans by infected female anopheles mosquito (Inigo and Manual, 2002). Four species of plasmodium have been emphazied as the causative agentsof malaria.However, the fifth specie known as *P. knowlesi* has been reported(Autino *et al.*,2012).Each year, about 250 million people worldwide suffer from malaria with an estimated death of about 1 million and this occurs mostly in the tropical and sub-tropical areas especially in Africa, Asia, Central and South America (Warren, 2010). It is also reported that malaria affects more than 3 million pregnant women every year in the developing countries (Abrams *et al.*,2005).Malaria parasite induces the activationof the cellsof the innate and adaptiveimmune response (Warren, 2010). In the maternal blood,malaria parasites elicit immune response by stimulating the immune cells such as the monocytes or macrophages,natural killer cells andhelper T-cells. These cells in the process secrete cytokine molecules (Elgert,2009). Different cytokines induce various transcription factors which in turn determine the fate of cells; either for proliferation, differentiation and survival or death (Quesenberry, 1995)

Ironis absorbed in the gastrointestinal tract and is essential for erythropoesis. In most cases, the amount of iron absorbed is not always enough for sustainance of pregnacy and may result in iron deficiency. The major reason for iron deficiency during pregnancy is that pregnancy places tremendous increase in the body's need for iron in other to match with the increase in plasma volume of the pregnant woman and the foetal needs for proper development (Bothwell, 2000).The need culminates in depleted iron stores. The progressive depletion of iron stores will eventually result in Iron Deficiency Anaemia (IDA) (Dreyfuss *et al.*,2000).

Body iron status is usually assessed by consideringHaemoglobin, red cell indicies and serum ferritin concentrations along with evidence of inflammation, infection and liver disease (Worwood and May, 2012). The assessment emphasizes onthe combination of parameters fromthe storage, transport and functional Iron compartments. The best combination would be estimations of haemoglobin or haematocrit, serum transferrin receptor and serum ferritin. Such a combination would reflect functional impairment, tissue avidity for iron and iron storage (WHO, 2001).Interestingly, cytokine and iron modulate the activities of each other.The mechanism whereby some cytokines for instance Interferon gamma (IFNγ), Interleukin - 6(IL-6), Tumor Necrois Factor alpha (TNFα), Interleukin 12(IL-12), and Interleukin 13(IL-13) regulate iron homeostasis is by affecting the expression of proteins involved in thestorage and release of iron (Ludwiczek *et al.*,2003).Furthermore, cytokines released during infection/ inflammation such as malaria parasitaemia induces impairmentin the normal physiological mechanism for transporting iron to target tissues and this appears to be mediated by Hepcidin (WHO, 2004)

This study tends to evaluate the levels of some cytokines and iron and their activitiesin pregnant and post-partum womenwith malaria parasitaemia in Aba, Abia State.

1.2 STATEMENT OF PROBLEM

The essence of this study was to contribute to solving the perennial problem of maternal/foetal morbidity and mortality frequently encountered in malaria endemic areas such as Nigeria.

There are cases where anaemia in pregnancy and in post-partum haspersisted inspite of necessary interventions (Guyatt and Snow, 2001). Other adverse conditions which sprouted, such as spontaneous abortion, intrauterine growth retardation, preterm delivery,foetal

distress, foetal death, stillbirth, Low Birth Weight, malaise and perinatal mortalitywere attributed to anaemia (Menedez *et* al., 2000; Unek*eet al*., 2007).

Conversely, adverse pregnancy and post partum conditions do occur without anaemia. Thorough investigation that would define the causes and suggest measures to prevent similar occurrences ought to be carried out before resting such cases.

It is ideal that a good clinical practice should aim at reaching specific and diagnostic conclusion and should be supported by current scientific knowledge. The result of this study of the associations of cytokines, iron and parasitaemia in the malaria vulnerable women in my environment may profer solutions to these problems.

1.3 AIM OF STUDY: To determine the relationship between the levels ofcytokines, iron and malaria parasitaemia among pregnant women infected with malaria

1.4 SPECIFIC OBJECTIVES: The specific objectives of this study are as follows;

(1) To evaluate the levels of pro-inflammatory (TNFα, IFNγ and IL-6) and anti-inflammatory (regulatory) (IL-4and IL-10) cytokines among pregnant andpost-partum women with malaria infection.

(2) To compare the parasite density and cytokine (TNFα, IFNγ, IL-4, IL-6, and IL-10) levels in the peripheral and placental blood.

(3) To determine the influence of age, gestational age and gravidity on the secretion of pro-inflammatory (TNFα, IFNγ and IL-6) and regulatory (IL-4and IL-10) cytokines among pregnant women infected with malaria.

(4) To determine the iron status and the effect of age, trimester and gravidity on the iron levels of the pregnant subjects.

(5) To correlateif there is any association between cytokines (TNFα, IFNγ, IL-4, IL-6, and IL-10), iron (Hb, MCV, SF, sTfR), acute phase protein (C-RP)and malaria parasite densityamong malaria infected pregnant women.

1.5 JUSTIFICATION

Either malaria parasitaemia or iron deficiency has been emphasized as major aetiological factors of anaemia in pregnancy (Nyakeriga *et al.,* 2005). Little observation also has been made on the co-existence of both factors. Again, these studies paid attention to the assessment of the aetiological factors of anaemia in pregnancy and pregnancy outcome(s) due to extreme severity of anaemia as the cause of maternal/infant morbidity and mortality.Moreover, some cases of poor foetal outcome or other adverse pregnancy outcome have been reported to have occurred and these were adduced to alteration of physiological mechanisms due to dysregulation of specific cytokines (Fried *et al.*,1998).

Most of the studies on malarial anaemia in pregnancy have been concentrated on few centers such as Ibadan, Lagos, Enugu, Calabar and Port-Harcourt zones of Nigeria inspite of the fact that the intensity of malaria transmission, socio-economic and cultural practices vary in different parts of the country. Besides, individual and governmental approach to implementation of preventive measures to malaria and anaemia in pregnancy differ with individuals and from one government to another and this is common in Nigeria.

In Nigeria, there is paucity of literature on molecular studies on malaria and anaemia in pregnancy and post partum caused by malaria infection. Again, there is deficit of published work on iron status. Furthermore, studies on interactions of cytokines, iron, acute phase proteins and parasitaemia have not been reported in Aba, Abia State, Nigeria, hence the need for this study.

CHAPTER TWO

2.1 LITERATURE REVIEW

2.1.1 MALARIA

Malaria has remained one of the global health problems and is endemic in parts of Asia, Africa, Oceania, central and South America (WHO, 2011).According to World Health Organization (WHO) malaria report 2011, a total of 216 million estimated malaria cases occurred in 2010of which 81% were reported in Africa followed by 13% from South East Asia and 5% from Eastern Mediterranean regions (WHO,2011). The number of death also reporteddue to malariain 2010 was estimated to be 655,000; of which 91% occurred in the Africa, 6% in South East Asia and 3% in Eastern Mediterranean regions (WHO, 2011). Malaria from the western countries is usually as a result of transportation from the tropical and the sub-tropical regions of the world where malaria is endemic (O'Brien *et al.*, 2006).

On the distribution of human pathogenic plasmodium, reports show a preponderance of P. *falciparum* in tropical Africa, *P.vivax* in South America while P. *falciparum* and P. *vivax* are prevalent in South-East Asia and Western Pacific. Although P. *malariae* may occur in any area, its prevalence is generally low. P. *ovale* is found in tropical Africa whereas P. *knowlesi* infection occurs only in certain forested area of South-East Asia (Autino *et al.*,2012).

P. falciparum is the most pathogenic of the human plasmodium and causes about 90 – 98% of malaria disease. It may invade about 30 – 40% of red blood cells and may exhibit a density of about 250,000 – 300,000parasite/ul of blood (Arora and Arora, 2010). The most outstanding and specific characteristic of *P. falciparum* is its sequestration and possible occlusion of the capillaries with aggregate of parasitized red cells. These may result in haemorrhage, damage and necrosis particularly in the brain and other vital organs such as the kidney and the heart (Warren, 2010).

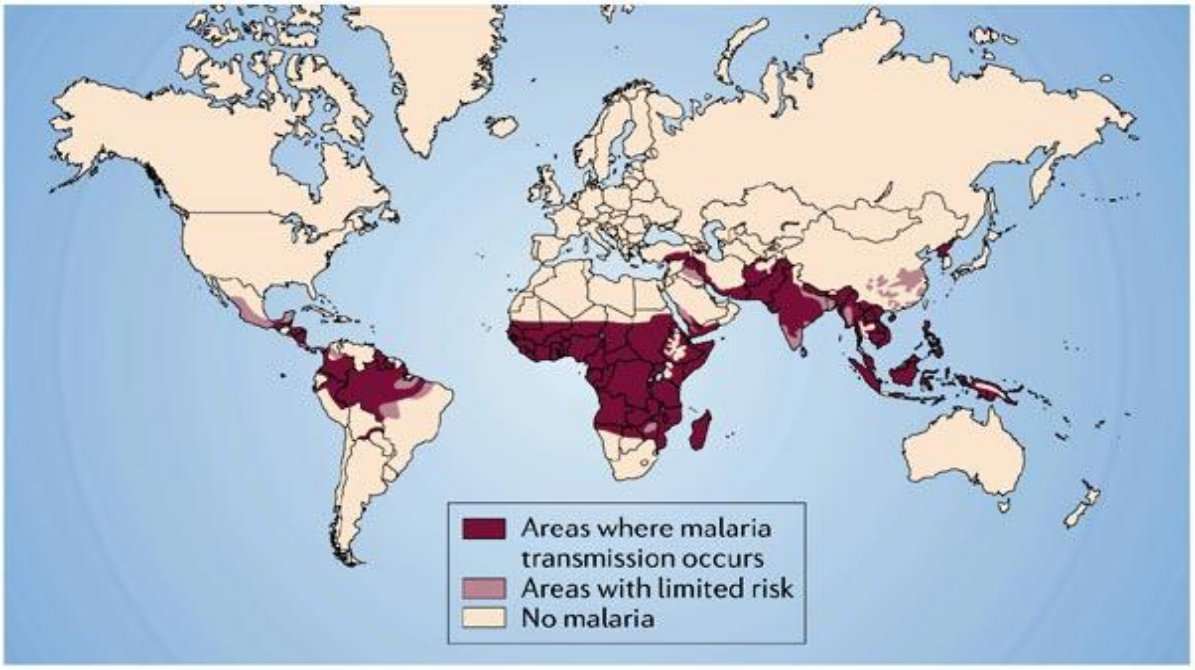


Fig.1.0. Map showing world malaria parasite distribution.(WHO, 2012)

2.1.2 Signs and Symptoms of Malaria

The sign(s) and symptoms that may be presented by those infected with malaria depend on the stage and severity of the infection. It could present as abrupt onset of fever, which may be accompanied by headache, myalgias, and arthralgias of about 2 weeks after mosquito bite (Warren, 2010). It may be frequently associated with non specific signs such as chills, nausea, vomiting and abdominal pain (Warren, 2010). It may also be followed by drenching sweat and patients may feel well before febrile episodes. The spleen and the liver could be enlarged and in most patients, anaemia is common. Other findings may include unrousable coma, convulsion, hypoglyceamia, metabolic acidosis, acute pulmonary oedema, respiratory disorder, haemoglobinuria and acute renal failure (Warren, 2010). Others includejaundice, circulatory collapse, hyperparasitaemia, electrolytedisturbance and spontaneous bleeding (WHO, 2000; Maitland *et al.*,2003; Mackintosh, *et al.*,2004; Warren, 2010).

2.1.3 CLASSIFICATION OF MALARIA

Malaria could be classified based on its severity,as severe and mild (uncomplicated) malaria (WHO, 2010). Severe malaria is defined when the asexual form of the parasite is seen on the peripheral blood andthe observation of any clinical presentation such asconvulsions,systolic blood pressure< 70mm Hg and 50mm Hg in adult and children respectively, acute renal failure (urine output<400ml/hr plus serum creatinine >3mg/dl), and bleeding disorders. The haemoglobin level may be<5g/dl while the parasite count may exceed10,000parasites/ul. Blood glucose may be low (<2.2mmol/L or 40mg/dl) and lactate level >5mmol/L. Also there might be jaundice with total bilirubin exceeding 2.5mg/dl(Daily, *et al.*,2007; Warren, 2010;WHO, 2010). On the other hand, mild malaria could be when asexual or the gametocyte stage is seen in the blood but without any clinical presentation. (Warren, 2010, WHO, 2010)

On another perspective, it could be classified as symptomatic and asymtomatic malaria. Symptomatic malaria refers to a period when a patient presents some clinical signs of the infection while asymptomatic malaria is when a patient harbours the parasite without manifesting any clinical sign (WHO, 2000). The classification of malaria as symptomatic is straight forward because patient shows observable clinical signs unlike asymptomatic and often sub-patent (undetectable by microscopy) malaria where symptoms are lacking (Bottius *et al.*,1996). The presentation of clinical signs may depend on the level of immunity a patient has acquired on previous exposure and sometimes due to anti-malaria administration(Laishram, *et al.*,2012). To an extent, a developed immunity is able to limit the blood parasite density to extremely low levels but may not clear the infection (Takem and D'Alessandro, 2013). This could account to why clinical signs and symptoms may fail to appear. As for asymptomatic malaria, there could be the detection of the parasites in peripheral blood with absence of malaria-related symptoms such as fever within the previous 48 hours (Duarte, *et al.*,2007; Leoratti *et al.*,2008; Males *et al.*,2008;DeMast *et al.*,2010).

2.1.4 PATHOGENESIS OF MALARIA

Malaria occurs basically by transmission of the sporozoites via a bite from an infected anopheles mosquito. Other means of acquiring the disease could be through blood transfusion (transfusion malaria), in utero transmission to the foetus through the placenta (congenital malaria) and by the use of contaminated syringes particularly in drug addicts (Arora & Arora, 2010). It commences with a life cycle involving asexual division (schizogony) which occurs in man (the intermediate host) and the sexual development (sporogony) which occurs in female anopheles mosquito (the definitive host).

Sporogony starts when mosquito, in an attempt to feed on blood from an infected person, sucks blood containing the sexual forms of the parasites. This is transported to the midgut where the gametocytes undergo fertilization and mature to an infective ookinete. On further

migration to the haemocele, the ookinete develops to oocyst that latter emerges as sporozites. The sporozite migrates to the salivary glands to initiate schizogony when it is dislodged into the vertebrates.

Schizogony begins when an infected female anopheles injects the sporozitesinto the subcutenous tissue and less frequently, directly into the blood stream (Miller *et al.*,2002; Schofield, 2007; Planche *et al.,* 2006). The sporozites circulates in the blood stream for less than one hour before migrating to the liver. In the liver cells, the sporozoites divide upto 1000-fold until mature tissue schizonts are formed; each schizont contains thousands of daughter merozoites. This cycle is called the exoerythrocytic schizogony and occurs without any clinical sign (Miller *et al.*,1991; Milner, *et al.*,2011). The schizontsrupture after 6 to 16 days to release the merozites which invade the red cells. The merozoites mature successively from ring forms through trophozoites to mature red cell schizonts over 24 hours in *P. knowlesi*, 48 hours in *P. vivax, P. ovale and P.falciparum* or 72 hours in *P. malariae* infections. Within the red blood cell, the parasite digests red cell protein, primarily haemoglobin to form polarizable crystal in the food vacuole known as haemozoin (WHO, 2013; Milner, *et al.*,2011).

Most of the released merozoites continue the asexual cycle and more red blood cells are infected while few differentiate into male or female gametocytes. The gametocytes cause no symptoms but can circulate in the bloodstream until they are ingested by female anopheline mosquito (Arora and Arora, 2010). Some parasites may remain dormant in the liver as hypnozoites and may cause a relapse by reactivating after many months. This is common with P. *vivax* and P. *ovale* infections but not with P. *falciparum* and P. *malariae*. (WHO, 2013; Imwong *et al.*,2007) On the other hand, there may be conditions when the infection is not eliminated by immune system or by therapy and the number of infected red blood cell begins to increase again with subsequent clinical symptom. This phenomenon is known as

recrudescence (Arora and Arora, 2010).The pathogenesis of malaria is complex and entails immunologic and non-immunologic mechanisms that result in alteration and dysfunction of some tissues or organs. Such dysfunction sometimes, leads to metabolic acidosis and localized ischemia (Miller *et al.*,2002, Abrams *et al.*,2005).Glycosylphosphtidylinositol (GPI) is a surface molecule found on the membrane of *P. falciparum.* It is a pathogenic factor and has the ability to induce the secretion of TNFα and IL-1 (Naik *et al*, 2000). It may bind to the membrane of uninfected erythrocytes to induce membrane alterations and may play a role in increased clearance of uninfected red blood cell (Brattig *et al.*,2008).

Infection with *P. falciparum* primarily may result in sequestration. Sequestration is the binding of infected red cell to the endothelia linings and may occur in blood vessels and capillaries of the brain, placenta and other internal organs. Sequestration and aggregation of infected erythrocyte can lead to removal of parasites from the peripheral circulation and may results in partial blood flow, breakdown of barrier endothelial, and inflammation (Newbold, *et al.*,1999). In the brain, it could contribute directly to cerebral oedema and raised intracranial pressure (Miller*et al.*,2002). Furthermore, *P. falciparum* parasitized red blood cells may adhere to the endothelial walls of the host in a phenomenon known as cytoadherence. Cytoadherence may be by any of the parasite receptor surface molecules such as thrombospondin (TSP), CD36, Intercellular adhesion molecule-1 (ICAM-1), E-selectin, chondroitin sulphate A (CSA) and hyaluronic acid (HO) (Carvalho *et al*, 2013; Beeson *et al*,2000).

Moreover, infected red cells may stick to uninfected red cells to form rosettes. Rosette formation is the adhesion of two or more uninfected erythrocytes to infected erythrocytes. It is mediated by an interaction between *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) and receptors such as complement-receptor 1on the surface of uninfected red cells (Chen *et al.*,2000; Francischetti, 2008). Rosetting may lead to damage of the subendothelium.

Damage of subendothelium of vascular wall(s) may in one aspect, initiate the activation of coagulation cascade. This in turn, may initiate the formation of thrombosis. On the other aspect, it may initiate bleeding (Kawthalker, 2008). The intracellular parasite derives energy by anaerobic glycolysis to yield lactic acid; contributing to hypoglycaemia and lactic acidosis (Daily,*et al.*,2007). Again the red cell membrane may appear less deformable, resulting in haemolysis and accelerated splenic clearance (Brattig, *et al.*,2008).

The clinical presentationof malaria occurs during the blood stage of the infection. Sequestration, Cytoadherence and Roseting may result in interwoven processes that inducethe generation of reactive oxygen species (ROS), release of cytokinesandanaemia. ROS is a collective noun used to describe oxidizing compounds such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), superoxides (O_2^-), lipid peroxides and other related oxidant radical species. They are produced as physiological responses to specific reactions. Their production is regulated by the antioxidant defense system. It is reported that at low concentration, ROS may contribute to the elimination of malaria parasites.However excess production of ROS may damage the vascular endothelial lining especially in celebral malaria(Kulkarni *et al.*,2003).

Within the erythrocytes, ROS could be sequestered by plasmodial degradation of haemoglobin (Pradines *et al.*,2005). Again ROS are generated through the interaction of the parasite and the phagocytic systems. Some of these radicals attack the plasma membranes and haemoglobin and can further damage uninfected erythrocytes. Erythrocytes are rich in polysaturated fatty acids and are more vulnerable towards oxidative stress. The reactive oxygen species can cause severe biochemical changes such as lysis of erythrocytes and alterations of erythrocytes antioxidants (Kumar et al., 2004). One of the consequencies of oxidative stress is the development of malaria anaemia (Das and Nanda, 1999; Kremsner *et al.,* 2000; Egwunyenga *et al.*,2004)

The number of exposure to mosquito bites by individuals had been confirmed to increase the level of immunity against malaria infection (Vanderberg and Fervert, 2004) and this increases with age. Thus in *P. falciparum* endemic areas, protective immunity against malaria infection is acquired slowly after a large number of infection and its maintenance requires a sustained exposure to infected mosquito (Akanbi *et al.*,2009). The level of immunity against malaria could be related to age of the individuals living in malaria endemic areas.

Moreover, it has been reported that a large number of changes can cause alterations on the shape and physiology of the red blood cells (Braga *et al.*,2000). Destabilization of red blood cells by antimalarial drugs has been reported (Guha *et al.*,2006) and could be due to rapid production and accumulation of ROS in most cases, causes overwhelming failure of the defense apparatus that sustains the intergrity of the erythrocyte membrane and the depletion of glutathione concentration.

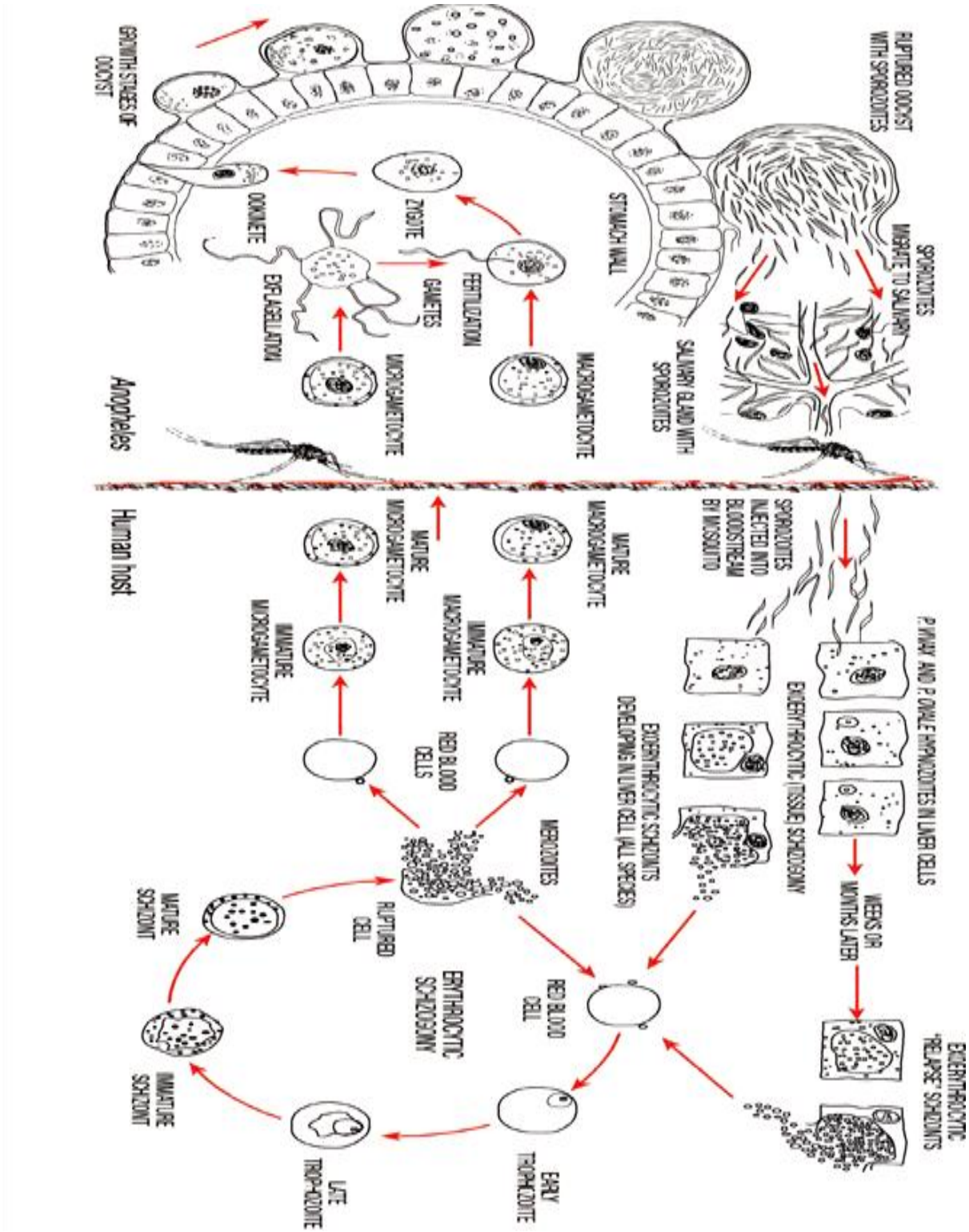


Fig 2.1: Diagram showing life cycle of malaria parasite (WHO, 2013)

2.1.5 IMMUNITY TO MALARIA

The immune response to malaria is triggered by phagocytosis of the parasites,haemozoins and the parasite toxins (Prato*et al.*,2005; Prato *et al.*,2008). It also depends on the intensity of transmission because individuals in malaria endemic or stable areas such as sub-Saharan Africa acquire some level of protection before adulthood while individuals in low and unstable transmission areas such as Southeast Asia fails to develop complete immunity and remains at risk of clinical infection (Dondrop *et al.*,2008; Osier *et al.*,2008; Doolan*et al.*,2009). Immunity to malaria could be innate (natural) or/and adaptive (aquired). The adaptive immunity could be humoral or cell-mediated (Warren, 2010).

The innate immunity is mainly through phagocytosis of the parasite by monocyte and granulocyte, often with induction of acute phase proteins and the activation of complements. There are some factors that helpred blood cells to resist succumbing to malaria. Such factors include the natureof haemoglobin molecule and red cell antigens. For example, genetic alteration of haemoglobin molecule in sickle cell is evolved in part to survival against P. *falciparum* infections(Aidoo, *et al*,2002; Williams*et al.*,2005; Arora and Arora, 2010).

Again, red blood cells of individuals with thalassemia appear to be susceptible to P. *falciparum* invasion but with significantly reduced parasite multiplication (Veenemans *et al.* 2008). This may be due to the variable degree of persistence of haemoglobin F, which is relatively resistant to digestion by malarial haemoglobinases (Clegg and Weatherall, 1999).In addition, absence of the Duffy blood group antigen on red cells (mostly seen in individuals from West and sub-Saharan Africa) is protective for P. *vivax* malaria (Arora and Arora, 2010).

Furthermore, some leucocytes such as Natural killer (NK) cells are activated whichsubsequently secrete cytokines. Most cytokines, IFNγ, for instance, facilitate the killing of parasites (Artavanis-Tsakonas and Riley, 2002).

The humoral immune response to malaria involves the activation of ß-cells and the synthesis of immunoglobulin molecules namely, immunoglobulin miu (IgM), immunoglobulin gamma (IgG), immunoglobulin alpha (IgA) and immunoglobulin epsilon (IgE)(Leoratti*et al.*,2008; Mibei*et al.*,2008; Arora and Arora, 2010). However, efficient protection against malaria through the humoral mechanism is not usually achieved because the parasite has little time to stay in the blood stream before entering into the liver. Also, in the blood stream, the parasite lives inside the red blood cell thus making it difficult for the organism to have direct contact with the antibodies (Arora and Arora, 2010). In addition, antibodies neither attack intracellular microbes nor enter the liver cells (Venugopal, 2007). Another reason adduced to the ineffectiveness of immunoglobulin molecules to ensuring protection against malaria is the incessant alteration in the malarial antigenic epitope occasioned by changes that occur at different stages of schizogony (Arora and Arora, 2010).

The cell mediated response appears to be the most effective adaptive mechanism through which protection against malaria is achieved (Prato *et al.*,2005; Arora and Arora, 2010; Warren, 2010). The cell mediated immune response is stimulated with the binding of malarial antigenic epitope and its presentation to the T-cells by the antigen presenting cells (APCs). This is accompanied by the induction of cytokine molecules (Venugopal, 2007). The recognition of either GPI, haemozoin or malaria toxins by the antigen presenting cells (APCs) induce the secretion of cytokines and other inflammatory mediators. Specifically, the ability to mount robust secretion of interferon-gamma has been associated with protection against parasitaemia (D'ombrain *et al.*,2008).

2.1.6. PATHOPHYSIOLOGY OF MALARIA

The pathophysiologic conditionsof malaria are anaemia and microvascular associated diseases notably cerebral malaria and placental defects.However, anaemia is the most prevalent. Anaemiaoccurs morewith *P. falciparum* infectionbecause the plasmodiuminvades

erythrocytes of all ages. *P. malariae* invades only the older erythrocyteswhile*P. vivax* and *P. ovale* infect only the reticulocytes (Mehta and Hoffbrand, 2001). Initially,the anaemia ranges from moderate to mild anaemia (Hb 8g/dl - 11g/dl). Highparasite density with severe haemolysis may result insevere anaemia (Hb <7g/dl).

Malarial anaemia may occur through different mechanisms: bydirect lysis of parasitized erythrocytes, the splenic removal of infected and uninfected red blood cells (red blood cells coated with immune complexes) and autoimmune lysis of coated infected and uninfected red blood cells. Other mechanisms are increased fragility of red blood cell, decreased incorporation of iron into haem and decreased production of red blood cells due to bone marrow suppression (Arora and Arora, 2010). Also, anaemia could result with the trapping and congestion of erythrocytes in the splenic sinusoids during splenic hyperplasia, (Warren, 2010).

Onimmune mediated lysis of both parasitized and unparasitized red blood cells. It is reported that specific antibodies are produced against parasitizedred blood cellswhich interacts to inducehaemolysis. Haemolysismay continue to occur even after the parasite clearance (Ouma *et al.*,2008). Moreover, antibodies may coatunparasitized cells and thus bind to malaria antigens on the red blood cell to form immune complexes. Immune complexes may trigger lysis and are removed by erythrophagocytosisresulting in anaemia (Warell *et al.*,2002).Furthermore, malarial anaemia could arise as a result of synthesis of cytokines. Cytokine dysregulation could up-regulate endothelial adhesion molecules and converts the anti-coagulant endothelium to a procoagulant surface. It has been reported that in response to cytokines such as TNFα or Interleukin 1 (IL-1) or bacterial endotoxin, endothelial cells synthesize tissue factor; the major activator of the extrinsic clotting cascade (Mitchell, 2010). Activation of the clothing cascade in turn may initiate the formation of disseminated intravascular coagulation (DIC) and haemorrhage (Olutoya and Mokuolu,2012). Again,

anaemia could occur especially in acute malaria as a result of increased nitric oxide (NO) production. It is posited that NO suppresses the early erythroid (Keller *et al.,* 2004).

As regards to microvascular associated diseases, the parasite may sequesterin any organ of the body however, its impact is commonly demonstrated in severe cerebral malaria and in acute renal failure (Das, 2008). The placental infection is another example of microvascular associated disease. High malaria parasite density and chronic infection induces dysfunction of the placenta with its attendant adverse consequences (Fried *et al.,* 1998; Menedez *et al.*, 2000; Suguitan *et al.*,2003; Bouyou-Akotet *et al.*,2004;Panda, *et al,* 2012). It is also posited that severe falciparum malaria may cause deformities in the genital tract to make conception impossible or if conception does occur, it may prevent normal implantation and development of the placenta. (Burrow *et al*, 2004)

2.1.7. MALARIA IN PREGNANCY

Some researchers have contributed that pregnant women are more susceptible to malaria than non pregnant women (Diagne *et al.*,2000; Nnaji *et al.*,2006; Akinboro*et al.*,2010; Ter-Kulie *et al.*,2010;Tay *et al.*,2013;). Again the majority of malaria infections is low-grade, frequently sub-patent and in most case asymptomatic, undetected and untreated (Shulman, 1999; Ter-Kuile*et al.*,2004). *P. falciparum* is the only human malaria parasite that is common in pregnancy and is the only parasite with a clear and substantial adverse effect on pregnancy and pregnancy outcome (Ismail et al., 2000; Steketee *et al.*,2001).

Some maternal factors associated with malaria include maternal age, parity, gestational age and gravidity (Takem and D'Alessandro, 2013).Their effect is associated with loss of immunity (Brabin, 1983). However, reports on the effectswere inconsistent. In one aspect,some reports contributed that adolescence has higher prevalence and higher risk of malaria infection than older women(Poespoprodjo *et al.*,2008; Ayoola *et al.*,2012). In

addition, primigravidae are at higher risk than multigravidae (Agbor-Enoh, *et al*, 2003; Shulman and Dorman, 2003; Kalilani *et al.*,2010; Valea*et al.*,2012; Matthew*et al.*,2013;Wogu *et al.*,2013;). Coulibaly *et al.*,(2007) reported that primigravidae had significantly higher risk of malaria infection than multigravidae.In Kenya, the rate of malaria infection with primigravidae was 85.7% while that for multigravidae was 51.7%(Brabin, 1983). In Ghana, Tay *et al.*,(2013)reported that age and gestational age of pregnant women were factors affecting malaria parasitaemia and recorded that primigravidiae were more affected than multigravidae. In Sokoto, primigravidae and secondegravidae demonstrated more vulnerability to malaria parasitaemia than multigravidae (Panti *et al.*,2012).Again, gestational age from several works haveshown that malaria infectionpeaks more in the first and second trimesters(Nduka *et al.*,2006; Saba*et al.*,2008;Wogu *et al.*,2013). However,Akinboro *et al.,* (2010) reported no correlation between malaria parasitaemia, age, gravidity and trimester.

2.1.8. PLACENTAL MALARIA

The placenta is the primary link between the mother and the foetus. It grows throughout pregnancy and separates the maternal and foetal circulation(Ashwood and knight, 2006). It nourishes the foetus, eliminates foetal wastes and produces pregnancy hormones (Ashwood and knight, 2006). The placenta is composed of large collection of foetal vessels called chorionic villi that sprout from the chorion to provide a large contact area between the foetal and maternal circulation. The villi are surrounded by the intervillous space. In mature placenta, the maternal blood enters the intervillious space through endometrial artries (spiral artries) and circulates around the villi allowing for exchange of gases and nutrients. The diffusion of gas and nutrient occurs through the villus capillary endothelial cells and thinnedout syncytiotrophoblast and cytotrophoblast. Under normal circumstances, there is no mixing between the foetal and placental blood (Ellenson and Pirog, 2010).

The placenta is also prone to malarial infection aside the peripheral blood in pregnant women, (Ukaga *et al.*,2007). Infected erythrocytes accumulate in the intervillous space, sometimes in higher density and binds to chondroitin sulfate-A in the syncytiotrophoblast (Matejevic *et al.,* 2001; Beeson*et al.*,2000). Placental infected erythrocytes express unique variant surface antigen (VSA) which mediate placental adhesion and binding to CSA and HO. However, the principal parasite ligand mediating adhesion to CSA and VSA on the infected erythrocytes surface is P. *falciparum* erythrocyte membrane protein-1 (PfEMP-1).Furthermore, the intervillous spaces are filled with macrophages that contain haemozoin, antibodies and cytokines (Ismail *et al.*,2000). Haemozoin probably remains for long period until it is diluted out by rapidly growing placenta (Rogerson and Beeson, 1999).

Chronic malaria parasite infection or high parasite density induces three specific changes in the placenta. Firstly,cellular immune response may result in the consumption of glucose and oxygen thatwould have gone to the foetus. Secondly,the cytotrophoblastic membrane thickens. This alludes to mechanical compromise of placental circulation whichresults in pathological legions and drastic reduction in the transport of nutrient to the foetus (Menedez *et al.*,2000; Panda*et al.*, 2012). Thirdly, is the disruption of the normal immune balance? This causes increased synthesis of inflammatory cytokines like TNFα, IL-2, and IFNγ (Fried *et al.*,1998; Suguitan *et al.*,2003;Bouyou-Akotet *et al.*,2004).In a study on pregnant women in Owerri, Imo state, South Eastern Nigeria, Ukaga *et al.*,(2007) observed a 29.9% prevalence of placental malaria. Furthermore, they reported that despite antimalarial therapy, a significant (P<0.05) relationshipexisted between placental malaria, gravidity, age and blood group. They alsoreported that primigravidae (15.4%) were the most infected with malaria followed by secondigravidae 9.6% while multigravidae had the least rate (4.9%) of infection. Again, younger women appeared more vulnerable to placental malaria than their older counterparts.

2.1.9 LABORATORY DIAGNOSIS OF MALARIA

Several methods have been adopted in the diagnosis of malaria infection and these include microscopic examination of stained blood films, centrifugation of blood,use of lateral-flow-immunochromatography [rapid diagnostic test (RDT) strip],and neucleic acid base detection (polymerase chain reaction) (Arora and Arora, 2010; Cheesbrough, 2010). However, microscopic examination of stained blood film is the gold standard for routine diagnosis (WHO, 2010).For the microscopic diagnosis of malaria parasite, blood film should be prepared from fresh blood samples. Two types of blood filmmay be used: thin and thick films. Thick blood film is more sensitive in detecting malaria parasite density than the thin film because blood is concentrated in the thick film and that allowsfor the examination of greater number of malaria parasites.

The thin film is air-dried, fixed with methanol and allowed to dry before staining. Thick filmsare allowed to airdry but fixed during staining.Best results are obtained when films are stained with a 2.5% Giemsa solution at a pH 7.2 for 45 minutes or 7.5% Giemsa for 15 minutes. A combined Wright-Giemsa stain can also detect malaria parasites butdoes not demonstrate schuffner's dots as reliably observed with Giemsa stain. If correctly stained, the cytoplasm of plasmodium parasiteappears blue, while the chromatin dot appears red. Malaria microscopy is prone to operator's error even with trained and experienced microscopists. Common errors that may arise in film reading are loss of identification of the parasite expecially in relatively low or sub-patent infection and over lying of an infected red blood cell by platelet. An elevated count could be obtained when precipitates arising from stains, dusts and microbial artifacts were enumerated as parasites.Therefore, the need for automation probably by using digital image analysis is required.

Estimation of parasite count is important in order to deal with discriminatory threshood of parasitaemia especially in asymptomatic cases. Besides, parasite count is important

expecially in*P. falciparum*infection which is always considered as potentially dangerous. This is because *P. falciparum* infects erythrocyte of any ageand has the potential to develop to high grade parasitaemia. Accurate estimation of malaria parasite density is necessary for patient's management, especially with increase in parasite resistance to available therapy and also in clinical trials and drugs efficacy studies. There are two acceptable methods of expressing the parasite count; either as the percentage of infected erythrocytes as inthin film or the number of parasites per unit volume of blood. The later is usually assessed on a stained thick film by counting parasites against 100, 200 or more leucocytes, then multiplying by either the patient's leucocytes count if available or a standard count of 8000µl. The use of assumed white blood cell count set by the WHO rather than the individual white blood cell count could lead to over estimation of parasite density in malaria infection (Jeremiah and Uko, 2007) or vice versa depending on several factors such as malaria burden, acceptable reference values for white blood cell (WBC) in an area and variability in the techniques adopted by film readers and other infections (O'Meara*et al.*,2005). However, no universal cut-off level has been adopted (Toure, *et al,* 2006; Dalla Marth *et al.*, 2007).Persons suspected of having malaria, whose blood films do not indicate the presence of the parasites should have blood films repeated approximately every 12-24 hours for 3 consecutive days. If the film still showsnegative, then the diagnosis of malaria parasite is unlikely.

Rapid Diagnostic Tests are based on the isolation and detection of many specific malarial parasite antigens from the blood of infected individuals and the development of corresponding antibodies directed to the target parasite antigens. The most important antigenic markers targeted for detection with RDT are histidine-rich protein II (HRP–II) and parasite lactate dehydrogenase (pLDH) (Sood, 2006). *P.falciparum* produces HRP – II and pLDH that are specific to it while *P.vivax, P.ovale* and *P.malariae* produces pLDH that are also specific but cannot be distinguished from each of the parasite species (Sood, 2006).A

new method for identification and quantitation of malaria parasite known as Quantitative Buffy Coat (QBC) has been developed by Becton and Dickson Inc. It involves staining of the centrifuged and compressed red cell layer with acridine orange and its examination under ultra-violet light. QBC is said to be fast, easy and more sensitive than the traditional thick smear examination (Arora and Arora, 2010).

In the diagnosis of malaria in pregnancy, infected subjects may be defined on the basis of pheripheralparasitaemia or placental infection (at the time of delivery). It is essential to identify malaria parasite in the placenta because peripheral microscopy may miss a large proportion of infected women whereas the parasites may be sequestrated in the placenta (Takem and D'Alessandro, 2013). Moreover, the placenta may contain large numbers of infected erythrocytes (as many as 65%) while the peripheral blood is free from parasites (Brabin, 1983). Conversely immigrants to malaria endemic areas often show high parasitaemia without heavy placental infection. The histological examination of the placenta is the most sensitive indicator of malaria infection compared with peripheral parasitaemia. The placental demonstration may show the trophozoites and/or the haemozoin (Shulman and Dorman, 2003). The presence of the trophozoites signifies active infection while haemozoin signifies either past, chronic infection or both (Ismail *et al.*,2000).In a study on pregnant and placental blood,it was observed that out of 123 pregnant women, 93 had placental infection without peripheral parasitaemia while 30 had peripheral parasitaemia without placental infection (Matteelli *et al.*,1994).Again, in a work on placental malaria Menedez *et al.*,(2000) observed 75.5% of the women with placental malaria of which 9.5% had the trophozoities, 40.3% had pigments while 25.7% had both trophozoites and pigment but there was no correlation between parasite density in the peripheral blood and that of the placenta.

2.1.9.1 PREVALENCE OF MALARIA IN PREGNANCY

Review of studies in sub-Saharan Africa between 2000 and 2011 (Takem and D'Alessandro, 2013) showed the prevalence of malaria among pregnant women attending antenatal clinic at 29.5% in East and South Africa and 35.1% in West and Central Africa. In Kenya, Ouma *et al.*,(2008) observed a prevalence of 18%. In Ghana, Tay *et al.*,(2013) recorded prevalence of 12.6%. In Cameroon, Achidi *et al.*,(2007) recorded the prevalence of 25.4%.

The hospital based data varieswithin the country. In Lagos metropolis, Western Nigeria, the prevalence of *P. falciparum*on pregnant women attending antenatal clinic in a one-year study stood at 88.5% (Iriemenam *et al.*,2011). Anorlu *et al.*,(2001) recorded 77.8% prevalence with 44.2% in primigravidae and 33.6% in multigravidae. In a study in Oshogbo, Southwestern Nigeria on 250 pregnant women on regular antenatal visits Akinboro *et al.*,(2010) reported the prevalence of malaria at 63.6% and mean parasite density of 461.33. The prevalence of malaria was highest among women in first pregnancy and between 26-30 years while the least prevalence was recorded in those above 40years. No correlated relationship was established between parasitaemia and age, gravidity and packed cell volume.

In Makurdi, Benue State, North central Nigeria Jumbo*et al.*,(2011) recorded the prevalence of 42.4% with *P.falciparum*. In South-South Nigeria, a prevalence of 26% and 20.3% were recorded at different periods in Port Harcourt while 70.1% was reported at Calabar (Buseri *et al.*,2008; Jumbo *et al.*,2011; Okafor *et al.*,2012; Wogu *et al.*,2013).

In South East Nigeria, different prevalence rates have been reported from various rural communities.Ogbodo *et al.*,(2009) in a study on 272 asymptomatic pregnant women in Ebonyi State, reported a prevalence of malaria parasite at 59.9% with the highest prevalence occurring in the first trimester. However, 47.2% had mild malaria while 15.3% had severe malaria. Furthermore, the distribution of malaria densities in different gravidity groups

showed significant inverse correlation. In addition, the prevalence of anaemia in the study was 62.4%. At Abakaliki Ebonyi State, the prevalence of malaria in pregnancy was 29% (Nwonwu *et al.*,2009). In Enugu, Enugu State, a prevalence of 21.3% was recorded (Ekejindu, 2006). In Owerri, Imo State a prevalence of 16% was reported (Uneke *et al.*,2007) while in Okigwe and Aba in Abia State, Nduka *et al.*,(2006) reported 52.6% and 56% malaria infected pregnant women respectively. Furthermore, anaemia was observed in 94.07% of the malaria infected pregnant women and it was significantly higher in women with higher parasitaemia.

2.1.9.2 EFFECT OF MALARIA ON PREGNANCY

Malaria exerts adverse effect on mothers, foetus and new born. The effect of malaria on the mother may range from negligible to severe conditions and this depends on the level of immunity the mother had acquired prior to pregnancy, the efficacy of the response during the periodand the parasite density (WHO 2004).However, the major effect is maternal anaemia and reduced birth weight of new born (Brabin and Rogerson, 2001; Guyatt and Snow 2001;Saba *et al.*,2008; Takem and D'Alessandro, 2013). Douamba *et al.*,in 2012,worked on asymptomatic malaria in pregnant women at Ouagadougou, Burkina Faso. They used Rapid diagnostic test and microscopy respectively as research techniques. Their finding revealed prevalence of *P. falciparum* asymptomatic infection in pregnant women at 20% and 30% respectively.Anaemia was reported at 61% and was significantly common with *P. falciparum* infected pregnant women compared with the uninfected pregnant counterparts. The parasite density was 4,058 parasites/µl. In Kenya, it was reported that malaria infection contributed to 69.1% of maternal anaemia (Ouma *et al.*,2008). In Ghana,Wilson*et al.*,(2010) recorded 36% cases of anaemia due to anaemia in pregnant women.

In a study on prevalence of anaemia in women with asymptomatic malaria parasitaemia at first antenatal visit at Calabar, Nigeria, Agan *et al.*,(2010) reported anaemia at 59.6% and

parasite density of 1297±1234 on primigravidae and 661±492 for multigravidae. They also reported significant association between severity of parasitaemia and degree of anaemia.

In another work on the impact of anaemia and malaria parasite infection in 414 pregnant women attending antenatal clinics in Calabar, (Okafor *et al.*,2012) reported a prevalence of malaria at 70.1% and anaemia at 61.1%. Among the apparently healthy, control, non-pregnant women, the prevalence of malaria was reported at 60.8% and anaemia at 38.3%. The primigravidae were more susceptible to parasitaemia especially *P. falciparum* with a mean parasite density of 1962.50 ± 220 parasite/µl of blood than the multigravidae with a mean parasite density of 446.7 ± 296 parasite/µl. Malaria parasite increased significantly with gestational age but anaemia was more prevalent in 2nd trimester than the other trimesters. There was a negative correlation between haemoglobin and parasite density in both pregnant and non-pregnant women. They concluded that malaria anaemia caused by *P. falciparum* had serious effect on pregnant women living in the study area. Two other different reports, recorded malaria anaemia at 20.3% and 71.6% (Buseri *et al.*,2008; Jumbo*et al.*,2011).

A study in three rural or semi-rural areas in South Benin, and on 3591 pregnant women from 1st trimester to delivery, Ouedraogo *et al.*,(2012) reporteda constant association between haemoglobin concentration and gravidity. They reported that haemoglobin concentration was significantly lower in primigravidae than multigravidae at first antenatal visit ($P<0.01$). Again, iron deficiency anaemia was morein multigravidae which also decreased slightly as pregnancy progresses from first antenatal visit till delivery. Moreover, an association has been suggested to exist between the parasite density and anaemia.This was shown in a study by Onyenekwe *et al.*,(2004). They recorded mean parasite density of 262±190 parasites/µl of blood in the blood of asymptomatic pregnant women and mean packed cell volume of 33.6 ± 41.

Apart from anaemia, some investigators have reported that women who become infected with malaria during pregnancy have an increased risk of delivering low birth weight babies following preterm delivery or intra-uterine growth retardation (Suguitan*et al.,*2003; Cottrell *et al.,*2007). Cases of perinatal mortality, spontaneous abortion or still birth due to malaria have been persistently reported (Coulibaly *et al.,*2007, Ukaga *et al.,*2007, Saba *et al.,*2008).

The foetus is not spared from the effect of maternal malaria as it causes the delivery of low birth weight babies (<2.5kg or 2500g) (Uneke *et al.,*2007). Low birth weight is either caused by preterm delivery (PTD) or foetal growth restriction (intra-uterine growth retardation). The molecular mechanisms underlying PTD differs from that of foetal growth restriction (FGR). It is suggested that acute infection, high parasitaemia and malaria anaemia areassociated with PTD (Menedez *et al.,*2000) whereas either maternal anaemia, chronic placental infiltrate or both factors and increased levels ofinflammatory cytokines such as TNFα and IL-8 are associated with FGR (Menedez, 1995; Steketee *et al.,*1996; Fried and Duffy, 1998). Mooremann*et al.,*(1999) showed that elevated TNFα and Interleukin-8 (IL-8) expression in placenta were associated with low birth weight. Low birth weight is a documented risk factor for poor neuro-sensory, cognitive and behavioural development, limited school performance and academic achievement (Taylor *et al.,*2000). Low birth weight infants have higher risk of dying during the post neonatal period than normal weight babies (Yasmin *et al.,* 2001; Bloland *et al.,* 1995).

2.1.9.3 ADAPTIVE IMMUNE RESPONSE IN MALARIA IN PREGNANCY

In pregnancy, immune response to plasmodium is reduced or depressed and this has been attributed to immunological and hormonal changes (Rogerson*et al.,*2007). The cell-mediated immunity is reduced by the inhibition of type-1 cytokines;as such protection to diseases by way of killing intracellular microbes such as malaria, tuberculosis, Human Immuodeficiecy Virus (HIV) and leshmaniasis is lost hence the risk of malaria and other infection increases

(Matteelli *et al.*,1994). Moreso,malaria causes anaemia (Ekejindu 2006; Buseri, 2008; Saba, 2008) and anaemia further depresses the immune system of pregnant women (Mor and Cardenas, 2010). In addition, the humoral arm seems not to mount efficient immune response to malaria because antibodies produced are directedagainst the surface of infected erythrocytes whereas the parasite is intracellularly located (Venugopal, 2007). Moreover, it is reported thatantibody synthesis occurs latein pregnancy. It is evensuggested to be absent mostly in the first trimester (Ismail *et al.*,2000). The issue becomes clearer with the report on gravidity dependent increased protection against developing severe placental malaria which stipulated that both primigravidae and multigravidae have antibodies at term and that antibody production increases with increasing gravidity (Ricke *et al.*,2000). The postulation wasstronger with the contribution that most primigravidae have low levels of antibodies prior to ~ 20 weeks of gestation while multigravidae lacks antibodies until ~12 weeks of gestation; and afterwards produces antibodies (Iona*et al.*,2001). Conclusively, the early onset of efficient antibody response in multigravidae and the delayed production in primigravidae appear to account for the gravidity dependent differential susceptibilities of pregnant women to placental malaria (Iona*et al.*,2001).The hallmark of the depressed immune system in pregnancy points to the importance of protective immunity that is specific for parasite variants to ensure the well-being of the mother and the foetus (Ricke *et al.*,2000).

2.2. CYTOKINES

Cytokines have been defined as small, non structural glycosylated or non-glycosylated multifunctional molecules (polypeptides and proteins) that are transiently secreted by cells in response to stimulus. They modulate the behaviour of target cells and mediate various physiological responses primarily that of host defence mechanism (Ikram *et al.*,2004). Similar definition is that cytokines are non-antibody molecules with multiple and overlapping

functions. Such functions include activation, proliferation, differentiation and maturation of many different immune cells (Dinarello, 2007, Elgert, 2009).

Cytokines are classified into three distinct functional classes although they may belong to multiple categories (Kumar, *et al.,*2004). They are cytokines of the innate immunity, the adaptive immune response and those that stimulate haemopoiesis. Cytokines of the innate immunity are produced principally by macrophages, dendritic cells and NK cells. They mediate inflammation and antiviral defense in response to microbes and other stimuli. The cytokines in this group include TNFα, IL-1, IL-12 and IFNγ. Those of the adaptive immune response are produced primarily by $CD4^+$ lymphocytes in response to antigen or other signals. They function to promote lymphocyte proliferation, activation and differentiation of effector cells. The main ones in this group are IL-2, IL-4, IL-5, IL-17 and IFNγ. The cytokines that stimulate haemopoiesis are called colony stimulating factors because they are assayed by their ability to stimulate formation of blood cell colonies from bone marrow progenators. Their functions are to increase leukocyte numbers during immune and inflammatory responses and to replace leukocytes that are consumed during such responses (Kumar, *et al.,*2004).Still on functional aspect, cytokines are also classified aspro and anti inflammatory cytokines. The pro-inflammatory cytokines promote inflammation. They include IFNγ, interleukin-2 [IL-2], and TNFα whereas the anti-inflammatory or regulatory cytokines suppresses or inhibit inflammatory response. These include interleukin 4 [IL-4], interleukin 10 [IL-10] and transforming growth factor beta [TGF-β]. Pro-inflammatory cytokines, also known as type-1 (T_H1) cytokines, drive immunity to cell mediated response whereas regulatory cytokines, also known as type-2 (T_H2), drive immunity to humoral response with subsequent production of antibodies (Venugopal, 2007).

Few cytokines induce the differentiation of sub-types. For instance;IFNγ and IL-12 are responsible for the differentiation of T_H cells (T_H0) into T_H1 subset; (IL-2, IL-12, IFNγ,

TNFα) while IL-4 is responsible for the differentiation of T_H0 to T_H2 subset; (IL-4, interleukin 5 [IL-5], IL-10, IL-13)(Venugopal, 2007). The third subset is called T_H17 subset. Interleukin- 17 (IL-17) is the major cytokine responsible for such differentiation. These include IL-6, IL-17, TNFα and TGFβ. T_H17 cytokines are powerful recruiters of neutrophils, eosinophil and monocytes (Steinmann, 2007). T_H17 cytokine subset plays a major role in several inflammatory diseases and profersa defense barrier against bacterial and fungal infections in which multiple inflammation is a prominent feature (Kumar *et al.*,2004).The rate at which cytokines drive immune response depends on their concentration. They are extremely potent even at very low concentration (10^{-9} – 10^{-15}M) (Venugopal, 2007). For example, at low concentration, IL-1, IFNγ and TNFα induce the expression of adhesion molecules on endothelial cells causing leucocytes in the circulation to adhere to the walls of the blood vessels at the site of infection (Elgert, 2009;Carl *et al.*,2006). But at higher concentration, TNFα or in conjunction with other inflammatory cytokines induce cachexia and intense fever (Warren, 2010). It has been reported that TNFα or IL-1 at an increased concentration is responsible for hypogylcaemia, anaemia and apoptosis (Venugopal, 2007).

In some pathological conditions, production of most cytokines is increased. This may directly or indirectly account to their rate of involvement andreflectsin the severity of the disease(s) and could serve as prognostic marker in disease conditions (Carl *et al.*,2006). For instance, excess production of TNFα and other cytokines depresseserythropoietic activity by inhibiting the growth of erythroid progenitor cells resulting in bone marrow suppression, ineffective erythropoiesis and dys-erythropoiesis and further increasing the severity of anaemia. In addition, it is also believed that excess secretion of TNFα in malaria parasitaemia causes acidosis by inducing changes in the body carbohydrate metabolism (Elgert, 2009).

Marked local inflammatory response causes cytokine to spill into the general circulation culminating in detectable levels in biological fluids such as serum, plasma and amniotic fluid

(Carl *et al.*,2006; Venugopal, 2007). Cytokine analysis provides useful information that establishes the presence of an activated immune response or asinflammatory marker as well as guide targeted to therapeutic regimen designed to reduce inflammation.

The cytokines secreted during malaria infection mediates the killing of malaria parasite and are critical intermediates for clinical manifestations(Mendis *et al.*,1990; Torre *et al.*,2002).Themechanisms whereby cytokines affect killing of malaria differ in intra-erythrocytic and intra-hepatic stages of the infection. For instance, it is reported that unidentified serum complementary factors mediates intra-erythrocytic stage involing TNFα and IFNγ while intra-hepatic stage mainlywith IFNγ is independent of serum complementary factors (Mendis *et al.*,1990).It is evident that the pathological alterations and outcome of diseases depend on reciprocal regulation of the pro and anti-inflammatory cytokines; high levels of pro-inflammatory (type 1) cytokines for example, in malaria, TNFα and IFNγ have been associated with severe pathologies whereas low levels of regulatory (type-2) cytokines such as TGFβ and IL-10 have been associated with acute malaria. Also, marked imbalance between type-1 and type-2 cytokines found in the blood could determine the severity of the infection (Perkins *et al.*,2000, Medina *et al.*,2011).

2.2.1 Interleukin-4 (IL-4)

IL-4 is a glycoprotein with molecular weight of about 15,000 – 20,000 Daltons. It is secreted by activatedT_H2 and mast cells. IL-4 is a regulatory cytokine and suppresses the cytokine secretion of T_H1 cells. It enhances the proliferation and activities of eosinophils and mast cells. As a result of its role in proliferation and activities of eosinophils and mast cells, it is suggested to play a central role in allergic reaction. Besides it induces class switching to IgE (Venugopal, 2007).

2.2.2 Interleukin-6 (IL-6)

IL-6 has a molecular weight of about 22,000 – 30,000 Daltons. It is produced primarily by macrophages (Warren 2010) and lots of other activated T-cells (Venugopal, 2007). It is a multifunctional cytokine andis involved in various biological processes eitheras pro or regulatory activities. However, it plays essential role in the hepatic synthesis of acute phase reactants (Warren. 2010), the regulation of inflammation as well as final differentiation of blood cells. Currently, it is known to induce the synthesis of hepcidin by the hepatocytes (Neimeth*et al.*,2003)

2.2.3 Interleukin 10 (IL-10)

IL-10 has a molecular weight of about 18,000 Daltons. It is produced by activated T_H2 cells, CD8+, Monocyte and B-cell. It is a potent anti-inflammatory cytokine that inhibits the action of most proinflammatory cytokines. It is the principal type-2 cytokine that attenuates cell mediated immune reaction and regulates a balance in favour of T_H2 or humoral immune response (Venugopal, 2007). IL-10 is known to inhibit the protective immune response against malaria parasites or suppressing severe pathology during plasmodial infection (Mamoru *et al.*,2011)

2.2.4 Interferon Gamma (IFNγ)

IFNγ is an 18,000 Dalton polypeptide that is secreted by activated T_H1 subset and NK cells (Venugopal, 2007). It is a potent activator of the phagocytic activity of macrophages, NK cells and neutrophils. It plays a crucial role in the clearance of intracellular pathogen (Stevenson *et al.*,1990;Gazzinelli *et al.*,1994) and tumour cells (Warren, 2010). Again IFNγ is required in the differentiation of T_H0 cells into T_H1 (Warren, 2010). IFNγ mediated responses have been shown to be involved in protection against infection with *P. falciparum* (Luty *et al.*,1999).It is a critical mediator of immunity to malaria and is generally associated

with protective mechanisms (Ferreira *et al.,*1986; Herrera *et al.,*1992*).* It is established that IFNγ has antiviral, anti-proliferative and immuo-modulatory activity (Kishimoto *et al.,*2002).

2.2.5 Tumour Necrosis factor alpha (TNFα)

Tumour Necrosis Factor alphais a proinflammatory cytokine produced predominantly by macrophages. It enhances the killing of intracellular parasites and may induce cachexia. The induction of cachexia is by inhibiting lipoprotein lipase in adipose tissue thereby reducing the utilization of fatty acids. In addition, it is a potent pyrogeninducing fever and septic shock (Warren, 2010). It is suggested that TNFα plays an important role in rheumatoid arthritis as it is elevated in such disorder (Venugopal, 2007).

2.3 CYTOKINES AND PREGNANCY

Report exists that under normal condition changes occur in the cytokine profile of the pregnant women and in transformations that take placein the maternal systemic and at thematernal-foetal interface to ensure successful delivery of healthy infants (Sacks, *et al.,*2003; Kraus *et al.,* 2010). During pregnancy, semi-allogenic foetal tissue is directly exposed to the maternal blood which incidentally invades the maternal decidua. This may involve an attack on the foetus by the immune system of the mother. However, the foetus escapes attack or rejection; being tolerated by the induction of several agonist such as the secretion of cytokines.Theseresults in the alteration of the immune system of the mother (Lashley *et al.,*2011).The altered immune system primarily causes sensitization of the maternalcells by the foetal cells (Lashley *et al.,*2011). The sensitization of the maternal cells may occur locally at the maternal-foetal interface via processing of major histocompatibility complex (MHC) alloantigens by antigen presenting cells and/or in the systemic maternal circulation via the entering of foetal cells. The foetal cells may consist of the

syncytiotrophoblast fragments, feotal deoxyribonucleic acid (DNA), the whole cell and debris from apoptotic cells.

Some reasons have been given to explain the changes that occur in cytokine profile during pregnancy. Firstly, it is stated that the cytokines in the plasma or serum initially are predominantly pro-inflammatory (Type1) where circulating monocytes are primed to produce more T_H1 cytokines (Sacks, *et al.,* 2003). These are crucial in immuno-surveillance against pathogens (Szoba *et al.*,2003).

As pregnancy progresses, the immune system especially at the maternal-foetal interface gradually becomes biased towards humoral defence mechanism, invert the cytokine ratio and becomes dominant with regulatory (Type 2) cytokines (Murphy *et al.*,2009). The systematic transition to type 2 cytokine is required to avoid overexpression of type 1 which could be harmful and may compromise the viability of the foetus (Wegmann *et al.*,1993). Also the shift to type-2 cytokine dominance is most vital forthe protection of the foetal allograft which allows pregnancy to grow without rejection (Germain *et al.*,2007). These reasons could account to why the foetal trophoblasts and maternal leukocytes secrete predominantly Interleukin 10 (IL-10) and cytolytic type responses to prevent initiation of inflammatory responses and damage of the maternal-feotal placental barrier (Lin*et al.*,1993; Bennet*et al.*,1999). Consequently, asuccessful pregnancy is characterized by predominance of T_H2 cytokines at the maternal foetal interphase which downregulates or suppresses the potential harmful effect of the T_H1 cytokine profile (Fried *et al.*,1998).

Furthermore, the changes that occur in the cytokine profile of normal pregnancy results in the balance in the ratio of T_H1/T_H2 cytokines. Some researchers posited that there is usually a balance in the level of T_H1/T_H2 cytokine in normal pregnancy (Bartha *et al.*,2003;Keelan *et al.*,2003; Parasisi *et al.*,2003). The balance between pro and anti-inflammatory cytokines is essential for implantation, placental development and pregnancy outcome (Niederkorn,

2006). Changes or imbalance in the ratio of T_H1/T_H2 cytokine in favour of T_H1 cytokine could be associated with adverse pregnancy outcome such as gestational complication, spontaneous abortion, foetal loss, preterm delivery and ecclampsia (Raghupathy *et al.*,2000). In a study in Kuwait and in determining the cytokine production by maternal lymphocytes during normal pregnancy (Raghupathy *et al.*,2000) shows that T_H2 cytokines (IL-4 and IL-10) were present at significantly greater concentration in women in first trimester of normal gestation than women undergoing unexplained recurrent spontaneous abortion. In contrast, the T_H1 cytokines (IL-2, IFNγ, TNFα and TNFβ) production were higher and statistically significant in pregnant women in unexplained recurrent spontaneous abortion in the first trimester. Again, the ratio of the various $T_H1$to T_H2 cytokines showed thatT_H1/T_H2 ratio in all the combination were higher and in favor of T_H1 in recurrent spontaneous abortion group than in the first trimester normal group. Overall they suggested a bias towards T_H1 in recurrent spontaneous abortion group.

The sources of plasma cytokines in pregnant women are the monocytes from the maternal circulation, uterus, endometrium and the trophoblast (Murphy *et al.*,2009). Other cells include activated cluster differentiation 4($CD4^+$) and cluster differentiation 8 ($CD8^+$) T cell (Sargent *et al.*,2006; Germain *et al.*,2007). Two different opinions exist as to the contribution of natural killer (NK) cells on the secretion of cytokines.An aspect maintains that NK cells secretes cytokines that are found in increased number at the placentation site in early gestation (Croy *et al.*,2006, Murphy *et al.*,2009) while the other opinion reported complete absent of NK cell (Ordi *et al.*,2001) and adduced that the secretion of IFNγ may have originated from other immune cells. Perhaps, the secretion is by NK cells, it could have come from the innateresponse (Artavanis-Tsokona and Riley, 2002). Howeverthe modification,the alteration that culminates in the shift to type-2 bias commences during the first trimester.In Canada, Aris *et al.*,(2007) studied the secretion of IFNγ and IL-6 as biomarkers of T_H1/T_H2

immune status through pregnancy in 35 healthy pregnant women from 10- 40 weeks of pregnancy. They contributed that throughout pregnancy, the concentration of IFNγ in maternal serum decreases significantly with increasing gestational age. The values mean and standard deviation (SD) were 3.4±1.0pg/ml, 1.6±0.6pg/ml, 0.35±0.35pg/ml at 10-20 week, 20- 30 weeks and 30- 40 weeks respectively. IL-6 increased significantly during pregnancy 0.73±0.42 pg/ml, 6.6±1.9 pg/ml and 17.2±5.2 pg/ml at 10-20, 20-30 and 30-40 weeks of pregnancy respectively $p<0.001$. Furthermore, assessment of ratio of T_H1/T_H2 was done using IFNγ/ IL-6 which showed prevailing T_H1 bias at the beginning of pregnancy and T_H2 bias at the end of pregnancy, ratio were 4.7 and 0.02 respectively. The cytokine ratio at the middle of the pregnancy was 0.24. Moreover, switching from T_H1 to T_H2 occurred from the middle of pregnancy (week 19).

In a study titled gestational age dependent expression of IL-10 and its receptor in human placental tissue and isolated cytotrophoblasts in Rhode Island(Sharma *et al.*,2000) reported that first and second trimester placental tissues from normal pregnancies expressed IL-10 predominantly whereas the levels of IL-2, IL-4 and TNFα were mostly below detection throughout pregnancy. Again, the IL-10 diminished significantly at term in placental tissues collected before the onset of labour and did not change appreciably after labour while TNFα and IL-1ß were significantly upregulated in response to labour associated conditions. In conclusion, IL-10 was expressed in the placenta in a gestational age dependent manner and its down regulation at term may be an important mechanism underlying the subtle changes associated with parturition. The samples were collected after elective pregnancy terminations in 1^{st} and 2^{nd} trimester, elective Cesarean Sectioning with no rupture of foetal membrane and after delivery via the vaginal route.Cytokine level varies with various cytokines in normal physiologic pregnancy and non-pregnant states. This is reported in a research study in Crete, Greece, on serum levels of pro and anti-inflammatory cytokines in non pregnant and pregnant

womenin labour and those that had abortion (Vassiliadis *et al.,*1998). The data showed that the cytokine levels differ in comparison to normal physiologic pregnant and non pregnant states and there exists a statistically significant differences ($P< 0.05\pm0.001$) in the mean values of IFNγ and IL-10. Also, IL-10 increased significantly after the first trimester. Furthermore, TNFα,thought to induce pregnancy failure,showed a stable production profile in all stages. Finally, IL-10 and IL-4; regarded as protective agents during pregnancy had constant values at the first and second trimesters while IL-10showed a peak production duringlabour.

2.4. CYTOKINES AND MALARIA IN PREGNANCY

In response to invading pathogens, the normal changes in the cytokine profile are truncated and reversed to T_H1 cytokine bias. The pro-inflammatory cytokines are up-regulated. One of such conditions that induce up-regulation or over-expression of pro-inflammatory cytokines, and change the T_H1/T_H2 cytokine balance with bias to T_H1 is maternal malaria (Clark *et al.,*2006).Malaria parasiteinfection of the peripheral blood and its sequestrationin the placenta elicits immune activation with predominance in T_H1 cytokine response (Suguitan Jnr *et al.,*2003). Itdisrupts the normal immune response in the systemic and in the placenta, inducing increased synthesis of pro-inflammatory cytokines such as TNFα, IL-2 and IFNγ, contributing to adverse maternal and postnatal outcome.Several investigations have reported increased levels of pro-inflammatory cytokine (IFNγ and TNFα) in women with malaria. Again, the condition is worsened because white blood cells; essentially important at controlling malaria parasite density, also induces the release of more inflammatory cytokines.Marked increase and persistent production of TNFα paralleled by decreased production of IL-10 and TGFβ is observed in severe or complicated malaria (Torre *et al.,*2002).

In a study on cytokine profile among Human Immunodeficiency Virus (HIV) and malaria co-infected pregnant mothers and their babies at post-delivery in Saki, Southwestern Nigeria, (Adeoti *et al.*,2012) reported prevalence of malaria at 57%. They indicated a direct relationship between cytokine (IFNγ, TNFα, IL-2 and IL-10) levels and malaria parasite infection. On another study on pro-inflammatory cytokine profile, in the blood of 96 pregnant women infected with malaria in Ekpoma, Edo State, Nigeria, Nmorsi *et al.*,(2010) observed a significantly increased serum concentration of IFNγ in infected pregnant women than their uninfected counterparts and the differences were statistically significant $P<0.05$. IL-12 was depressed in the infected than the uninfected but was not significant $P>0.05$. IL-6 was significantly elevated in infected pregnant women than the uninfected pregnant women $P<0.05$. In the placenta, the mean ± SD concentration of IL-6, IL-12 and IFNγ from infected pregnant women were significantly higher than the uninfected subjects. In addition, in the sera from peripheral blood, the mean concentration of IFNγ for multigravidae was statistically lower than primigravidae counterparts $P< 0.05$. The mean ± SD concentration of IL-6 among the infected multigravidae was 52.5±39.6 pg/ml while the primigravidae was 109.5±38.8 pg/ml; the difference was statistically significant$P< 0.05$. In Ghana, Wilson*et al.*,(2010) studied the level of 27 biomarkers in 30 healthy pregnant subjects, of which 27 were asymptomatic to malaria and fifteen (15) healthy uninfected subjectsserved as control. They showed that IL-10 and Granulocyte Colony Stimulating Factor(G-CSF) were elevated and statistically higher ($P<0.05$) in the asymptomatic group when compared with the control group. On the other hand, IFNγ and TNFα showed marginal changes though not significant($P>0.05$).

Another study on serum anti-inflammatory interleukin profile by Nmorsi *et al.*,(2010) in Ekpoma, Edo State, Nigeria and among 96 pregnant women showed the level of parasitaemiaas mild and moderate. The concentration of IL-10 in the peripheral blood was

elevated in mild infection than moderate infection and the difference was statistically significant at P<0.05.IL-4 was elevated in mild infection than moderate infection while depressed levels of IL-5 were observed with mild infection than moderate infection. In the placenta, increased concentration of IL-4 was recorded with moderate infection compared with mild infection (*P*<0.05). A depressed level of IL-5 was seen in mild infection than moderate infection. On the other aspect, evaluation of iron by degree of parasitaemia showed the mean haemoglobin level to be higher in mild infection than moderate infection.

In a work on cytokine profile in peripheral, placental and cord bloodamong 87 women in an area of unstable malaria transmission in Eastern Sudan, Bayoumi *et al.*,(2008) posited that IFNγ, IL-4 and IL-10 levels were significantly higher in peripheral and placental sera of uninfected than in the sera from infected women. The levels of these cytokines were not significantly different between primigravidae and multigravidae. However, strong positive correlation existed between peripheral and placental cytokines. Furthermore, IFNγ concentration was significantly lower in the cord sera from uninfected women in comparison to the infected ones. Also cord sera in all groups showed lower levels of the three cytokines of study. They concluded that the immune responses that occur in placental, peripheral and cord blood were influenced by the malaria infections irrespective of parity. Also the immune response during *P. falciparum* infection was not different in the peripheral and placental compartment.In Sudan, Bayoumi *et al.*,(2009) worked on the susceptibility to cortisol, prolactin, and cytokinein 82 pregnant study subjects. They showed that IL-10 was significantly higher in pregnancy in uncomplicated P. *falciparum* malaria than the control subjects while IFNγ was significantly lower. On parity, the values obtained shows that parity did not appear to influence the immune responsewhen compared with the control subjects (P>0.05).

Again,in a study on 239 pregnant subjects in Yaounde, Cameroun, Suguitan*et al.*,(2003) showed the level of parasitaemiaas significantly higher in the placenta than in the peripheral blood ($P<0.05$). In addition, the levels of IFNγ, TNFα and IL-10 were significantly higher in the plasma from malaria positive placenta than in the plasma from uninfected placentas. The mean values of IFNγ, TNFα, IL-10 and IL-4 were 9.7pg/ml, 10.6pg/ml, 16pg/ml and 9.0pg/ml respectively for uninfected placentas where as the mean values of IFNγ, TNFα, IL-10 and IL-4 (11.9pg/ml, 13.4pg/ml, 32.0pg/ml and 8.3pg/ml respectively) were obtained from infected placentas. However, no significant difference was observed between malaria infected and uninfected placentas with IL-4.

In a work in Mangochi, Malawi, and in determining placental cytokine expression using cytokine mRNA in 23 malaria infected and 21 uninfected primigravidae,(Mooremann *et al.*,1999) reported a significantly($P< 0.05$) increased expression with TNFαand a significantly($P< 0.05$) decreased expression with IL-6. There was no significant difference ($P> 0.05$) in the values of IL-10 between the infected and uninfected placentas.None of the placentas contains RNA for IFNγ and IL-4.In astudy on cytokine profile among 30 women (10 primiparus and 20 multiparus) in Libreville and Lambarene, Gabon, (Bouyou-Akotet *et al.*,2004) showed differences in the mean values of TNFα in pheripheral, placental and cord blood of malaria infected and uninfected subjects. Again the mean value of TNFα by gravidity differs between the systemic and local circulation and differs also in multigravidae.
On the effect of parasite density on the rate of cytokine production, inconsistent reports exist on the rate of cytokine secretion. While some studies adopted that the rate at which cytokinesare expressed is a function of the parasite density, others indicated that parasite density does not alter the rate of cytokineexpression (Achidi*et al.*,2007; Nmorsi *et al.*,2010).

In South Western Cameroon and in a study on malaria parasitaemia and systemic cytokine bias in 174 pregnant women attending antenatal clinic, (Achidi *et al.*,2007) reported mean parasite density of 565 parasites/μl. The study also showed parasite density to be significantly ($P<0.05$) higher in younger than older women. In addition, the mean value of IFNγ was higher than the mean value of IL-4 for those in first trimester. The reverse was observed as pregnancy progresses irrespective of parasite density. Using IL-4/IFNγ to determine the ratio of T_H1/T_H2, it was shown that cytokine profile was bias towards type-2 responses in 112(84.3%) of 132 pregnant women. Furthermore, the cytokine pattern was unaffected by maternal age, gestational age, gravidity or parasitaemia.

In Tanzania, a retrospective assessment of biomarkers on*P. falciparum* was carried out on 121 pregnant women (42 infected and 79 uninfected) by Boston *et al.*,(2012) showed that the levels of TNFα and IFNγ were unaffected by infection and there were no differences between infected and uninfected individuals. The results also showed that IL-10 increased significantly ($P< 0.05$) in infected pregnant women irrespective of gestational age. But, following antimalarial treatment, the amount of IL-10 decreased to the background level. With regard to effect of gravidity, the levels of IL-10 were significantly higher ($P< 0.05$) in infected primigravidae and multigravidae but not in secondigravidae (Boston *et al.*,2012).

In a study in Kenya, titled malaria elicits type-1 cytokines in human placenta: IFNγ and TNFα associated with pregnancy outcomes, (Fried *et al.*,1998) reported that normal placentas displayed a bias towards type-2 cytokines; type-1 cytokines (IFNγ and TNFα) were absent in placentas not exposed to malaria but present in a large proportion in placentas from holoendemic areas. The concentration of TNFα and TGF-ß were significantly higher while that of IL-10 was significantly lower in placentas from holoendemic areas on comparison with those not exposed to malaria. They concluded that maternal malaria decreased IL-10

concentrations and elicits IFNγ, IL-2 and TNFα in the placenta shifting the balance towards type-1 cytokines.

In Yaounde, Cameroon, and in a study on cytokine changes in the placenta of 308 women,Suguitan *et al.*,(2003) observed that 110 of the study subjectswere infected with malaria.They showed that IFNγ was elevated in malaria infected placentathan uninfected placenta. Meanwhile, TNFαwas not higher in malaria infected placenta compared to that of uninfected placenta. Again, mean values of IL-4 was higher in infected placentas than that of uninfected placenta. Finally, mean values of IL-10 was significantly ($P<0.05$) higher in infected placentas thanuninfected placentas. Again in a study on the effects of *P.vivax* gestational malaria on the clinical and immune status of pregnant women in North Western Colombia,Yasnot *et al.*,(2013) reported that out of 35 pregnant women (15 Gestational malaria positive and 20 gestational malaria negative), proinflammatory cytokines (TNFα, IFNγ and IL-1β) were higher in the infected placenta than the uninfected placenta although IFNγ and IL-1β did not show any significant difference. The anti-inflammatory cytokines (IL-6, IL-10, TGF-ß) concentrations were lower in the infected placenta than the uninfected. In the peripheral blood, the proinflammatory cytokines were higher in the infected than uninfected group while the anti-inflammatory cytokines (IL-6, IL-10) except TGF-ß were higher in the infected pregnant women than the uninfected group. However, the changes were not statistically significant. The technique used in the measurement of cytokines was real time polymerase chain reaction (PCR);

2.5 IRON METABOLISM

Iron is the fourth most abundant element in the earth's crust. It occurs in trace amount in human beings- about 0.004% of the body's mass. It is essential in the synthesis of haemoglobin and myoglobin as well as the proper functioning of some enzymes (Pippard and Hoffbrand, 2001). Normal iron metabolism follows a regulated process such that after

ingestion, it is absorbed primarily in the duodenum, the principal site of iron absorption in man, although absorption is possible in any part of the small intestine (Pippard and Hoffbrand, 2001).About 18umol (1mg) is absorbed each day and the rate of iron absorption seems to be influenced by oxygen tension in the intestine, marrow erythropoeitic activity and the size of the body iron stores (Crook, 2012). The body iron is obtained from dietry sources mostly from meat and dark green vegetables. The dietry iron occurs as heam and non heam sources. However, non heam iron is the predominant form. Cereals, vegetables and cooking utensils are the major sources of non heam iron whereas meat appears to be the major source of heam iron (Pippard and Hoffbrand, 2001).

Absorption of heam and non heam iron assumes different processes. Absorption of non heam iron is such that the iron which exists as the oxidized form (Fe^{3+}) must be reduced to the ferrous state (Fe^{2+}) before it is transported across the intestinal epithelium. The normal gastric acidity provides an optimal environment for the reduction of ferric iron to the ferrous form.The enzyme that facilitates the reduction of iron from ferric to ferrous state is a heam oxygenase known as duodenal cytochrome B (Dcyt B) (Nadadur *et al*., 2008). The absorbed ferrous iron is imported across the apical membrane of the enterocytes by divalent metal transporter I. divalent metal transporter I is the only known intestinal iron importer (Hoffbrand and Moss, 2011). Heam iron which exists in the ferrous state could be absorbed directly by the enterocytes (Hoffbrand and Moss, 2011). The ferrous iron (Fe^{2+}) is transported across the enterocytes through the basolateral membrane being bound by ferroportin. Once internalized in the enterocytes, $Fe^{2+}$is later oxidized to the ferric state (Fe^{3+}). The oxidation from ferrous to ferric state is medicated by copper oxidase protein called hephaestin and in the plasma by Ceruloplasmin before it is bound to transferrin (Hoffbrand and Moss, 2011). Free iron is toxic until it is bound to transferrin (Crook, 2012). The iron bound to transferrin is transported to the transferrin receptor (TfR) located on the surface of cells especially cells

with increased need for iron. It is reported that the expression of TfR in the proximal small intestine is increased in the cells that have higherneed for iron such as the proliferating crypt cells (Chua *et al.*,1998).

The diferric transferrin binds to TfR to form a ligand complex. The complex enters the cell by receptor mediated endocytosis. This is followed by a series of reaction that results in the release of iron in the cytosol. The apotransferrin-TfR is recycled back to the cell surface (Pippard and Hoffbrand, 2001). Virtually all cells have TfR on their surface but more appears on the erythroid. In the marrow, not all the erythroblasts develop and mature successfully. Some die in the marrow and the iron salvaged by macrophages (Hoffbrand and Moss, 2011).

The amount of iron absorbed is seriously regulated to create metabolic balance to avoid iron overload because there is no effective excretory mechanism for iron. Iron is lost by desquamation mostly sloughing of enterocytes and from the skin. The daily loss by these routes is about 1mg. Urinary excretion is minimal as circulating iron is protein bound and not water soluble. When there is iron insufficiency, disturbance of the homeostatic process and impaired supply to the erythriods, iron deficiency will occur. Iron deficiency becomes haematologically evident only when no stainable iron is detected in the reticuloendothelial cells in bone marrow films. Conversely, iron overload is likely when reticuloendothelial storage capacity is exceeded and stainable iron is demonstrated in the parenchymal cells in liver biopsy (Crook, 2012). The classic anaemia that occurs in iron deficiency is hypochromic microcytic anaemia. However, early or mild cases of iron deficiency show microcytosis without hypochromia (Antelman *et al.*, 2000; Brugnara, 2003).

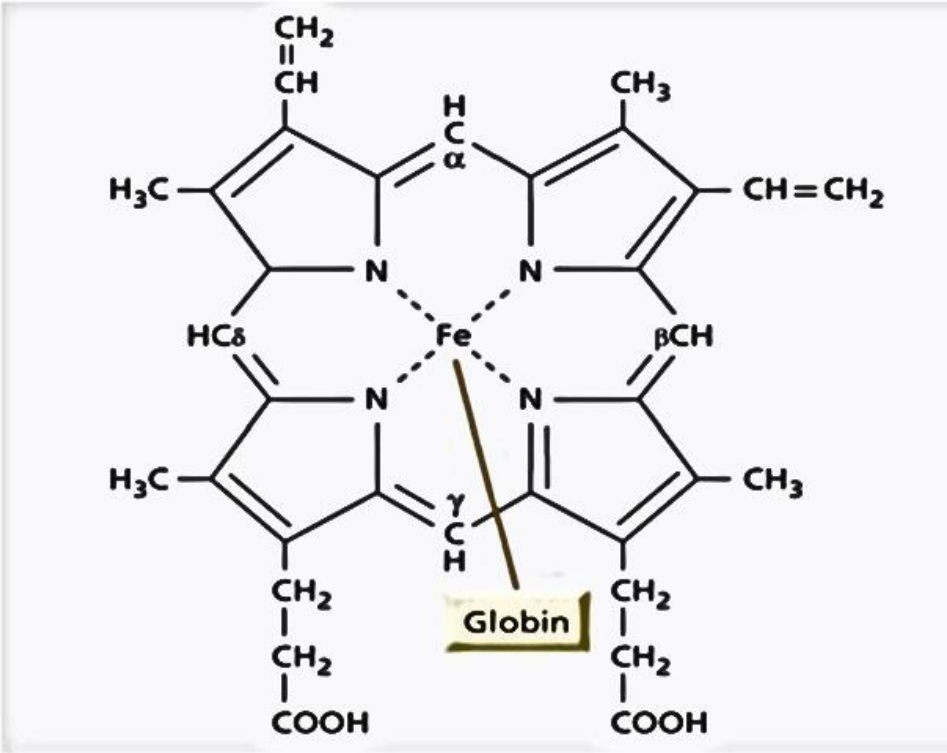


Figure 2.2: Structure of haem molecule (Hoffbrand and Moss, 2011).

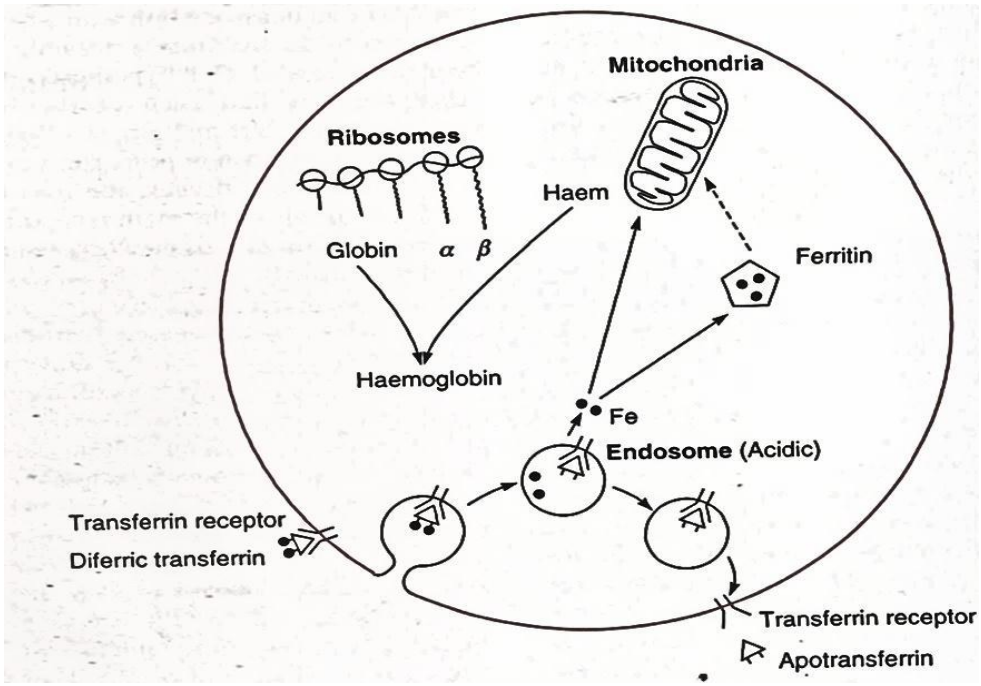


Fig 2.3: Incorporation of iron from plasma transferrin into developing red blood cells. Uptake of transferrin iron is by receptor mediated endocytosis (Pippard and Hoffbrand, 2001).

2.6 STORED IRON

Both ferritin and haemosiderin provides the stored iron. Haemosiderin, unlike ferritin, is water insoluble non-crystalline protein iron complex, probably formed by the partial digestion of ferritin aggregate by lysosomal enzymes. Ferritin is not only found in the spleen, liver and bone marrow but also in the mucosal cells of small intestine, the placenta, kidneys, testes, skeletal muscles, red cells and plasma (Crichton, 1973). It also exists in the sideroblast found in the bone marrow (Hoffbrand and Moss, 2011).In the liver most of the ferritin is stored within the parenchymal cells whereas in other tissues (spleen and bone marrow) it is stored mainly in the mononuclear phagocytes or macrophages. Ferritin consists of apoferritin (protein moiety or shell, free of iron) with molecular mass of 440 –500KDA. The shell encloses a core of ferric hydroxyl phosphate, which can hold up to 3000 - 4500 ferric atoms.

In normal subjects, the major function of ferritin is closely that of providing the store iron, which may be used for haem synthesis (Mark, 2002).Normally very small amount of ferritin (largely derived from the storage pool of body iron stores) circulates in the plasma and its level is a good indicator of the adequacy of body iron store. Serum ferritin measurement is clinically significant in detection of iron overload (very high values approaching 5,000 microgram per litre) and in the monitoring of iron status of pregnant women, blood donors and in renal dialysis. It may be used to evaluate some clinical conditions not related to iron storage such as leukemia, acute or chronic infection, inflammation and Hodgkin’s disease (Rogers, 1996).

2.7 SOLUBLE TRANSFERRIN RECEPTOR (sTfR)

Transferrin is the iron transport protein, providing iron to cells through its interactionwith the specific memberane receptor (TfR) (Beguin, 2003). It is a disulphide-linked, transmembrane glycoprotein with two identical polypeptide chains, each weighing 95,000 Daltons. TfR plays an important role in the regulation of iron metabolism by mediating the cellular uptake of

transferrin bound iron (Fe^{3+}). An inverse relationship occurs between TfR and body iron stores such that when iron stores are low, receptor expression is enhanced to acquire more iron. However, when the body iron stores become replete, down-regulation of the receptor expression occurs (Chua *et al.*,1995). In the same vein, soluble transferrin receptor (sTfR) is a truncated monomer of the transmembrane receptor (Beguin, 2003). sTfRcirculates in the plasma at a concentration proportional to the total body mass of TfR (Beguin *et al,* 1988).It is reported that increased concentration of sTfR reflects an impaired supply of iron to the bone marrow however in absence of iron deficiency (ID), its concentration reflects erythroid activity(Pippard and Hoffbrand,2001). Several researchers have reported thatsTfR is not influenced by chronic or acute inflammation; the assay has been reported to be normal in the majority of patients with inflammation (Skikne *et al.*, 1990; Carriaga *et al.*,1991).It is believed that potentially increased cytokine production does not greatly affect the TfR response caused by iron depletion(Punnonen *et al.*, 2013).Therefore, it could be used to distinguish between iron deficiency anaemia (IDA) and anaemia of chronic diseases (ACD).

2.8 REGULATION OF CELLULAR IRON TRANSPORT

Regulation of iron level in the body is important in order to provide the amount of iron required by the cells to carry out metabolic activities and to prevent excessive accumulation of iron or its toxicity to the cells. Due to the fact that there is no effectively regulated mechanism of iron excretion, iron homeostasis (maintaining normal iron level) is tied to recycling of regulated processes involving intestinal absorption, cellular transport and storage. The regulatory mechanisms are influenced by iron stores, hypoxia and rate of erythropoiesis (Hoffbrand and Moss, 2011).

Two models have been proposed to explain the regulatory mechanism of iron absorption. These are the crypt programming model; (mucosal intelligence) and the hepcidin programming model (Siah *et* al., 2006).

The crypt programming model borders on the sensing of iron by the receptors on the crypt cells. The crypts are located on the intestine villi. The model proposes that the crypt cells senses body iron levels which in turn regulates the amount of absorption of dietary iron by mature villus enterocytes. The absorbed iron is transported across the basolateral membrane by divalent metal transporter I (DMT1) (Siah *et al*., 2006).

The second model, the hepcidin model proposes hepatic synthesis of hepcidin in response to infection/ inflammation, hypoxia and iron replete states. Hepcidin interacts with the villus enterocyte to regulate the rate of iron absorption. It also regulates the retention and release of iron by enterocytes (Siah *et al.*, 2006).

At the cellular level, the intracellular iron concentration regulates the interaction of cytosolic iron regulatory proteins (IRP) 1 and 2 with iron regulatory (responsive) element (IRE). The mechanism is that in iron replete state, IRP1 contains iron- sulphur (4Fe- 4S) which does not allow IRP1 to bind to IRE. On the contrast, in iron deficiency or absence of iron stores, the amount of 4Fe- 4S is relatively low, allowing effective binding of IRP1 with IRE. The site of IRP1 binding with IREs is either at the 5' (upstream unsaturated region) or 3' (downstream unsaturated region) on the mRNA. Binding at 5'upstream unsaturated region increases the translation of ferritin mRNA and δ-aminolevulinic acid synthase (ALA-S) but inhibits the translation of TfR1mRNA. On the other hand, binding at 3' downstream unsaturated region increases the translation of TfR1mRNA but inhibits the synthesis of ferritin mRNA and ALA-S. The TfR1mRNA increases iron uptake while ferritin mRNA increases store iron (Pippard and Hoffbrand, 2001; Bentler, 2004; Siah *et al.,* 2006; Hoffbrand and Moss, 2011).

At the storage compartment, hepcidin the major iron regulating hormone binds to its putative receptor ferropotin. Such binding increases the retention and reduces the release of iron by

macrophages. The contrast is the case in anaemia and hypoxia (Ganz, 2003; Neimeth *et al*., 2003).

2.9 PATHOPHYSIOLOGY OF IRON DEFICIENCY ANAEMIA

The disorders of iron metabolism manifest in form of iron deficiency and iron overload (Virgil *et al.,*1990). Iron deficiency is when the body's iron store isdepleted and with restricted supply of iron to various tissues. This may occur in three stages. The first stage refers to decreased iron in the stores without any effect on the functional activity of the organs. Although this stage may be characterized by low serum ferritin levels however, it may be difficult to detect as traditional laboratory parameters may not have changed significantly and marked overlap in values may exist between normal and iron deficient subjects. Further depletion of iron at this stage may lead to the second stage known as iron deficient erythropoiesis.Iron deficient erythropoiesis occurs when iron depletionresults in functional deficiencies of some organs (Expert Scientific Working Group, 1985). The clear consequence of iron depletion at this stageis a reduction in oxygen transport and reduction in oxidative activity. Serum (soluble) transferrin receptor (sTfR) assay is the most sensitive index to detect early stage of iron deficiency (Baynes *et al.,*1993).

The last but severe end of iron depletion is iron deficiency anaemia. Iron deficiency anaemia is identified using the red cell profile.It is reported thatall the iron status indicators are abnormal in iron deficiency anaemia (Lynch, 2012).

2.9.1 IRON REQUIREMENT IN PREGNANCY

Approximately,1200mg - 1240mg of iron is required by the pregnant woman whilethe average need for a menstruating woman for the same period of time is about 400mg. Therefore the increased requirement is about 800mg. Theneed for iron is reduced in the first

trimester because of the absence of menstruation, thereafter the need rises steadily (Bothwell, 2000).

The increased need for iron is due to increases in the red cell mass, iron needs of the foetus, placenta and umbilical cord. It is also required to compensate for iron lost to parasitic infections such as malaria and some helminthic infectionsand gastrointestinal blood loss (Bothwell, 2000; Dreyfuss *et al.*,2000).

In developing countries however, the diets of most women do not contain sufficient amount of bioavailable iron required for pregnancy especially during the second and third trimesters (even if iron stores are adequate at the beginning of pregnancy). Therefore, maternal subjectsare prone to the risk of iron deficiency (WHO, 2001).

2.9.2 ASSESSMENT OF IRON STATUS AND ANAEMIA IN PREGNANCY

According to the World Health Organization (WHO), anaemia in pregnancy is a state in which the total circulating haemoglobin concentration is < 11g/dl (110g/L); or packed cell volume (PCV) < 33% (0.33L/L) (WHO, 2001). It is classified in terms of it's severity as mild, moderate and severe based on the haemoglobin concentration of the blood; 10g/dl-11g/dl, 7g/dl-9.9g/dl and <7g/dl respectively (WHO 2001). Anaemia is literally and interchangeably used for iron deficiency anaemia but they are not synonymous (Scholl, 2005). Anaemia is as a result of insufficient iron for the synthesis of haemoglobin and may be caused by several factor notably blood loss through heavy menstruation, gastrointestinal tract infection, malignancy, autoimmune disorders, nutritional factors (including iron deficiency) and genetic disorder such as sickle cells but iron deficiency anaemia is more specific and is caused by loss of large amount of blood or impairment in the absorption of iron in diets.

Estimates from WHO indicated that anaemia in pregnancy in developing countries stood on the average of 56%; with a range of 35% to 100% from different regions (WHO, 2001). For

example, the prevalence of anaemia among pregnant and non-pregnant women in India was up to 88% and 74% respectively (Agrawal and Tejwani, 1999). In contrast, studies from industrialized countries show that 2-45% of women become anaemic during pregnancy (WHO, 2001). Throughout Africa, about 50% of pregnant and 40% of non-pregnant women were anaemic (WHO, 2001). West Africa is the most affected and South Africa the least affected. In Latin America and the Caribbean, anaemia in pregnant and non-pregnant women was about 40% and 30% respectively (WHO, 2001).

Iron status can be assessed using biochemical, haematologic and histological indices.The biochemicalparameters are serum iron (SI), total iron binding capacity (TIBC) and percentage transferrin saturation. Other parameters are transferrin receptor assay, Zinc protoporphyrin, serum/red blood cell ferritin and soluble transferrinreceptor-ferritin Index (sTfR–F). The haematologic parametersare red blood cell counts, haemoglobin concentration, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red blood cell distribution width (RDW) (Pippard and Hoffbrand, 2001).Recently newer red cell indices have been incorporated; these are percentage hypochromic red cells, and reticulocyte iron (CHr) (Hoffbrand and Moss, 2011). Currently, attempts are being made to evaluate the usefulness of hepcidin estimation (Pasricha *et al.*,2011). Virtually all the parameters have their respectivelimitations and are affected by some confounding factors,such as diurnal variation,infections and inflammation. Haemoglobin estimation is not suitable enough due to hypovolaemia and haemodilution (Milman, 2006; Milman, 2008).Haematocrit lacks standardization, is affected by dehydration, elevated on storage and by inflammation (Levin and Rocco, 2006). Increased erythropoiesis affects the accuracy of MCV and thus underscores its adoption as a sole index of iron status. Serum Iron (SI), TIBS and %TS are affected by infection, inflammation and circardianand diurnal rhythm in the release of iron.

Histological assessment of bone marrow iron remains the gold standard for assessing iron stores (Northrop-Clewes, 2008) but because of the invasiveness of the procedure and large volume of sample required, alternative method is sought which is the estimation of plasma ferritin. However, ferritin is an acute phase reactant and is grossly affected by inflammation. For the assessment of iron status,no measurement of aniron parameter is ideal for all diagnostic and clinical circumstances as no single parameter monitors the entire iron compartments. Also changes may develop sequentially that may affect a particular body compartment (Pippard & Hoffbrand, 2001).Therefore, a combination of parameters to measure various iron compartments are adopted. Northrop-Clewes (2008) posited the final recommendation of the WHO/CDC consultative group on assessment of iron status of a population to include estimations of Hb, serum ferritin and sTfR. Haemoglobin is a measure of anaemiaeven though not all anaemias are caused by iron deficiency while plasma ferritin is an important indicator of iron stores.A relationship exists between serum ferritin and storage iron, which calls for the use of serum ferritin assay as a dependable parameter in the evaluation of iron status (Mark, 2002). The relationship is that one microgram (1µg) of serum ferritin corresponds to between eight and ten milligram (8-10mg) of storage iron. Ferritin estimation is particularly useful especially in apparently healthy people because it is usually the earliest laboratory measure to change in iron deficiency states.Under normal conditions, serum ferritin levels less than 15µg/l are consistent with depleted iron reserves and levels below 12µg/l are associated with IDA (Hoffbrand and Moss, 2011; (Worwood and May, 2012)**.**Reference values vary with sex (men 15–300ng/ml (median 100ng/ml) and women (pregnant women inclusive) 15– 200ng/ml (median 30ng/ml) (Lewis& Bates, 2012). A combination of low values of serum ferritin and haemoglobin suggests iron deficiency anaemia (Schwartz and Thurnau, 1995). Soluble transferrin (sTFR) reflects the intensity of erythropoiesis or the cellular supply of iron.Values of sTfR assay exceeding the reference

range are seen in early ID and may be found in women with depleted iron stores. The unit and reference range are specific to methods (Worwood and May, 2012).

Furthermore, Iron status is affected by infection such as malaria, Innocent*et al.*,(2008) contributed that malaria negatively affects iron status and causes redistribution of iron in patients. They observed a significant correlation between serum iron and malaria. Again, the link between malaria and hepcidingrossly influences iron metabolism. This is due to hypoferemia, the end result from the interaction of malaria and hepcidin (Ganz, 2003; Neimeth, 2003).

Defining iron status where chronic inflammation existsmay not always be clear.Itrequires the measurement of one or more acute phase reactants such as C-reactive proteinor/and α-1-acidglycoprotein (AGP). Also, in conditions where inflammation coexists with IDA, characterizing iron status may not be apparent enough. However,Soluble Transferrin – Ferritin Index (sTfR-F Index) has been suggested as an appropriate measure.Soluble Transferrin – Ferritin Indexis the ratio of sTfR assay and the log of ferritin.This is illustrated as

$$\text{sTfR-Ferritin Index} = \frac{sTfR}{\log Ferritin}$$

Assessment of iron status of pregnant women is important in order to identify normal and abnormal conditions that might influence pregnancy outcomes. Normal iron status implies a level of erythropoiesis in which the supply of iron is not limited to cope with normal physiological needs (Worwood and May 2012) while abnormal iron status reflects a deficiency that may alter the physiological mechanisms of iron. Abnormal iron status could show in the form ofID, IDA, inflammatory anaemia and inflammatory anaemia in association with IDA (Bleyere *et al.*,2013). Moreso, it is vital to ascertain whether iron supplementation would be beneficial to the pregnant subjects. Iron supplementation increases maternal iron

status or iron stores and may improve pregnancy outcome. However, there are reports that in some subjects prophylactic iron supplementation might not increase the maternal haematocritrather may impose some risk to the pregnant mother because increased haematocrit might counteract haemodilution and result in increased blood viscosity. Also, high maternal iron store is said to be associated with unfavorable pregnancy outcome (Lao *et al.*,2000) in that increased iron stores has been linked to maternal complications such as geatational diabetes and increased oxidative stress. In supporting this idea, Scholl, (2005) posited that high levels of haemoglobin, haematocrit and ferritin are associated with increased risk of foetal growth restriction, preterm delivery and preeclampsia.

Assessment of iron status appears to be difficult especially in pregnancy because of changes in maternal physiology such as expansion of plasma volume, increased erythropoiesis and increased demand for iron by foetal-placental unit and inflammation. Moreover, the physiological changes that occur throughout gestation may differ markedly between individuals (Van den Broek*et al.*,1998).

The exact data on iron status and the prevalence of iron deficiency anaemia among pregnant women in Nigeria is lacking. This is due to the fact that various scholars adopt different criteria in their definitions. Also,iron statusparameters vary withtrimesters.

In Calabar, Nigeria, Usanga *et al.*,(1994) worked on 140 pregnant women and affirmed that 79.1 percent of thesubjects were anaemic of which 22.6 percent were due to iron deficiency anaemia. The study also recorded anaemia in 9.3% of the control group. The criteria for their study were based on estimated values of haemoglobin combined with serum iron and percent transferrin saturation. Haemoglobin values less than 12g/dl was adopted for non pregnant women while haemoglobin values less than 11g/dl was adopted for pregnant subjects. Also serum iron less than 12.5 µmol/l and percent transferrin saturation less than 16% were

adopted for pregnant subjects. Evidence of reduced iron stores among the pregnant women was more among the 19-24 years age group

In a study on 288 elite young Hausa primigravidae at less than 24 weeks of gestation in Zaria, Guinea Savanna, Northern Nigeria, Fleming *et al.*,(1984) reported that 99 subjects (43.4%) were anaemic (Hb less than 11.0g/dl). The criteria for defining iron status were red cell indices (MCV and MCH). Iron deficiency was diagnosed in 18% of all and 25% of anaemic women. About 14% of all subjects were folate deficient. Increased MCV and MCH correlated with anaemia while low serum folate showed weak association with anaemia and malaria. Serum vitamin B_{12} was normal or high in 145 subjects in whom it was measured; 3% had congenital elliptocysis, but did not contribute to anaemia. The common cause of anaemia was malaria and it was diagonised in 28% of all and 40% of all the anaemic subjects.

In another study on 66 elite non-pregnant and 95 pregnant (27 elite and 68 non-elite) Nigerian women in Zaria, Guinea Savannah, Isah *et al.*,(1985) observed anaemia in 46% non-pregnant, 37% pregnant elite and 52% non-elite pregnant women. The paremeters studied were haemoglobin concentration, serum iron, Total Iron Binding Capacity (TIBC), transferrin saturation (TSAT), free erythrocyte protoporphyrin and serum ferritin. The mean SF (28.9ug/L) in pregnant elite was significantly lower than 33.6ug/L obtained in non-elite pregnant women. Iron deficiency was diagnosed at 54%, 30% and 25% in non-pregnant, elite pregnant and non-elite pregnant women respectively. On parity, the prevalence of anaemia falls with increasing parity, being 52% in primigravidae and 40% in multigravidae. In contrast, prevalence of ID increased with parity, 18% and 35% in primigravidae and multigravidae respectively. Furthermore, anaemia and ID were seen most frequently in the third trimester.In a rural area in South Western Nigeria, Oluboyede and Ogunbode, (1976) in a study on iron deficiency anaemia; evaluation of diagnosis and treatment with imferon found that 87.5% of 32 pregnant women with PCV of 30% or less had iron depletion and 84.5% had

megaloblastic changes. The criterium used in diagnosis was staining of bone marrow iron. Parental iron gave statistically significant rise in PCV (P<0.001) in those who received iron therapy compared with control group with no iron therapy.

Van den broek and Letsky (2000),in South Malawi,reported 55% prevalence of iron deficiency among 150 study subjects. They used serum ferritin as the single predictor of iron deficiency and a cutoff of <30µg/l (the most appropriate cutoff for diagnosing iron deficiency in their study environment). They reported that iron deficiency was the most common micronutrient deficiency associated with anaemia during pregnancy. They also demonstrated an absence of stainable iron in bone marrow aspirate. Also, 44% of the pregnant women had haemoglobin levels less than 10.5g/dl, 23% were iron deficient with no evidence of folate, vitamin $B_{12,}$ or vitamin A deficiencies; 32% were iron deficient with one or more other micronutrients deficiencies; 26% were not iron deficient but had evidence of one or more of the other micronutrient deficiencies, most often vitamin A; and 19% were not deficient in any of the micronutrients studied.

In Cote d'Ivoire, Bleyere *et al.*,(2013) assessed iron status of 112 adolescents during pregnancy and reported that iron metabolism was highly altered and the degradation was more significant in third trimester of pregnancy (P<0.05). Findings of the study showed that haemoglobin, haematocrit and mean cell volume were decreased during pregnancy and the decrease was significant in third trimester of pregnancy (P<0.05). Significant decrease (P<0.05) was shown in the saturation coefficient of transferrin (SCT) and serum ferritin estimation. In contrast, mean values of sTfR and TIBC were increased above the reference values and these changes were more during in the third trimester. Iron stores were progressively decreased throughout the third trimester. Furthermore, 77.7% of the adolescents were shown to be anaemic in the third trimester. Severe anaemia was prevalent

throughout pregnancy 23.3%, 15.2% and 8% in the 1st, 2nd and 3rd trimesters respectively. They also reported few adolescents to be with normal iron status during pregnancy.

A work on iron status of pregnant Seychellois women showed an increased incidence of iron deficiency. The criteria for defining iron deficiency were ferritin <15ng/ml and sTfR >28nmol/l. IDA was present in 6% of the subjects at enrollment and 20% at delivery. Mean values of maternal serum ferritin (normal range 15-300ng/ml) was 50.2 ± 49.4 ng/ml at enrolment while at delivery, it was 39.4±42.1ng/ml. The study also showed that parity had a negative effect on sTfR and ferritin at delivery. Mean values of sTfR (normal range 8.7-28.1nmol/l) was 23.0±7.0nmol/l at enrolment while at delivery, it was 36.6±17.2 nmol/l (Duffy *et al.*,2009).

Erhabor *et al.*,(2010) showed the effect of P. *falciparum* malaria on the indices of anaemia among 50 pregnant women in the Niger Delta of Nigeria. In the study, the mean haematological values was significantly lower among parasitized pregnant women compared to non parasitized pregnant subjects (p= 0.01). The incidence of anaemia among the parasitized and non parasitized pregnant subjects was 66% and 48% respectively. A positive correlation was obtained between the level of parasitaemia and anaemia (r= 0.67, p= 0.04).
In a work in Rawa, Pinindi, Haq *et al* (2008) determined iron status of 100 non-anaemic pregnant females during their third trimester, 47% were found to have serum ferritin levels below the reference range where as 18% had SF levels at lower limits of the normal. Conclusively, 65% of non-anaemic females were found to have iron deficiency and with increased levels of sTfR.In determining the prevalence and severity of anaemia in 570 pregnant women at Enugu, Nigeria, (Chukudebelu and Obi, 1979) found 33.7 percent to be anaemic.By severity, 25 (50%) were mildly anaemic (haemoglobin values 9-10g/dl); 19 (38%) moderate anaemia (haemoglobin values 8.9g/dl) while 6 (12%) had severe anaemia (haemoglobin values below 8g/dl). Of theanaemic subjects, 14 (28%) were primigravidae

while 36 (72%) were multiparous. By gestational age, one of the subjects was anaemic in the first trimester representing 1.9% of the anaemic subjects while 20 subjects were anaemic during the second trimester and 31 were anaemic during the third trimester, representing 38.4% and 59.6% respectively.

Iloabachie and Meniru, (1990) studied the incidence of anaemia among 144 pregnant women attending antenatal care in Enugu, Eastern Nigeria. The incidence of anaemia at booking was 30.6% (using haemoglobin value less than 10g/dl) and 67.7% using the World Health Organization minimum values of haemoglobin less than 11g/dl. By adopting the World Health Organization standard, mild anaemia (haemoglobin 10g/dl – 10.9g/dl) was observed in 44 subjects, moderate anaemia (8g/dl – 9.9g/dl) was 27.5%while severe anaemia (below 8g/dl) stood at 2.6%. Thirteen subjects (9%) had anaemia due to malaria. Also, in the study, there was no definite relationship between anaemia at booking and either age or parity, rather anaemia increased with gestational age. The most common aetiologic factors of anaemia were iron and folic acid deficiency.

The study by Saadiya, *et al.*,(1990) in Karachi, Pakistan on 709 pregnant women aged 16-45 years found 17% of the women to be anaemic. Anaemia was defined as haemoglobin concentration below 10g/dl. Thirty six out of 232 primigravidae (16%) were anaemic while 63 out of 412 gravidae 2-6 (16%) were anaemic. Anaemia was common among gravidae 7 and above of whom 23 out of 61 (38%) were anaemic. Anaemia was more in the third trimester (80 out of 345 [23%]) than any other gestational age.

In awork on 533 pregnant women in Sagamuand using packed cell volume (PCV≤ 30%) as the criterium for establishing anaemia,(Olodeoku, 1991) found 29.1% of the pregnant women to be anaemic. In classifying the anaemia by severity, majority of the anaemic subjects 124 (23.3 %) were mildly anaemic (packed cell volume 27–30%). Twenty nine (5.4%) were

moderately anaemic (packed cell volume 19 – 26%) while 2 (0.4 %) were severely anaemic (packed cell volume <19 %). On parity, 42 (30.2%) of 139 primiparous women were anaemic. He indicated that the percentage of patients that were anaemic did not change appreciably with increasing parity except in women para 5 and above. A study on anaemia in pregnancy in Aba, (Aluka *et al.*,2001) showed that 300 (29%) of the 1,034 pregnant women were anaemic. Forty-three percent of the anaemic subjects were in the ages of 26–30 years. Mild anaemia occurred in 82 (97.6%) of the anaemic subjects while moderate anaemia was recorded in 2 (2.4%). There was no case of severe anaemia. Out of the anaemic subjects, 51.3% presented anaemia in the second trimester, 44.6% in the third trimester while 4.1% presented in the first trimester. Primigravidae women (28.7%) had the highest prevalence followed by those who had 3 (19%) deliveries.

2.9.3 MODULATIONBETWEEN CYTOKINE AND IRON

Immunomodulation can be defined as a biologic response whereby some modifying substances affect the function of immune system. The modifying substance, called the immunomodulator, may either enhance or suppress the immune function (Venugopal, 2007). It is possible that cytokines and iron may suppress or enhance the expression of each other thus modulating the immune system. Cytokinemay suppress or enhance the level of ironin the bodythrough various pathways that effect change in iron homeostasis and metabolism. These mechanisms involve the processes of absorption, transport,storage andrelease of iron bythe macrophages and are as follows: IFNγincreases the expression of divalent metal transporter-1 in the gut enterocytes and macrophages and stimulate the uptake of ferrous iron (Fe^{2+}). Again, IFNγ, TNFα, IL-1 and IL-6 stimulate hepatic synthesis of hepcidin, which inhibits duodenal absorption of iron(Hillman and Ault, 1995; Ludwiczek *et al.*,2003; Ganz, 2003).Also, proinflammatoy stimuli - IFNγ and lipopolysaccharides (LPS), increases the uptake of non-transferrin bound iron (NTBI) via stimulation of divalent metal transporter-.

NTBI induces the retention of iron within monocytes by down regulating the synthesisof ferroportin; a process that is also affected by Hepcidin. In addition, IL-10 stimulates TfR-mediated iron uptake by activated monocytes; the effect in time leads to hypoferraemia and haemosiderosis of the macrophage. Furthermore, TNFα, IL-1, IL-6 and IL-10 induce the synthesis, storage and retention of ferritin within macrophages. Again, IL-1 induces the neutrophils to release lactoferrin which returns its bound iron to macrophages instead of the erythrons (Hillman and Ault, 1995; Ludwiczek *et al.*,2003*)*.

Similarly, iron has been reported to modulate cytokine secretion (Foster *et al.*, 2000). It is reported that iron directs immune response towards T_H2 response; a pattern that is believed to be unfavourable for the killing of many intracellular pathogens (Weiss *et al.*,1997). In addition, iron chelation could increase the T_H1 mediated immune response in *P.falciparum* infection (Weiss *et al.*,1997); an effect that is associated with more efficient clearance of the parasite (Gordeuk*et al.*,1992).

2.9.4INTERACTIONS OF ACUTE PHASE REACTANT, CYTOKINE AND IRON

Acute phase response involves a large number of non-specific, physiological and metabolic changes that occur within hours after an inflammatory stimulus. The stimuli could be bacterial, viral andmyocardial infections, trauma and cancers (Ceciliani *et al.,* 2002; Venugopal, 2007). During acute phase responses, some proteins may be synthesized by the hepatocytes and their concentrations could be upregulated beyond the normal plasma level (Warren, 2010). Such proteins are known as AcutePhase Proteins (APP). Theseinclude cardiovascular reactive protein (C-RP), serum amyloid P component (SAA), ferritin, α-1-antichymotrypsin (ACT), α-1 acid glycoprotein (AGP), fibrinogen, haptoglobin, mannose binding protein and hepcidin (WHO, 2004; Venugopal, 2007). The acute phase response is usually transient and its main purpose is to prevent further damage to the tissue affected and to remove harmful molecules (Northrop-Clewes, 2008). Their levels provide an idea about

the extent or degree of inflammatory response (Venugopal, 2007). An explanation has been given on the synthesis of acute phase reactants as it is thought to be the result of interactions of cytokineincluding IL-1, TNFα, IL-6 and sometimes with iron. For example,Interleukin-1 (IL - 1) stimulates the synthesis of ferritin. This is trapped by apoferritin in the phagocytic cells leading to adequate or high amount of iron in the macrophages, or in the store with a corresponding reduction of iron to be transported to bone marrow (Fairbanks and Beutler,2011; Cazzola, *et al.,* 1997; Pippard and Hoffbrand, 2001; Mark, 2002). In HIV/AIDS patients also, ferritin synthesis is elevated. In this condition, the presence of the virus elicits reactive haemophagocytosis. Reactive haemophagocytosis is a disorder caused by inappropriate activation of the mononuclear phagocytes characterized by benign and generalized proliferation of histiocytes with the release of cytokines (Cline, 1994; Imashuku, 1997).Another classical example of the interactions of acute phase proteins, cytokine and iron is in the hepatic synthesis of Hepcidin. Thehepatic sinusoids are lined by endothelial cells and Kupffer cells. Exposure of the endothelial cells and Kupffer cells to microbes or high iron-saturated transferrin (Fe/Tf) causes the release of IL-6 and possibly other cytokines that act on hepatocytes to induce the synthesis and secretion of hepcidin (Neimeth*et al.,*2003).

2.9.5IRON STATUS AND ANAEMIA OF INFLAMMATION

Anaemia of Inflammation is a term used to describe the hypoproliferative anaemias. Hypoproliferative anaemias are anaemias that occur due to diminished production or diminished response to erythropoietin. Most cytokines such as IFNγ and TNFα are incriminated in the inhibitory activity against erythropoietin in the kidney or its response at the erythriods.It is reported that hypoproliferative anaemias occur as a result of systemic illness or inflammation (Cullis, 2011). It was formerly known as Anaemia of Chronic Diseases (ACD). The cause of the anaemia of inflammationis multifactorial and it is posited that systemic illnesses, infectious and non-infectious inflammatory disorders may

induceabnormal antigenic activation of immune cells and/with sequestration of iron in macrophages which result in anaemia; hence the name anaemia of inflammation (Ganz, 2003). During acute phase response, proinflammatory cytokines are released, the vascular system and the proinflammatory cells are also activated leading to the production of more cytokines and other inflammatory mediators that diffuse into the extracellular fluid compartment and circulate in the blood. Anaemia of inflammation is characterized by reduced iron uptake, disturbed iron metabolism,hypoferraemia and iron limitations to the erythron (Weiss and Goodnough, 2005). The diversion of iron from circulation,its retention at the storage site of reticuloendothelial cells and liver and inappropriate release of iron to the erythrons, results in iron restricted erythropoisis which terminates with reduced haemoglobin synthesis. The hallmark of anaemia of inflammation is the dysregulation of iron homeostasis, impaired red blood cell production and shortened red cell life span. There are no uniform criteria for diagnosis of anaemia of inflammation. However, combinations of iron parameters have proved useful. These include MCV, MCH, CHr and hypochromic red blood cells. Other parameters are ferritin, transferrin saturation, sTfR and sTfR/Log Ferritin ratio. The diagnostic feature that may show anaemia of inflammation are low values of serum iron, normal or raised serum ferritin, normal sTfR assay, sTfR-Ferritin ratio <1.0 and elevated values of acute phase protein. sTfR-ferritin ratio >2.0 suggests that iron stores are deficient with or without anaemia of inflammation(Roy, 2010).

2.9.6 IRON STATUS AND HEPCIDININ PREGNANCY

Hepcidin, a 25 amino acid peptide,was recently discovered as the hormone/acute phase protein that controls or regulates iron metabolism and anaemia of inflammation (Neimeth*et al.,* 2003). It is converted from prohepcidin in a process mediated by furin (a prohormone convertase) (Ganz, 2003). Hepcidin is found in human plasma and urine, and it is synthesized primarily in the liver (Krause *et al.*,2000). It regulates iron metabolism by inhibiting duodenal

iron absortion in the gut, and by affecting mobilization of iron from liver and spleen (Malyszko *et al.*,2006). Plasma hepcidin regulates iron absorption (by binding to ferropotin) in the duodenum and iron released from macrophages in the spleen and other reticulo-endothelial cells (Neimeth*et al*, 2003;Haurani, 2006). Ferroportin is found in all cells that export iron into plasma and is the only known cellular exporter of iron. Hepcidin binds to the iron exporter, ferroportin, inducing its internalization and degradation thereby affecting iron status. Ferroportin is necessary for materno-foetal iron transfer and iron efflux from duodenal enterocytes, macrophages and hepatocytes (Neimeth*et al.*,2004). Consequent to down regulation of iron absorption by hepcidin is that functional iron deficiency may occur even when iron stores are present (Gasche *et al.*,2004). During infections/inflammation such as malaria, most cytokines that are expressed induces the synthesis of Hepcidin (WHO, 2004).As would be expected of an iron regulatory hormone, the production of hepcidin is homeostatically regulated by plasma iron concentrations and iron stores, predominantly, through a transcriptional mechanism. It is posited to be expressed in an iron-replete state or iron overload (Pigeon *et al.*, 2001; Neimeth*et al.*,2004). Increased hepcidin released in response to increased iron concentration generates a negative feedback loop that limits iron absorption and retains iron in the stores (Ganz, 2003). Hepcidin levels seem to change rapidly in response to changes in iron status. This is demonstrated in a study on urinary hepcidin (Krause *et al.*,2000). In normal human volunteers, urinary hepcidin levels increased up to 15-fold within 24 hours after 65 mg of oral iron supplementation. In iron deficiency anaemia, urinary hepcidin levels are low or undetectable.

In a study in Bangladesh on hepcidin and iron status of 149 pregnant women,(Schulze *et al.*,2008) observed a correlation between hepcidin and some iron parameters. Urinary hepcidin used in the study showed apositive correlation with ferritin (r= 0. 33, p<0.001) but no significant correlationexisted between urinary hepcidin and sTfR, and between urinary

hepcidin and haemoglobin.In a similar study in Poland, Rams *et al.*,(2008) demonstrated a possible role of prohepcidin in anaemia in pregnancy. Recruited in the study were 37 healthy pregnant women in third trimester, 34 anaemic pregnant women in third trimester, 20 healthy pregnant women in first trimester and 30 healthy female volunteers. The study showed elevated prohepcidin in pregnancy which was also related to ferritin and sTfR. The study also showed that prohepcidin, IL-6, high Sensitivity C-Reactive protein (hsC-RP), sTfR, TIBC and TSAT were significantly higher in anaemic pregnant women than non - anaemic pregnant women in their third trimester. In anaemic pregnant women, prohepcidin showed negative correlation with haemoglobin (r= -0.34, $p< 0.005$), haematocrit (r= -0.39, $p< 0.05$) and total protein (r= -0.34, $p< 0.05$). Prohepcidin was positively correlated with hsC-RP (r= 0.49, $p< 0.05$) in pregnant females in their first trimester. Furthermore,innon-anaemic pregnant women (first and third trimesters) prohepcidin correlated negatively with sTfR (r= -0.27, $p< 0.05$) and positively with ferritin (r= 0.26, $p< 0.05$) while in the healthy volunteers prohepcidin corelated positively with ferritin (r= 0.37, $p< 0.05$).

2.9.7 C- REACTIVE PROTEIN (C-RP)

C-RP has been described as a substance that was present in the sera of acutely ill patients. It binds to the C-polysaccharide on thecell wall of *Streptococcus pneumoniae* and agglutinates the organism (Ridker and Henneens, 2000). The substance was shown to be a protein and given the name cardiovascular reactive protein, commonly referred to as C-reactive protein. C-RP consists of five identical non-glycosylated polypeptide subunits non-covalently linked to form a disc-shaped cyclic polymer with a molecular weight of approximately 115kda. Tumor necrosis factor -alpha and IL-1 induce the expression IL-6 which later stimulates the hepatic synthesis of C-RP.It was subsequently shown to be an acute phase reactant and important in non-specific host defence and binds to polysaccharides present in many bacteria, fungi and protozoal parasites and polycations such as histones (Kimberly and Vesper, 2003)

Cardiovascular Reactive Protein (C-RP)is a notable APP often used to assay samples for infection/inflammation. It has also shown to be predictive of future events in patients with acute coronary syndromes; stable angina and coronary artery defects (Roberts and Moulton, 2001).Elevated C-RP appears to be a better predictor of risk of heart attack than elevated cholesterol levels (Warren, 2010).It may enhance inflammatory and prothrombotic responses (Fred and Allan, 2006).

CHAPTER THREE:

3.1 SUBJECTS AND METHOD

3.1.1 STUDY AREA

This study took place at Aba, Abia State, Nigeria. Aba is located on latitude $05^{o}10'N$ and longitude $07^{o}19'E$ with a population estimated at 1,020,900 (National Population Commission, 2006). It is a cosmopolitan town in Abia State and the second largest commercial city of South Eastern Nigeria. The area experiences malaria transmission throughout the year with the majority of clinically evident infection occurring following the long and short rains that generally occur during the months of April-July and September - November respectively. The stagnant water in the drainages in the metropolis provide adequate ecological habitat for the breeding of mosquitoes. The mosquitoes are less susceptible to insecticides and the majority of the study population sleep outside mosquito nets. Apart from the ethnic Igbos, there are other ethnic groups living in Aba. The subjects therefore represent a sub-group of mothers in Nigeria.

3.1.2 STUDY POPULATION

Subjects: Two groups of subject were recruited for the study. They were asymptomatic pregnant and immediate post partum women. They were within 17–44 years. Each of the groups was further sub-divided into parasitaemic and aparasitaemic group based on the detection of malaria parasite in the peripheral blood of the pregnant subjects and blood from the placenta of the immediate post-partum. The pregnant subjects were on enrollment for antenatal care while the immediate post-partum had their delivery shortly before the collection of specimen. Both groups were drawn at antenatal care and delivery units of Abia State University Teaching Hospital (ABSUTH) and Living Word Mission Hospital (LWMH). ABSUTH and LWMH have standard health facilities, frequently used by pregnant women

and are manned by qualified and experienced health professionals. The malaria parasitaemic pregnant women were 144 in number and considered as the first group of the test subjects while the malaria aparasitaemic pregnant women were 62 in number and considered as the 2nd batch of the test group. The malariaparasitaemic immediate post-partum were 30 in number and taken as the third group of the test subjects while the malaria aparasiteamic immediate post-partum were 20 in number and taken as the 4th batch of the test group. Similarly, 40malaria aparasitaemic apparently healthy non-pregnant women of the same agewith the test groups were recruited as controls and served as the 5th group. The control subjects were drawn from the Students of School of Nursing, ABSUTH.

3.2 STUDY DESIGN

The study was a cross-sectionalresearch work that involved pregnant and immediate post partum women. Samples were collected from the pregnant subjects on enrollment for antenatal care. Again blood from the placenta was collected from them shortly after delivery. For the pregnant and non pregnant (control) subjects, the samples were collected between 9.00 am 11.00 am. The samples collected were screened for malaria parasite and HIV and those that tested positive were referred to their respective hospitals for necessary intervention. Based on the pattern of recruitment and information conveyed by the various parameters, the study was designed into 4 modules.

3.2.1 Module I: Assessment of Immune Stimulation

Samples were screened for malaria and HIV.Subsequent analysis was carried out only on HIV negative samples. The degree of malaria parasitaemia was quantitatively determined by the parasite count.Cytokine assay establishes the presence of immune stimulation. It describes the activity of all leucocytes of bothinnate andadaptive immune responses. The cytokines evaluated were IL-4, IL-6, 1L-10, TNFα and IFNγ.

3.2.2 Module II: Determination of some iron parameters and Red Cell profile

Iron parameters were carefully selected to evaluate iron at the store, transport and functional levels. Those assessed were serum ferritin (SF) and soluble transferrin receptor (sTfR). Values of sTfR and SF were used to calculate sTfR – F Index. Full blood count with differential and red cell indices evaluates only the innate immune response. It was used in the determination and classification of anaemia.

3.2.3 Module III: Assessment of some metabolic and surrogate parameters

The parameters measured were levels of C-reactive protein, glucose and creatinine. C-RP was adopted not only to assess infections/inflammation but as a surrogate marker to show the influence of inflammation/ infection on SF.Serum ferritin would be elevated in infection/ inflammatory response. The glucose estimation was to show the effect of parasitaemia on glucose metabolism while creatinine evaluation assesses the normal functioning of the kidney.

3.2.4 Module IV: Determination of Association.

In this study, the levels of association or relationship were determined by correlation.Correlationmeasures the strength of relationships between variables. The variables may be expressed in different units and could be located on any axis.ScatterDiagramand Karl Pearson's coefficient of correlation were the graphical and mathematical methods respectively used. The Pearsonian coefficient of correlation is denoted by the symbol 'r' (Nwachukwu and Egbulonu, 2000; Pillai and Bagavathi, 2012).

3.3 SAMPLE SIZE

The sampling method for the study was Convenience Sampling and thesample size of 206 was determined using 16% prevalence of malaria in pregnancy in South Eastern Nigeria (Uneke, 2007). It was calculated by simple formula (Daniel, 1999).

$$N = \frac{Z^2 P\,(1-P)}{D^2}$$

Where N = sample size

Z= z statistic for a level of confidence

P= expected prevalence or proportion

D= precision

Z= 1.96 for the level of confidence at 95%

P= 16% (0.16) prevalence of malaria in pregnancy in South Eastern Nigeria (Uneke, 2007).

D= 5% (0.05)

N= 206.

3.3.1 SELECTION AND ENROLMENT CRITERIA

The participants were those that met and responded favourably to the enrolment criteria. The criteria were stated in the questionnaire which also tallied with the information on the subject's folder. The criteria were as follows: The study groups had not complained of bleeding prior to 32 weeks of gestation and had not been transfused. Again, the participants had no history of gastric and duodenal ulcer. They had no report of malignancy, liver diseases, hypertension and pre-eclampsia. Again, their HIV/ AIDS status recently showed negative. Also recruited were those without chronic diseases or other known causes of inflammation. Moreover, the pregnant subjects were those with singleton pregnancies (based on fundal height estimation and last menstrual period and ultrasound scan) from 8 weeks of gestation. The gestational term of the pregnant groups especially those that reported at second and third trimester were also confirmed with ultrasound scan. The pregnant and non pregnant groups had not taken anti-malaria drugs and iron supplements for about four (4) weeks. The immediate post partum had uncomplicated pregnancies and gave birth via vaginal

route.These criteria were applied in order to ensure proper diagnosis, eliminating other sources of immune stimulation aside pregnancy and malaria and to avoid bias in interpretation of iron parameters.

3.3.2 EXCLUSION CRITERIA

Subjects who were apparently known with underlined chronic illness such as: diabetes mellitus, cardio-vascular diseases, tuberculosis, liver diseases and other inflammatory or pathological disorders that may alter immune response and iron levels were excluded. Also those that had not lived in Aba for more than a year were excluded from the study. Again, subjects on hormonal drugs were not recruited. Others excluded from this study were HIV Sero-positive subjects and those with multiple gestations. The pregnant and control non-pregnant women that were on iron supplement/fortification within the period were excluded. Also excluded were those whose informed consent could not be obtained.

3.4 ETHICAL APPROVAL

Approval for the study was obtained from the Ethics Committee of ABSUTH and LWMH as well as informed consent of the participants.

3.5 SPECIMEN COLLECTION

Peripheral blood was obtained from pregnant and non pregnant women by venipunctureand was carried out by the phlebotomist while blood from the placenta was obtained within 30 minutes after deliveryby biopsy pool method. For the biopsy pool method,a block of tissue (5cm x 5cm x 5cm) was excised from the cleaned maternal side of the placenta, resulting in a large pool of intervillous blood at the excision site. About 8 mIs of the blood was quickly aspirated with a graduated sterile pipette. This was carried out by the Matrons.

3.6 METHOD OF INVESTIGATION AND PARAMETERS STUDIED

Out of 8mlsof blood collected, 2mls were dispensed into 2 drops of 10% Ethylene Diamine Tetra Acetic acid container and mixed for the examination of malaria parasite and haematology parameters (full blood count [Haemoglobin estimation, haematocrit, total red blood cell count, total leukocyte count and differential] and red cell indices [mean cell haemoglobin concentration, mean cell volume, and mean cell haemoglobin]). The remaining 6mls were allowed to clot in a pyrogen free tube and centrifuged at 3000rpm for 10mins with a bench top laboratory centrifuge. About 500µl of serum was used for HIV screening and estimation of biochemical parameters: glucose, creatinine and C - reactive protein. The serumwas stored at 2- 8°C and the assay conducted within 24 hrs.The remaining2500µl of the serum was stored at -20^{o}C in pyrogen free containers. Aliquot of the serumwere used for estimationsofsolubletransferrin receptor (sTfR), serum ferritin (SF) and sTfR/log ferritin ratio and Cytokines; TNFα, IFNγ, IL-4, IL-6 and IL-10. The Rapid Diagnostic Strip for malaria was purchased from SD Bioline, Korea. The Giemsa stain was locally sourced from Glanson Chemicals, Awka. Determine strip and immunocomb confirm for HIV screening were respectively procured from Alere Medical Coy. Japan and Orgenics, Isreal. The kits for the estimation of glucose and creatinine were purchased from Randox laboratory, UK. The kits and reagents for cytokine analysis were procured from Abcam plc UK while the kits for the assay of sTfR and SF were sourced from Monobind INC USA. C - reactive protein was purchased from Agappe Laboratory, Switzerland. The automated machine and reagents for haematological investigations were supplied by the Sysmex Corporation, Kobe, Japan while the kits for analysis of cytokines were sourced from Abcam Company, UK. During the assays, the manufacturers' Standard Operation Procedure (SOP) for each investigation and the operation of the machine were strictly followed.

3.7 SITE OF STUDY

The full blood count and red cell indices, HIV screening and malaria parasite estimation were carried out at the Haematology unit while biochemical parameters were determined at the Chemical Pathology Unit of ABSUTH. The cytokines were determined at the Pathology Unit LWMH and New Covenant Medical Laboratories and Blood Bank Services Ltd. Aba.

3.8 LABORATORY METHODS

3.8.1 SCREENING FOR MALARIA PARASITE USING RAPID DIAGNOSTIC TEST By (SD BIOLINE INC., 2013) BATCH NO. 05FK30-02-8

The SD BIOLINE Malaria Ag P.f/Pan test is a rapid, qualitative and differential test for the detection of histidine-rich protein II (HRP-II) antigen of *Plasmodium falciparum* and common *Plasmodium* lactate dehydrogenase (pLDH) of *Plasmodium* species in human whole blood.

Principle:

The SD BIOLINE *P.falciparum*/*P.vivax* test contains a membrane that is pre-coated with recombinant malaria *P.falciparum* capture antibody on test band 1 region and with recombinant *P.vivax* capture antibody on test band 2 region. Whole blood of the subject is mixed with colloid gold conjugate. During testing, the mixture migrates upwards along the membrane chromatographically to the test region (1,2) and forms a visible line as the antigen-antibody colloid gold complex. To serve as a procedural control, a coloured line appears at the control region. This validates the test performance. The malaria antigens were detected at region 1 and 2 are HRP-II and pLDH respectively.

PROCEDURE

With the aid of a calibrated sterile pipette, 5µl of whole blood was transferred into the sample well. Four drops of the assay diluent was added to the bottom of the well and mixed. The SD BIO-strip was dipped into the mixture and the complex allowed to flow by capillary method.

The result was read within 20 – 40mins. A pink line at the positive and control band indicated a positive reaction whereas only one pink line at the control band indicated negative reaction. No pink line at both positive and negative control bands indicates an invalid result.

3.8.2 MICROSCOPIC DETECTION OF MALARIA PARASITE USING GIEMSA STAINED THICK FILM (Cheesbrough, 2010)

PRINCIPLE

Giemsa stain is one of the Romanosky stains with two component substances, namely; the eosin and methylene blue dyes. It differentiates the nucleus and cytoplasm of leucocytes, platelets and malaria parasites. The eosin stains the chromatin dot of malaria parasite dark red. The Schuffner's dot of *P. vivax* and *P. ovale* appears red while the Maurer's dot of *P. falciparum* shows red mauve. The methylene blue stains the cytoplasm of the parasite blue. The stain is diluted with water buffered to pH range of 7.0-7.2. The buffered water used in diluting the stain induces lysis of red cells leaving the malaria parasite and leucocytes more visible.

PROCEDURE

The procedure is as described by (Cheesbrough, 2010). Two drops of blood was closely droped on a clean greese free slide. A faint spread of the blood was made to obtain a diameter of about 5mm. The preparation was allowed to air dry at room temperature with the slide flat, the film upwards and protected from dust. Immediately before use, the stock Giemsa stain was diluted 1 in 10 with buffered water at pH 7.2. The dilution was by measuring 45ml of buffered water, pH 7.1- 7.2 in a 50ml cylinder and 5 ml of Giemsa stain (to 50ml mark) was added and gently mixed. The slide was placed face downwards in a shallow tray supported on two rods, in a Coplin jar. The diluted stain was poured into the shallow tray Coplin jar and allowed to stain for 30 minutes.

The stain was washed off from the staining container using clean water.

The back of each slide was cleaned and placed in a draining rack for the preparation to air-dry. The examination of the slide was done using X100 objective lens.

3.8.3 QUANTITATIVE PARASITE COUNT (THICK FILM) (WHO, 1991)PRINCIPLE/PROCEDURE:

The average number of asexual parasitic forms (trophozoites , schizonts) per microlitre of blood in a thick film is counted in relation to the subjects total white blood cell count or the standard number of leucocytes (8000cell/ul). A thick film was made and allowed to air dry. Giemsa stain diluted 1:10 was applied and allowed to stain for 40 minutes. It was washed with water and allowed to air dry. The film was examined with X100 objective lens. The numbers of asexual parasitic forms (trophozoites, schizonts) present were counted over 200 leukocytes.

CALCULATION:

$$\frac{The\ nos.of\ observed\ asexual\ parasites\ x\ total\ \ wbc\ count/\mu l}{200\ leukocytes} = parasite/\mu l$$

The total WBC count used was that of the subjects.

GRADING:

Parasitaemia was grouped as mild or low (<999 parasite/ul of blood) and moderate infection (1,000 – 9,999 parasite/ul of blood) and high or severe infection (>10,000) (Ketema and Bacha, 2013).

3.8.4 HIV SCREENING USING DETERMINE AS DESCRIBED BY (ALERE, 2013)

Catalogue No. 240852/R7: Ref. No. 7D2343.

The Alere Determine HIV-1/2 is an *In Vitro*, qualitative immunoassay for the detection of antibodies to HIV-1 and HIV-2 in human serum, plasma or whole blood. The test is intended as an aid to detect antibodies to HIV-1/HIV-2 from infected individuals.

PRINCIPLE: This is an immunochromatographic assay. Serum/plasma added to the test pad migrates and mixes with the selenium colloid-antigen conjugate. This mixture continues to migrate to the immobilized recombinant antigens and sythethic peptides at the patient's window site. Antibody to HIV-1 and/or HIV-2 binds to the antigen-selenium colloid forming a red line at the patient window site. If antibodies to HIV-1 and/or HIV-2 are absent, the antigen-selenium colloid flows across the patient window and no red line is formed. To ensure the validity of the test, a procedural control bar is incorporated in the device.

PROCEDURE:

Apply 50µl of serum to the strip pad. Wait until serum is absorped into the sample pad and observe the flow along the test strip. The result is read within 10 minutes.

3.8.5 HIV SCREENING USING HIV 1 AND 2 AS DESCRIBED BY (ORGENICS, 2013). Plate Ref. No 10434503; Lot No. 131015; Card Catalogue No. 10434103; Card Lot No. 131003

The immunoComb II HIV 1 and 2 CombFirm test is a solid-phase enzyme immunoassay (EIA). The solid phase is a card with 12 projections (teeth). Each card has 6 pairs of teeth, with six antigen spots per pair (3 spots on each tooth). The left tooth of each pair carries an upper spot sensitized with human immunoglobulin (Internal Control) and two protein markers p24 (gag) and p31 (pol). The right tooth has three env-derived protein spots gp41, gp120 and gp36.The Developing Plate has 6 rows (A-F), each row containing a reagent solution ready for use at a different step in the assay.

PRINCIPLE: The assay is of the antibody capture format. Antibodies in the sample reacts with anti-HIV IgG present in row A. The complex binds to the HIV antigens on the teeth of the card. Unbound components are washed leaving the anti-HIV IgG-antibody captured on the teeth. The human immunoglobulin on the upper spots (internal control) reacts with anti-human IgG antibodies labeled with alkaline phosphatase (AP). Unbound components are

again removed by washing. The bound alkaline phosphatase reacts with chromogenic components to show a visible gray-black spot on the surface of the teeth of the card.

Procedure

The developing plates, cards, reagents and specimens were brought to room temperature (22°-26°C) and the row plate perforated. About 50µl of the specimen, positive control and negative control were added to wells of row-A respectively. The card was inserted correspondingly. The card was severally withdrawn and inserted for thorough mixing with the printed side of the card at sight. It was left for exactly 30 minutes.

The card was taken out from row A and excess liquid adsorbed with the tip of the teeth placed on an absorbent paper and transferred to row B. This was vigorously agitated for 2 minutes. Row C was perforated and after adsorption, the teeth was transferred into it and allowed to stay for 30 minutes. The teeth were further transferred to row D and E where they were allowed to stay for 2 minutes in each row. This was again washed. After adsorption of the excess liquid, the card was inserted into row F, mixed and allowed to stay for 10 minutes. The reaction was stopped, the card returned to row E and allowed to stay for 1 minute. Finally, the card was withdrawn and allowed to air dry. The result was read and interpreted according to the manufacturer's instruction.

3.8.6QUANTITATIVE MEASUREMENT OF IL-4 BY ELISA TECHNIQUE AS DESCRIBED BY (ABCAM, 2013) Batch No. ab100570, Lot No. GR55997-1

PRINCIPLE: This assay is of the sandwich format. Antibody specific for human IL-4 is coated in a well for the detection of IL-4. IL-4 in the specimen binds to the immobilized Antibody. The wells are incubated and washed before adding Biotinylated anti-human IL-4 Antibody. After incubation, unbound Biotinylated Antibody is washed. An enzyme complex known as HRP-conjugated Streptavidin is added and incubated. On addition of TMB substrate solution, a blue colour develops which is proportional to the amount of bound IL-4.

The stop solution changes the colour to yellow, and the intensity of the colour is measured at 450nm.

PREPARATION OF STANDARD

Serially diluted standards were prepared immediately prior to use.

After a brief spin of the vial of lL-4 Standard, a concentration of 110ng/mL stock standard was prepared by adding 400µL of Assay Diluent A (for serum/plasma samples) into the vial. After that, standard of 7 different concentrations were prepared from the stock standard (100ng/mI) to obtain values of 200pg/ml, 100pg/ml, 50pg/ml, 25pg/mI, 12.5pg/ml, 6.25pg/ml and 3.13pg/ml respectively. The preparation was as follows - 7 scrupulously cleaned sterile tubes were labeled 1-7. In the first tube, 2µl of 110ng/ml (110,000pg/ml) of stock standard was added to 1098µl of Assay Diluent A. This was gently mixed and a concentration of 200pg/ml was obtained. Then 200µl of the standard in tube no. 1 was added into tube 2 containing 200µl of Diluent A to obtain a concentration of 100pg/ml. Again, 200µl of 100ng/ml standard in tube 2 was added into tube 3 containing 200µl of Diluent A to obtain a concentration of 50pg/ml. The same steps were taken upto the 7th tube to achieve concentrations of 25pg/ml, 12.5pg/ml 6.25pgIml and 3.l3pg/ml respectively. The assay diluent A served as the zero standard (0pg/ml).

ASSAY PROCEDURE:

All materials (the standards, prepared reagents, and samples) were brought to room temperature and the assay conducted in duplicate. One hundred microlitres (100µl) of each standard and samples were added into the appropriate wells and gently mixed. It was covered and incubated for 2.5hours at room temperature. The solution was discarded and washed 4 times with 300µl of wash solution. The liquid were completely removed after each wash. On completion of the last wash, the plate was inverted and blotted against a clean absorbent paper.A one hundred microlitre (100µL) of IX Biotinylated IL-4 detection antibody was

added to each well and incubated for 1 hour at room temperature. The solution was again discarded, the washed steps repeated and wells blotted from any liquid. A 100µL of IX HRP-Streptavidin solution was added to each well. This was incubated for 45minutes at room temperature. Again, the solution was discarded; the washed step repeated accordingly and left over liquid was blotted. This was followed by the addition of 100µL of TMB one-step reagent. It was incubated in the dark for 30 minutes at room temperature. The reaction was stopped on addition of 50µL of stop solution to each well and a yellow coloured complex developed which was read immediately at 450nm.

CALCULATION

The mean of the absorbance for each set of duplicate standards, control and samples were taken and subtracted from the average optical density of zero standards. A standard curve on log-log graph paper was plotted with the concentration of the standards on the x-axis and absorbance on the y-axis and values of samples and controls interpolated.

SENSITIVITY

The minimum detectable dose of lL-4 was < 5pg/mL.

3.8.7QUANTITATIVE MEASUREMENT OF IL- 6 BY ELISA TECHNIQUE AS DESCRIBED BY (ABCAM, 2013) Batch No. ab46027, Lot No. GR140910-1

PRINCIPLE: The assay is of the competitive format. A monoclonal antibody specific for IL-6 is coated into the wells of the microtiter strips. IL-6 in the specimen and Biotinylated monoclonal antibody are added simultaneously. Both compete for the antibody binding site in the well. Unbound complex is washed; Enzyme Streptavidin-HRP is added and incubated. A TMB substrate solution is added which acts on the bound enzyme to produce a blue colour which is proportional to the amount of bound IL-4. The stop solution changes the colour to yellow, and the intensity of the colour is measured at 450nm.

PREPARATION OF REAGENTS AND STANDARDS

The reagents and standards were prepared according to the manufacturer's directive. For the IX Standard Dilute Buffer and Wash Buffer, a 1 in 10 dilution was made using distilled water and a 1 in 200 dilution using IX Wash Buffer were prepared respectively. Each of the solutions was gently mixed to avoid foaming. The lyophilized Control vial was reconstituted with the Standard Diluent for human serum while the IX Biotinylated anti-IL-6 was prepared by making a 1 in 20 dilution using Biotinylated antibody diluent. Again, an IX Streptavidin-HRP concentrate was prepared by adding 500µl of HRP diluent to the streptavidin-HRP vial. Further dilution of the already prepared streptavidin-HRP concentrate was made in the ratio of 1 in 15 using HRP diluent. As regards the preparation of standards, the Standard Diluent for human serum was used. Seven tubes were scrupulously cleaned and labeled accordingly. In the first tube, a 200pg/ml was made on reconstituting the vial. A 100µl of the diluted standard diluent was added into each tube assigned 2- 6. Then, 100µl of the standard from tube 1 was added into tube 2 and mixed thoroughly to obtain a concentration of 100pg/ml. A 100µl was lifted from tube 2 and transferred into tube 3. The content was gently mixed to obtain a concentration of 50pg/ml. The same step was repeated to the last tube (6th tube) to obtain concentrations of 25pg/ml, 12.5pg/ml and 6.25pg/ml respectively.

ASSAY PROCEDURE

All materials, the Standards, prepared reagents, and samples were brought to room temperature and the assay conducted in duplicate. One hundred microlitres (100µl) of each Standard, IX Control solution, samples and blank were added into the appropriate wells. A 50µl of IX Biotinylated anti-IL-6 was later added, covered and incubated for 1 hour at room temperature. The solution was discarded and washed 4 times with 300µl of wash solution. The liquid were completely removed after each wash. On completion of the last wash, the plate was inverted and blotted against a clean absorbent paper. A one hundred microlitre

(100μL) of IX Streptavidin-HRP solution was added to each well. The wells were covered and incubated for 30 minute at room temperature. The solution was again discarded and the normal process of washing was repeated.Any possible liquid in the well after the last wash was blotted. One hundred microlitre of chromogen TMB substrate solution was added into each well and incubated in the dark for 12 - 15 minutes at room temperature. The reaction was terminated on the addition of 100μl of the stop reagent and the yellow colour developed was read at 450nm.

CALCULATION

The mean of the absorbance for each set of duplicate standards, control and samples were taken and subtracted from the average optical density of zero standards. A standard curve on log-log graph paper was plotted with the concentration of the standards on the x-axis and absorbance on the y-axis and values of samples and controls interpolated.

SENSITIVITY

The minimum detectable dose of IL-6 using was <2pg/ml.

3.8.8QUANTITATIVE MEASUREMENT OF IL-10 BY ELISA TECHNIQUE AS DESCRIBED BY (ABCAM, 2013) Batch No. ab46034, Lot No. GR115656-3

PRINCIPLE:

The assay is of the competitive format. Antibody specific for IL-10 is immobilized in microtiter wells for the detection of IL-10. IL-10 present in the specimen and Biotinylated monoclonal antibody specific for IL-10 are added simultaneously. Both compete for the antibody coated in a microtiter well. After washing to remove the unbound IL-10/Biotinylated monoclonal Antibody, an enzyme complex Streptavidin-HRP is added and incubated. The unbound complex is washed and a TMB substrate solution is added. A blue colour develops. The stop solution changes the colour from blue to yellow. The intensity of

the colour is directly proportional to the concentration of lL-10 present in the samples. It is measured at 450nm.

PREPARATION OF REAGENTS AND STANDARDS

The manufacturer's directive was strictly followed in the preparation of the reagents and standards used to assay IL-10. The reagents that were prepared include IX Standard Diluent Buffer, IX Wash Buffer, IX Control Solution, IX Biotinylated anti-IL-10, IX streptavidin-HRP solution and various concentrations of the standard. As for the IX Standard Diluent Buffer, a 1 in 10 dilution was prepared using distilled water. As for IX Wash Buffer, a 1 in 200 dilution was prepared with distilled water. The reagents were gently mixed to avoid foaming. The lyophilized Control vial was reconstituted with the standard diluent for human serum. The solution was gently mixed to avoid foaming and allowed to stand for 5 minutes before it was used. Also, the preparation of IX Biotinylated anti-IL-10 was done with a dilution of 1 in 26.5 using Biotinylated Antibody Diluent. In addition, the IX streptavidin-HRP concentrate was prepared by adding 500µl of HRP diluent to the Streptavidin HRP vial. The already prepared IX streptavidin-HRP concentrate was further diluted with HRP diluent in the ratio of 1:67 dilutions. Similarly, the vial containing the standard was reconstituted with Standard Diluent for human serum to obtain 400pg/ml. Different concentrations of the standard were achieved by double diluting 400pg/ml to obtain 200pg/ml, 100pg/ml, 50pg/mI, 25pglml and l2pg/ml in 6 test tubes respectively. The Standard Diluent served as the zero standards.

ASSAY PROCEDURE:

All materials, the standards, prepared reagents, and samples were brought to room temperature and the assay conducted in duplicate. One hundred microlitres (100µL) of each Standard, IX Control Solution, Samples and Blank were added into the appropriate wells. A 50µl of IX Biotinylated anti-IL10 was later added, covered and incubated for 1 hour at room temperature. The solution was discarded and wells washed 4 times with 300µl of wash

solution. The liquid were completely removed after each wash. On completion of the last wash, the plate was inverted and blotted against a clean absorbent paper. A one hundred microlitre (100µl) of IX Streptavidin-HRP solution was added to each well. The wells were covered and incubated for 30 minutes at room temperature.The solution again was discarded and the normal process of washing as stated earlier was repeated. This was followed by blotting of any possible liquid in the well after the last wash was done. One hundred microlitre of chromogen TMB substrate solution was added into each well and incubated in the dark for 12 -15 minutes at room temperature.This was followed with the addition of 100µl of the stop reagent and the colour developed was read at 450nm.

CALCULATION

The mean of the absorbance for each set of duplicate standards, control and samples were taken and subtracted from the average values of the optical density of zero standards. A standard curve on log-log graph paper was plotted with the concentration of the standards on the x-axis and absorbance on the y-axis and values of samples and controls interpolated.

SENSITIVITY

The minimum detectable dose of IL-10 was <5 pg/ml.

3.8.9QUANTITATIVE MEASUREMENT OF IFN*γ* BY ELISA TECHNIQUE AS DESCRIBED BY (ABCAM, 2013) Batch no. ab46025, Lot No. GR146867-4, GR1168084

PRINCIPLE:

This assay is of the competitive format. A monoclonal antibody specific for IFNγ is coated into wells for the detection of IFNγ. Samples containing IFNγ and Biotinylated monoclonal antibody specific for IFNγ are added simultaneously and incubated. After washing, the enzyme Streptavidin-HRP is added to the bound IFNγ/ Biotinylated antibody complex. A TMB substrate solution is added and a blue colour develops which is proportional to the

amount of bound IFNγ. The stop solution changes the colour to yellow, and the intensity of the colour is measured at 450nm.

PREPARATION OF STANDARD

The manufacturer's directive was strictly followed in the preparation of the reagents and standards used to assay IFNγ. The reagents were IX Standard Diluent Buffer, IX Wash Buffer, IX Control Solution, IX Biotinylated anti- IFNγ and IX Streptavidin-HRP solution and various concentrations of the standards. For the IX Standard Diluent buffer, a 1 in 10 dilution was made using distilled water. As for IX wash buffer, a 1 in 200 dilution was made with distilled water. Each of the reagents was gently mixed to avoid foaming. The lyophilized Control vial was reconstituted using the standard diluent for human serum. The solution also was gently mixed to avoid foaming and allowed to stand for 5 minutes before it was used. Again, the preparation of IX Biotinylated anti-IFNγ was done with a dilution of 1 in 26.5 using Biotinylated Antibody Diluent. In addition, the IX streptavidin-HRP concentrate was prepared by adding 500µl of HRP diluent to the streptavidin HRP vial. The already prepared IX streptavidin-HRP concentrate was further diluted with HRP Diluent in the ratio of 1:67 dilutions. Similarly, the vial containing the standard was reconstituted with standard diluent for human serum to obtain 400pg/mI. Different concentrations of the standard were made by double diluting the 400pg/ml to obtain 200pg/ml, 100pg/ml, 50pg/ml, 25pg/ml and 12.5pg/ml in 6 test tubes respectively. The standard diluent serves as the zero standards.

ASSAY PROCEDURE

All materials, the standards, prepared reagents, and samples were brought to room temperature and the assay conducted in duplicate. One hundred microlitres (100µl) of each standard, IX control solution, samples and blank were added into the appropriate wells. A 50µl of IX Biotinylated anti-IL-10 was later added, covered and incubated for 1 hour at room temperature. The solution was discarded and washed 4 times with 300µl of wash solution.

The liquid were completely removed after each wash. On completion of the last wash, the plate was inverted and blotted against a clean absorbent paper. One hundred microlitres (100µl) of IX streptavidin-HRP solution was added to each well. The wells were covered and incubated for 30 minutes at room temperature. The solution was again discarded and the normal process of washing earlier carried out was repeated. Retained liquid in the well after the last wash was blotted on an absorbent paper. One hundred microlitre of chromogen TMB substrate solution was added into each well and incubated in the dark for 12-15 minutes at room temperature. The reaction was terminated on the addition of 100µl of the stop reagent and the colour developed was read at 450nm.

CALCULATION

The mean of the absorbance for each set of duplicate standards, control and samples were taken and subtracted from the average optical density of zero standards. A standard curve on log-log graph paper was plotted with the concentration of the standards on the x-axis and absorbance on the y-axis and values of samples and controls interpolated.

SENSITIVITY

The minimum detectable dose of IFN gamma was <5 pg/mL.

3.9.1QUANTITATIVE MEASUREMENT OF TNFα BY ELISA TECHNIQUE AS DESCRIBED BY (ABCAM, 2013) Batch No. ab100654, Lot No. GR154836-1, GR150780-1, GR151204-1

PRINCIPLE:

This assay is of the sandwich format. Antibody specific for human TNFα is coated in a well for the detection of TNFα. TNFα in the specimen is bound by the immobilized Antibody. The wells are incubated and washed before adding Biotinylated anti-human TNFα Antibody. After washing away the unbound Biotinylated antibody, the enzyme HRP conjugated streptavidin is added. The wells are again washed and a TMB substrate solution added. A

colour develops which intensity is in proportion to the amount of bound TNFα. The stop solution changes the colour from blue to yellow and is measured at 450nm.

PREPARATION OF REAGENTS AND STANDARDS

The manufacturer's directive was strictly followed in the preparation of reagents and standards used to assay TNFα. The prepared reagents were IX Assay Diluent Buffer, IX Wash Buffer, IX Biotinylated TNFα Detection Antibody, IX streptavidin-HRP solution and different concentrations of the standard. For IX-Assay Diluent Buffer, a 5X Assay Diluent was diluted 1 in 5 with distilled water. For IX Wash Solution, 380mls of distilled water was added to 20mls of 20X Wash Buffer Concentrate to yield 400mls of IX Wash Buffer. As for the preparation of IX Biotinylated TNFα detection antibody, the vial containing IX Biotinylated anti-human TNFα was briefly spun and 100µl of IX Assay Diluent B added to the vial, to yield the Antibody Concentrate. The solution was gently mixed and a further dilution of 1 in 80 dilution of the concentrate was made using IX Assay Diluent B. For the IX HRP streptavidin solution, another brief spun of the 600X HRP streptavidin concentrate vial was done and a 600 fold dilution made with IX Assay Diluent B prior to use by dissolving 20µl of 600X HRP streptavidin concentrate into a tube with 12ml of IX Diluent B. Similarly, serial dilutions of the standards were prepared immediately prior to use as follows; After a brief spin of the vial of TNFα Standard, a concentration of 50ng/mL Stock Standardwas prepared by adding 160µL Assay Diluent A (for serum/plasma samples) into the vial. Then, standard of 7 different concentrations were prepared from the stock standard (50ng/ml) to obtain values of 6000pg/ml, 2400pg/ml, 960pg/ml, 384pg/ml, 153.6pg/ml, 61.44pg/ml and 24.58pg/ml. The steps were as follows: 7 scrupulously cleaned sterile tubes were labeled 1 to 7. In the first tube, 80µl of 50ng/ml (50,000pg/ml) of stock standard was added to 586.7ml of assay diluent A. This was gently mixed and a concentration of 6000pg/ml was obtained. A 300µl of Assay Diluent A was pipetted and added into the remaining tubes either from tube 2

to 7. A 200µl of the standard in tube 1 (6000pg/ml) was added into tube 2 to obtain a concentration of 2400 pg/ml. The same steps were taken to the 6th tube to achieve concentrations of 960.0pg/ml, 384/ml, 153.6pg/ml, 61.44pg/ml and 24.58pg/ml respectively while assay diluent A serves as the zero standard (0pg/ml).

ASSAY PROCEDURE

All materials, the standards, prepared reagents, and samples were brought to room temperature and the assay conducted in duplicate. One hundred microlitres (100µL) of each standard and samples were added into the appropriate wells. The plate was covered and incubated for 2.5 hours at room temperature. The solution was discarded and washed 4 times with 300µl of wash solution. The liquid was completely removed after each wash. On completion of the last wash, the plate was inverted and blotted against a clean absorbent paper. One hundred microlitres (100µl) of IX Biotinylated TNFα Detection Antibody was added to each well and was later incubated for 1 hour at room temperature with a gentle shake. Again the solution was discarded and washed 4 times with 300µl of wash solution. The liquid was completely removed after each wash. On completion of the last wash, the plate was inverted and blotted against a clean absorbent paper. A 100µl of IX Streptavidin-HRP solution was added to each well. The wells were covered and incubated for 45 minutes at room temperature. The solution was again discarded and the normal process of washing was repeated and blotting of remaining liquid in the well was done after the last wash.One hundred microlitre of chromogen TMB substrate solution was added into each well and incubated in the dark for 30 minutes at room temperature. The reaction was terminated on the addition of 50µl of the stop reagent and the colour developed was read at 450nm.

CALCULATION

The mean of the absorbance for each set of duplicate standards, control and samples were taken and subtracted from the average optical density of zero standards. A standard curve on

log-log graph paper was plotted with the concentration of the standards on the x-axis and absorbance on the y-axis and values of samples and controls interpolated.

SENSITIVITY

The minimum detectable dose of TNFα is typically less than 30pg/ml

3.9.2QUANTITATIVE ASSAY OF sTfR BY ELISA TECHNIQUE AS DESCRIBED BY (MONOBIND INC., 2013). Catalogue No. 8625-300

PRINCIPLE:

Immunoenzymometric sequential assay (TYPE 4):

A specific anti sTfR antibody with different and distinct epitope recognition is coated in a microtiter well. Monoclonal Biotinylated antibody and serum containing the native antigen are added simultaneously. A reaction result forming an antibody antigen complex. The biotin attached to the antibody binds to the streptavidin coated in the microwells resulting in immobilization of the complex. The complex is incubated and later on washed to remove the unbound antibody-antigen biotinylated complex.

The interaction is illustrated by the following

$$Ag_{(sTfR)} + Ab_{(m)} \underset{K_{-a}}{\overset{K_a}{\rightleftharpoons}} Ag_{(sTfR)} - {}^{Btn}Ab_{(m)}$$

where,

${}^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

$Ag_{(sTfR)}$ = Native Antigen (Variable Quantity)

$Ag_{(sTfR)} - {}^{Btn}Ab_{(m)}$ = Antigen-Antibody complex (Variable Quan.)

K_a = Rate Constant of Association

K_{-a} = Rate Constant of Disassociation

$Ag_{(sTfR)} - {}^{Btn}Ab_{(m)}$ + Streptavidin C.W. ⟶ immobilized complex (IC)

Streptavidin C.W. = Streptavidin immobilized on well

Immobilized complex (IC) = Ag-Ab bound to the well

After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation. Another antibody (directed at a different epitope) labeled

with an enzyme is added. An interaction occurs to form an enzyme antibody-antigen-Biotinylated-antibody complex in the wells. Excess enzyme is washed. The substrate is added and a blue colour develops that is proportional to the concentration of the native antigen.

The intensity of the colour is measured at 450nm. Different serum references of known antigen concentration were used to determine the concentration of the antigen in the specimen.

$$(IC) + {}^{Enz}Ab_{(x - sTfR)} \; {}^{Enz}Ab_{(x - sTfR)} \underset{K_{-b}}{\overset{K_b}{\rightleftharpoons}} IC$$

Where,

${}^{Enz}Ab_{(x - sTfR)}$ = Enzyme labeled Antibody (Excess Quantity) EflZAb(ferritin) ${}^{Enz}Ab_{(x - sTfR)}$ –

IC = Antigen-Antibodies Complex

K_b = Rate Constant of Association

K_{-b} = Rate Constant of Dissociation

PREPARATION OF WASH BUFFER

The preparation of the wash buffer was done following the manufacturer's directive; 980ml of distilled water was added to 20ml of the wash buffer to obtain 1000ml of the solution.

PROCEDURE

All reagents, serum references and control were brought to room temperature 20 – 27^{o}C. The assay was carried out in duplicates. Ten microlitres of the appropriate serum reference control and specimen were pipetted into the appropriate well. A 100µl of sTfR Biotin Reagent was added to all the wells and the microplate was gently mixed for 30 seconds, covered and incubated for 45 minutes at room temperature. The content of the wells were discarded and the plate blotted with absorbent paper. Later 300µl of wash buffer was added and well washed 3 times. A 100µl of Anti-sTfR Enzyme Reagent was added to all the wells which were covered and incubated for 30 minutes at room temperature. The content of the microplate were again discarded and the plate blotted with absorbent paper. Another 300µl of the wash buffer was added and the wells were again washed. The washing was again repeated

for two (2) additional times for a total of three (3) washes. This was followed by the blotting of the plate on an absorbent paper. One hundred microlitre (100μl) of substrate solution was added to all wells, allowed to stand without shaking and incubated for 15 minutes at 37^{o}c. The reaction was terminated on the addition of 50μl of stop solution. The mixture was gently mixed for 15 – 20 seconds. The colour produced in each well was read within 30 minutes at 450nm.

CALCULATION

The mean absorbance for each duplicate serum reference versus the corresponding soluble transferrin concentration was interpolated.

Expected Values for the sTfR Test System

1.8 – 4.6mg/L ⟺ 25.13 – 64.22nmol/L

Conversion between mg/L and nmol/L

(nmol/L + 13.96 = (mg/L ⟺ (mg/L x 13.96 = nmol/L)

SENSITIVITY

The sTfR AccuBind™ has a sensitivity of 0.055nmol/L.

3.9.3QUANTITATIVE ASSAY OF SERUM FERRITIN BY ELISA TECHNIQUE AS DESCRIBED BY (MONOBIND INC., 2012). Catalogue No. 2825-300

PRINCIPLE:

Immunoenzymometric Sequential Assay (TYPE 4):

A specific anti-ferritin antibody with different and distinct epitope recognition is coated in a microtiter well. Monoclonal Biotinylated antibody and serum containing the native antigen are added simultaneously. A reaction result forming an antibody antigen complex. The biotin attached to the antibody binds to the streptavidin coated in the microwells resulting in immobilization of the complex. The complex is incubated and later on washed to remove the unbound antibody-antigen complex.

The interaction is illustrated by the following

$$Ag_{(ferritin)} + Ab_{(m)} \underset{K_{-a}}{\overset{K_a}{\rightleftharpoons}} Ag_{(ferritin)} - {}^{Btn}Ab_{(m)}$$

Where,

${}^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

$Ag_{(ferritin)}$ = Native Antigen (Variable Quantity)

$Ag_{(ferritin)} - {}^{Btn}Ab_{(m)}$ = Antigen-Antibody complex (Variable Quan.)

K_a = Rate Constant of Association

K_{-a} = Rate Constant of Disassociation

$Ag_{(ferritin)} - {}^{Btn}Ab_{(m)}$ + Streptavidin C.W. ⟶ immobilized complex (IC),

Where,

Streptavidin C.W. = Streptavidin immobilized on well

Immobilized complex (IC) = Ag-Ab bound to the well

After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decanting the mixture. Another antibody (directed at a different epitope) labeled with an enzyme is added. An interaction occurs to form an enzyme antibody-antigen-Biotinylated-antibody complex on the surface of the wells. Excess enzyme is washed. The substrate is added and a blue colour develops that is proportional to the native antigen concentration. The intensity of the colour is measured at 450nm. Different serum references of known antigen concentration were used to determine the concentration of the antigen in the specimen.

$$(IC) + {}^{Enz}Ab_{(ferritin)} \underset{K_{-b}}{\overset{K_b}{\rightleftharpoons}} {}^{Enz}Ab_{(ferritin)} - IC$$

Where,

${}^{Enz}Ab_{(ferritin)}$ = Enzyme labeled Antibody (Excess Quantity) EflZAb(ferritin) ${}^{Enz}Ab_{(ferritin)} - IC$ = Antigen-Antibodies Complex

K_b = Rate Constant of Association

K_{-b} = Rate Constant of Dissociation

Preparation of reagent: The reagents were prepared as directed by the manufacture. The reagents prepared were the wash buffer and the working substrate solution. For the wash buffer, a 1 in 50 dilution was made on addition 20mls of the wash buffer to 980mls of distilled water. As for the working substrate, equal volumes of substrate solution A was mixed with equal volumes of substrate solution B.

Test procedure: All reagents, serum references, controls and test specimen were brought to room temperature (20 – 27^{o}C) and the assay carried out in duplicate.Twenty-five microlitre (25µl) of the appropriate serum reference, control or specimen was pipetted into the respective wells. A 0.100ml (100µl) of the Ferritin Biotin Reagent was dispensed, close to the bottom of the coated well and the microplate gently mixed for 20-30 seconds, covered and incubated for 30 minutes at room temperature. The contents of the microplate were discarded and blotted with absorbent paper. 300µl of the washed buffer were added and the wells washed 3 times. This was followed by the blotting of the plate on an absorbent paper. A 100µl of the Ferritin Enzyme Conjugate was added to each of the wells. Care was taken to avoid the well being shaken while incubation was done for 30 minutes at room temperature. The contents of the microplate were again discarded and blot dried with an absorbent paper. Also 300µl of wash buffer was added and the washing process duly repeated for 3 times. A 100µl of working substrate solution was added to all wells. Again uttermost care was taken to avoid the wells being shaken after the addition of the working substrate. The plate was incubated at room temperature for fifteen (15) minutes. A 0.050ml (50µl) of stop solution was added and the wells mixed gently for 16-20 seconds. The absorbance of each well was read at 450nm.

CALCULATION OF RESULTS

The absorbance for each duplicate serum reference versus the corresponding ferritin concentration in ng/ml was interpolated.

EXPECTED RANGE OF VALUES

Female 10 – 124ng/ml

SENSITIVITY

The minimum detectable dose is 0.17ng/ml.

3.9.4 ESTIMATION OF FULL BLOOD COUNTS AND RED CELL INDICES USING SYSMEX AUTOMATED 3 PARTS HAEMATOLOGY ANALYZER MODEL KX-21N MANUFACTURED BY SYSMEX, KOBE, JAPAN.

Principle: Four basic principles of determination are integrated in the system. These are the electric impedance hydrodynamic focusing, light scattering, fluorescence/flow cytometry and non-cyanide haemoglobin methods. Impedance is generated when non-conducting particles passes through the sensing zone. Scattering of light occurs when light reaches a surface of any material and changes its orientation. Fluorescent dyes absorb light at a narrow wavelength and re-emits light at a long wavelength.

3.9.5HAEMOGLOBIN ESTIMATION

Haemoglobin is determined by spectrophotometric method. The red blood cells are haemolyzed and the haemoglobin converted to a single stable form such as oxyhaemoglobin or sodium-lauryl sulphate (SLS) haemoglobin. A beam of light that passes through the sample and a 525nm filter is measured by a photo-sensor. The signal is then amplified and the amount of haemoglobin is measured and compared to the blank reference reading. The HB was calculated per the following equation and expressed in g/l.

$HB(g/L) = \text{constant} \times Log_{10}$ (Blank photocurrent/sample photocurrent).

3.9.6.ESTIMATION OF RED BLOOD CELLS (RBC)

Red blood cells and platelets are counted by direct current (electric) impedance method. The method is based on the measurement of transitory electrical resistance produced by particles.

An electrode is submerged in the liquid on both sides of an aperture to create an electrical pathway. In impedence method, cells such as red blood cells, platelets and white blood cells are regarded as completely non conducting particles. Blood cells suspended in electrolyte solution, passes through an aperture (sensing zone), and a change of impedance (electrical resistance) is produced which in turn generates an electrical pulse. The amount of pulse generated indicates the number of particles that passes through the aperture and the amplitude of each pulse is proportional to the volume of each cell. Each pulse is amplified and compared to the internal reference channel which only accepts the pulse of certain amplitudes. If the pulse generated was above the RBC/PLT lower threshold, it will be counted as an RBC/PLT.

3.9.7.ESTIMATION OF TOTAL LEUKOCYTE COUNT

The impedance or/and light scattering methods are adopted in counting white blood cell. In the impedance method, the red blood cells are haemolyzed and the same process in counting red blood cells/platelets is applied. With the light scattering method, it is observed that when light reaches the surface of any material, the light changes its orientation and scattering occurs. The light scattering occurs at all angles between 0^o and 360^o. Light scattering from the side (side scattered light) or angle scattered light provides information on the internal quality of a cell while the forward scattered light determines the size of the cell. On this note, white blood cells can be analyzed with regard to their size and structural complexity (form, cytoplasmic granularity and density of the nucleus). The scattered light is detected by a photomultiplier and the rate at which it is generated is proportional to the number of cell counted and the surface area of the cells.

3.9.8. ASSESSMENT OF DIFFERENTIAL LEUKOCYTE COUNT

The fluroscence flow cytometry technology is applied in the differentiation of WBCs and immature WBCs,. A fluroscence dye is applied to stain the cells. Fluroscence dye absorbs

light at a narrow wavelength and re-emittes light of a long wavelength. The amount of light absorbed is proportionate to the concentration of the substance analyzed. After sample aspiration, a part of the whole blood sample was diluted to 1:50 with lysing reagent stromatolyser-4DL and then stromatolyser-4DS dye was added. After a predefined response time the stained sample was introduced into the detector where forward light scatter and side fluorescent emission were measured. With Sysmex KX-2IN The cells are stained and computed into 3 leukocyte population namely neutrophol count, lymphocyte count, and mixed leucocyte count as well as percentage neutrophil, percentage, lymphocyte and percentage mixed leucocyte.

PROCEDURE

Sysmex machine was inspected (for instrument, reagent, waste bin and printer paper). The machine was switched on and machine calibrated before use. The control sample was analysed along with each batch of sample. The analysis commenced once the instrument displayed “Ready” status on the LCD screen. The mode was selected and samples moved to the aspiration unit.

The sample was thoroughly mixed and the tube carefully set to the sample probe. The start button was switched on and a little delay observed till the buzzer sounds two times- “beep, beep” – and the LCD screen displays “Analyzing”. The tube was opened and the sample aspirated through the sample probe. The tube was removed after a beep sound indicating completion of aspiration and the aspiration cap returns to clean the aspiration probe while the analysis is going on. On completion of analysis, the result was displayed on the LCD screen. The unit returns to the Ready status, being ready for analysis of the next sample(s). Results were printed out on the in-built printer. The shut down was initiated to clean the detector and dilution lines. Finally the power soure was switched off.

3.9.9. ESTIMATION OF GLUCOSE AS DESCRIBED BY (RANDOX 2007) Ref. No. GL364; Lot No. 122957

The random blood glucose level was evaluated using the glucose oxidase-perioxidase method as described by (Cheesbrough, 2010).

ASSAY PRINCIPLE

The enzyme glucose oxidase catalyses the oxidation of glucose with the production of gluconic acid and hydrogen peroxide. Addition of the enzyme peroxidase and a chromogenic oxygen acceptor, such as 0-dianisidine, results in the formation of a coloured compound. Its intensity is proportional to the glucose concentration and is measured at 500nm.

$$\text{Glucose} + 2H_2O + O_2 \xrightarrow[\text{Glucose oxidase}]{\text{Peroxidase}} \text{Gluconic acid} + 2H_2O_2$$

O-Dianisidine (colourless) + H_2O_2 ⟶ Oxidized O-Dianisidine (coloured)(oxygen acceptor) + H_2O.

PROCEDURE

Clean test tubes were labeled accordingly as samples, standard and blank. About 0.05ml of samples, standard and distilled water were pipetted into their corresponding tubes. Also, 2mls of the commercially prepared reagents were added to each of the tubes, mixed and incubated at 37^{o}C for 10 minutes. Absorbance was measured against reagent blank at 510nm. Glucose concentration was calculated as:

$$\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard}\ x\ concentration\ of\ standard$$

3.9.9.1 ESTIMATIONOF CREATININE AS DESCRIBED BY (RANDOX, 2013) Ref. No. CR510; Lot No. 276534

PRINCIPLE:

Creatinine reacts with picric acid to produce a coloured compound known as alkaline creatinine picrate. The intensity of the colour produced or change in absorbance is proportional to the creatinine concentration.

PROCEDURE

Clean test tubes were properly labeled as sample, standard and blank. About 1000ml of working reagent was added to each tube, followed by 100ml of sample, standard and distilled water into their corresponding tubes. They were mixed and the absorbance taken 60 seconds after adding the sample, standard and distilled water. This serves as the first reading (T1). Exactly 60 seconds after the first reading, the second reading (T2) was taken. The absorbance was read at 505nm. The serum creatinine was calculated as:

$$\text{Creatinine concentration (mg/dl)} = \frac{(\text{T2-T1})\text{of sample x standard concentration}}{(\text{T2-T1})\text{of standard}}$$

3.9.9.2. QUANTITATIVE DETERMINATION OF C-REACTIVEPROTEIN

C-RP was determined using immunoturbidimetric method as described by (Otsuji *et al.*,1982).

PRINCIPLE:

This is a latex enhanced turbidimetric immuno assay. C-RP binds to specific anti- C-RP antibodies, which have been adsorbed to latex particles to produce turbidity. The turbidity is proportional to the concentration of C-RP in the sample. The actual concentration is then determined by interpolation from a calibration curve prepared using calibrators.

PROCEDURE

Six calibrators were prepared using NaCl (0.9mg/dl) as diluent, the corresponding dilution factor used to multiple the concentration of the calibrator to obtain the C-RP concentration for each tube dilution. About 450µl of Reagent 1 was added to the assigned tubes for calibrators, samples and blank respectively. Also, l50µl of Reagent 2 was added to the labeled tubes. Then, 5µl of the diluted calibrators and the samples were respectiveely added to the designated tubes and 5µl of distilled water was added into the blank. The tubes were mixed and absorbance of calibrators, samples and blank respectively were read immediately after 2 mins at 578nm.

CALCULATION

The exact value was obtained by subtracting the absorbance value of the sample from that of blank and extrapolated from the calibration curve.

3.9.9.3. STATISTICAL ANALYSIS

The results were expressed as mean and standard deviation. Student's t-test and analysis of variance (ANOVA) were used for comparison of differences in various groups.Level of significance was set at $p<0.05$. The tests ofassociation wereperformed using Pearsonian Correlation.A two sided P-value of less than 0.05 were considered statistically significant for tests of association. All statistical analysis were performed using Statistical Package for Social Sciences (SPSS) version 21.0

CHAPTER FOUR

4.1 RESULTS

Two hundred and six (88%) out of234 pregnant and 50 (96%) out of 52 immediate post partum women thattested negative to HIV infection were enrolled for this study. The two groups were further stratified into 4 groups. Group 1 comprised of 144 (69.9%) malaria parasitaemic pregnant women while group 2 were 62 (30.1%) aparasitaemic pregnant women. Group 3 were made of 30 (60%) post partum women with placental malaria while group 4 were 20 (40%) post partum women with placenta uninfected by malaria. The control samples, 40 uninfected non pregnant women and constituted the 5^{th} group. All that participated as test-subjects and control groups were asymptomatic and apparently healthy pregnant and nonpregnant womenrespectively. They were between 17 – 44 years. Resultsare presented as tables, charts and graphs.

Table 4.1 showed comparison ofsome cytokine levels of malaria infected pregnant women. The cytokines include Interferon Gamma(IFNγ), Tumour Necrosis Factor Alpha(TNFα), Interleukin-4(IL-4), Interleukin-6(IL-6) and Interleukin-10(IL-10). The values of the cytokines were expressed as mean±SD and in pg/ml.

The value22.94 ± 12.71of IFNγfor malaria infected pregnant womenwas significantly higher ($p<0.005$) compared with 5.98 ± 3.11 and 4.69 ± 2.64of the malaria uninfected pregnant and uninfected non-pregnant groups respectively.

The mean±SD(5.98±3.11) of IFNγ for uninfected pregnant women showed no significant difference ($P>0.05$) when compared with 4.69±2.64 for uninfected non-pregnantwomen.The mean±SD (21.12±12.57) of TNFαfor the infected pregnant subjects was significantly higher ($P<0.05$) when compared with 10.03±3.04 and 4.66±0.78 of the uninfected pregnant,and

uninfected non-pregnant groups respectively.Again, the mean±SD (10.03±3.04) of TNFαfor uninfected pregnant groupwassignificantly higher ($p<0.05$) compared to 4.66±0.78 for the uninfected non-pregnant group.

The mean±SD (9.66 ± 7.05) of IL-4 for malaria infected pregnant womenwas significantlyhigher ($P<0.05$) than7.17±3.91 and 2.13±0.36 for the uninfected pregnant and uninfected non-pregnant women respectively. The mean±SD (7.17±3.91) of IL-4 for uninfected pregnant women was significantly higher ($P<0.05$) when compared with 2.13±0.36 for the uninfected non-pregnant women.

The mean±SD (32.11±27.92) of IL-6 for the infected pregnant women was higher and statistically significant ($P<0.05$) compared with8.68±8.41 and 2.33±0.58 for uninfected pregnant and uninfected non-pregnant women respectively. The mean±SD (8.68±8.41) of IL-6 for uninfected pregnant women was higher and significantly different ($P<0.05$) compared with 2.33±0.58 for uninfected non-pregnant women.The mean±SD of IL-6 for uninfected non-pregnant women was significantly higher ($P<0.05$) than2.33±0.58for uninfected non-pregnant women

The mean±SD (35.19±28.82) of IL-10 for the infected pregnant women was significantly higher ($P<0.05$) compared with14.76±6.17 and 6.45±4.15 for uninfected pregnant and uninfected non pregnant women respectively.The mean±SD (14.76±6.17) of IL-10 for uninfected pregnant women was significantly higher ($P<0.05$) when compared with 6.45±4.15 for uninfected non-pregnant women.

TABLE 4.1: COMPARISON OF MEAN±SD OF CYTOKINE LEVELS OF MALARIA INFECTED PREGNANT WOMEN.

GROUPS	Cytokines (pg/ml)				
	IFN-γ	TNF-α	IL-4	IL-6	IL-10
G1 (n = 144)	22.94 ± 12.71	21.12 ± 12.57	9.66 ± 7.05	32.11 ± 27.92	35.19 ± 28.82
G2 (n = 62)	5.98 ± 3.11	10.03 ± 3.04	7.17 ± 3.91	8.68 ± 8.41	14.76 ± 6.17
G5 (n = 20)	4.69 ± 2.64	4.66 ± 0.78	2.13 ± 0.36	2.33 ± 0.58	6.45 ± 4.15
F(p) Value	64.00 (0.00)	30.26 (0.00)	15.17 (0.00)	23.12 (0.00)	21.47 (0.00)
G1 vs G2	0.001*	0.001*	0.008*	0.001*	0.001*
G1 vs G5	0.001*	0.001*	0.001*	0.001*	0.001*
G2 vs G5	0.277	0.001*	0.001*	0.001*	0.001*

Key
α-level set at 0.05
*(P< 0.05) = Significant
P> 0.05= Not Significant
G1 = Malaria Infected Pregnant women
G2 = Malaria Uninfected Pregnant women
G5 = Malaria Uninfected Non-Pregnant women

Table 4.2 showed comparison of some cytokine levels of malaria infected post partumwomen. The cytokineinclude, TNFα, IL-4, IL-6 and IL-10. The values were expressed as mean±SD and in pg/ml.

The mean±SD (11.71±6.55) of IFNγ for malaria infected post partum was significantly higher (P<0.05) when comparedto 5.58±2.86, and 4.69±2.68 for uninfected post partum and uninfected non-pregnant women respectively.The mean±SD (5.58±2.86) of IFNγ for uninfected post partum women was higher than 4.69±2.68 for uninfected non pregnant women but did not show any difference when compared statistically.

For TNFα, the mean±SD (19.35±10.94) for malaria infected post partum was significantly higher (P<0.05) when compared with 12.36±6.81 and 4.66±0.78 for uninfected post partum and uninfected non-pregnant women respectively.Again, the mean±SD (12.36±6.81) for uninfected post partum women and was significantly higher (p<0.05) when compared with 4.66±0.78 for uninfected non-pregnant women.

For IL-4, the mean±SD (14.86±6.37) for infected post partum womenwas significantly higher(P<0.05) when compared with 2.13±0.36 for uninfected non-pregnant women but showed no significant difference P>0.05 with 12.03±5.01 for the uninfected post partum women. Also the mean±SD (12.03±5.01) for uninfected post partum women was significantly higher (P<0.05) on comparison with 2.13±0.36 for uninfected non pregnant women respectively.

For IL-6, the mean±SD(34.27±13.78) for malaria infected post partum women was higher and showedno significant difference (P>0.05) when compared with 26.99±12.65 for malaria uninfected post partum women and significantly higher (P<0.05) when compared with 23.42±0.45 and 2.34±0.58 for infected and uninfected non-pregnant women respectively.

Also, the mean±SD (26.99±12.65) for uninfected post partum women showed significantly higher value (P<0.05) when compared with 2.34±0.58 for uninfected non-pregnant women.With IL-10, the mean±SD (55.57±43.13) for malaria infected post partum women was significantly higher (P<0.05) when compared with 16.60±4.88 and 6.45±4.13 for uninfected post partum and uninfected non-pregnant women. Again, the mean±SD (16.60±4.88) for uninfected post partum women showeda higher stignificant value (P<0.05)compared with 6.45±4.15 for the uninfected non-pregnant women.

TABLE 4.2: COMPARISON OF SOME CYTOKINE LEVELS OF MALARIA INFECTED POST PARTUM WOMEN

GROUPS	Cytokines (pg/ml)				
	IFN-γ	**TNF-α**	**IL-4**	**IL-6**	**IL-10**
G3 (n = 30)	11.71 ± 6.55	19.35 ± 10.94	14.86 ± 6.37	34.27 ± 13.78	55.57 ± 43.13
G4 (n = 20)	5.58 ± 2.86	12.36 ± 6.81	12.03 ± 5.01	26.99 ± 12.65	16.60 ± 4.88
G5 (n = 20)	4.69 ± 2.68	4.66 ± 0.78	2.13 ± 0.36	2.34 ± 0.58	6.45 ± 4.13
F(p) Value	155.91 (0.00)	17.14 (0.00)	47.36 (0.00)	42.482 (0.00)	19.34 (0.00)
G3 vs G4	0.001*	0.037*	0.306	0.233	0.001*
G3 vs G5	0.001*	0.001*	0.001*	0.001*	0.001*
G4 vs G5	0.730	0.001*	0.001*	0.001*	0.001*

Key
α-level set at 0.05
*(P< 0.05) = Significant
P> 0.05= Not Significant
G3 = Malaria Infected Post-Partum women
G4 = Malaria Uninfected Post-Partum women
G5 = Malaria Uninfected Non-Pregnant women

Table 4.3 shows comparison of cytokine levels, parasite density and leucocyte count between peripheral and placental blood among malaria infected pregnant and post-partum women.

The mean±SD (22.94±12.71)of IFNγ in the peripheral blood was significantly (P<0.05) higher than that of placental blood (11.71±6.55). On the other hand, the mean±SD (21.12±12.57)of TNFα in the peripheral blood showed no significant difference (P>0.05) when compared with 19.35±10.94 in the placental blood. Values of cytokines were expressed in pg/ml, parasite density as parasite/µl, total leucocytes count and differential leucocyte count as Absolute Values in $\times 10^9$/l.

As for IL-4, the mean±SD (9.66±7.05)in the peripheral blood was significantly (P<0.05) lower than 14.86±6.37 in the placental blood.

ForIL-6, the mean±SD 32.11±27.92 in the peripheral blood showed no significant difference (P>0.05) with 34.27±13.78 in the placental blood.

However,the mean±SD(35.19±28.81) of IL-10 in the peripheral blood was significantly (P<0.05) lower than 55.57±43.13 in the placental blood.

The mean±SD (658.56±484.55)of the parasite density in the peripheral blood was lower and showed no significant difference (P>0.05) with 762.47±459.62 in the placental blood.

As for total leucocyte count, the mean±SD (7.09±2.02)in the peripheral bloodwas significantly (P<0.05) lower than 11.00±4.80 in the placental blood.

The mean±SD (3.9±0.91)of neutrophil count in the peripheral blood was significantly (P<0.05) lower than the placental blood (5.0±0.78).

The mean±SD (2.3±0.77)of lymphocyte count in the peripheral blood was significantly (P<0.05) lower than 4.9±0.89 in the placental blood. Furthermore, the mean±SD (0.9±0.41) of mixed differential leucocyte count in the peripheral blood was significantly (P<0.05) lower than 1.1±0.25 from the placental blood.

TABLE 4.3: COMPARISON OF MEAN±SD OF CYTOKINE LEVELS, PARASITE DENSITY & LEUCOCYTE COUNT BETWEEN PERIPHERAL AND PLACENTAL BLOOD AMONG MALARIA INFECTED PREGNANT AND POST-PARTUM WOMEN.

Parameters	Peripheral Blood (n = 144)	Placental Blood (n = 30)	P- values
IFNγ (pg/ml)	22.94 ± 12.71[a]	11.71 ± 6.55[b]	0.001
TNFα (pg/ml)	21.12 ± 12.57[a]	19.35 ± 10.94[a]	0.862
IL-4 (pg/ml)	9.66 ± 7.05[a]	14.86 ± 6.37[b]	0.001
IL-6 (pg/ml)	32.11 ± 27.92[a]	34.27 ± 13.78[a]	0.922
IL-10 (pg/ml)	35.19 ± 28.81[a]	55.57 ± 43.13[a]	0.082
MP (p/ul)	658.56 ± 484.55[a]	762.47 ± 459.62[a]	0.082
WBC ($x10^9$/L)	7.09 ± 2.02[a]	11.00 ± 4.80[b]	0.001
NEUT($\times10^9$/l)	3.9±0.91[a]	5.0±0.78[b]	0.001
LYMP($\times10^9$/l)	2.3±0.77[a]	4.9±0.89[b]	0.009
MXD($\times10^9$/l)	0.9±0.41[a]	1.1±0.25[b]	0.001

KEY
α-level set at 0.05
a and b are superscripts used to show significant difference.
Values not sharing the same superscript means there is a significant difference
Values sharing the same superscript means there is no significant difference

Table 4.4 shows the comparison of influence of age on cytokine level of malaria infected pregnant women. Five age groups: G1, G2, G3, G4 and G5 were designated to represent 20-24, 25-29, 31-34, 35-39 and 40-44 years respectively. Values of cytokines were expressed in pg/ml.

For IFNγ, the mean±SD (21.00±10.25) for Group 1 showed no significant difference (P>0.05) when compared to 22.83±13.80, 22.94±12.63, 26.28±13.86 and 21.37±10.33for Groups 2, 3,4 and 5 respectively. Overall different values of mean±SD for IFNγ was recorded for various groups however, within the groups they showed no significant difference (P>0.05) ForTNFα, the mean±SD (19.49±7.99)for Group 1 showed no significant difference (P>0.05) when compared with 19.19±10.69 for group 2 and also was insignificantly lower when compared to 21.38±13.89, 23.16±12.89 and 28.84±16.37 for groups 3, 4 and 5 respectively.Different values of mean±SD for TNFα was recorded for various groups however within the group they showed no significant difference (P>0.05)

As for IL-4, the mean±SD (6.94±4.38) for group 1 was lower but statistically insignificant (P>0.05) on comparison with8.46±5.53, 10.94±6.56, 10.77±13.03 and 9.96±7.33 for group 2, 3, 4 and 5respectively. Different values of mean± SD for IL-4 was recorded for various groups and within the group however they showed no statistical difference (P>0.05)

Similarly with IL-6, the mean±SD (32.64±29.43)for malaria infected group 1 pregnant women showed no significant difference (P>0.05) when compared to 29.60±23.40, 33.36± 29.83, 26.33± 24.15 and 45.12±39.70 for groups 2, 3, 4 and 5 respectively.Different values of mean±SD for IL-6 was recorded and within groups, however they showed no statistical significant difference (P>0.05).

For IL-10, the mean±SD (17.55±7.73) for group 1 of malaria infected pregnant womenshowed significant difference (P<0.05) when compared to 34.26±26.99 and 40.74±31.46 for groups 2 and 3 respectively but showed no significant difference (P>0.05) to

30.89±29.82 and 37.10±31.65 for groups 4 and 5 respectively.Overall, different values of mean±SD of IL-10 for groups 2, 3, 4 and 5 showed no significant difference (P>0.05) when compared within groups.(See appendix IIIa)

Table 4.5shows comparison of the inflence of age on cytokine levels of malaria uninfected pregnant women. Five groups namely G1, G2, G3, G4 and G5 were designated to represent 20-24yrs, 25-29yrs, 30-34yrs, 35-39 and 40-44yrs respectively. Values of cytokines were expressed in pg/ml.

For to IFNγ, mean±SD 4.67±3.41, 5.92±2.76, 5.70±3.11, 7.40±3.54 and 8.33±2.12 was recorded for Groups 1 - 5 respectively andthey showed no statistical difference (P>0.05) within the groups.

As for TNFα, the mean±SD (7.39±2.12) of group 1 was lower (P<0.05) when compared to 9.75±2.36, 10.75±3.39, 10.33±2.53 and 9.90±3.61 for groups 2, 3, 4, and 5 respectively. However, it was significantly different (P<0.05) when compared with group 2 and statistically insignificant when compared with the rest of the groups (P>0.05). Overall, no significant difference (P>0.05) was recorded within the other groups.

Considering IL-4, the mean±SD (9.30±1.87) of group 1 was higher and significantly different (P<0.05) when compared with 5.90±3.00 for Group 2. Similarly, it was higher on comparison with 7.79±4.37, 5.65±3.95, 6.63±5.35 forgroups G3, G4 and G5 respectively but no statistical difference (P>0.05) was recorded within the group.

Considering IL-6, the mean±SD(11.49±2.94) for group 1 was slightly higher when compared with 7.18±5.27, 10.10±11.07, 4.44±3.87 and 7.40±4.59 for groups 2, 3, 4 and 5 respectively. However, significant difference (P<0.05) existedwhen compared with group 4. Comparison with the rest of the groups yielded no significant difference (P>0.05).

On IL-10, group 2 recoreded the highest mean±SD value (15.88±8.14) but was statistically insignificantly (P>0.05) when compared with the rest of the groups. The mean±SD (15.61±5.84) of group 3 however, was statistically significant (P<0.05) when compared to 11.19±1.95 for group 4. On the other hand, the values of mean±SDfor all the other groups showed no significant difference when compared with each other (P>0.05).

(See appendix IIIb)

Table 4.6 shows comparisonof the influence of gestational age (by trimesters) on the cytokine levels of uninfected pregnant women. Group1 represents women in their first trimester while groups 2 and 3 represent women in their second and third trimesters respectively. Values of cytokines were expressed in pg/ml.

With respect to IFNγ, the mean±SD(8.80±1.46) for group 1 was higher than 6.78±2.96 and 4.40±2.81 for groups 2 and 3 respectively. But while group 1 showed no significant difference on comparison with group 2 women (P>0.05), it however showed significant difference on comparison with group 3 subjects (P<0.05). Again, the mean±SD (6.78±2.96) for group 2 women was significantly different (P<0.05) when compared with 4.40±2.81 for group 3.

For TNFα, the mean±SD (11.98±1.60) for group 1 women was higher than those for groups 2 and 3 women but wasnot significantly different (P>0.05) when compared with 11.20±2.92 for group 2 andthere was a significant difference in comparison with 8.15±2.40 for group 3. Again statistical difference (P<0.05) existed between the mean values for groups 2 and 3.

As for IL-4, mean±SD (4.74±4.82) of group 1 women was lower and showed no significant difference (P>0.05) when compared to 6.96±4.60 and 7.93±2.40 for groups 2 and 3 respectively.

The mean±SD (4.50 ± 4.77)of IL-6for group 1 was lower and showed no significant different (P>0.05) with 5.79 ± 4.94 and 13.23 ± 10.40 for groups 2 and 3 respectively. On the other hand, significant difference (P<0.05) existed on comparison with groups 2 and 3 women. Considering IL-10, the mean±SD 11.86±6.61, 14.41±6.64 and 15.79±5.42 forGroups 1, 2 and 3 respectivelyshowed no statistical significant difference (P>0.05) on comparison within and between the groups.

TABLE 4.6:COMPARISON MEAN±SD OF THE INFLUENCE OF GESTATIONAL AGE (BY TRIMESTER) ON THE CYTOKINE LEVELSOF UNINFECTED PREGNANT WOMEN.

AGE GROUPS	IFNγ (pg/ml)	TNFα (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
G1(n=5)	8.80±1.46	11.98±1.60	4.74±4.82	4.50±4.77	11.86±6.61
G2(n=32)	6.78±2.96	11.20±2.92	6.96±4.60	5.79±4.94	14.41±6.64
G3(n=25)	4.40±2.81	8.149±2.40	7.93±2.40	13.23±10.40	15.79±5.42
F(p) Value	25.669	12.949	1.680	12.723	6.667
	(.000)	(.000)	(.141)	(.000)	(.000)
G1 vs G2	0.235	0.941	0.913	0.990	0.956
G1 vs G3	0.003*	0.017*	0.707	0.096	0.801
G2 vs G3	0.036*	0.001*	0.904	0.026*	0.954

Key:
α-levelset at 0.05. P<0.05
G1=1st trimester Malaria uninfected pregnant women
G2=2nd trimester Malaria uninfected pregnant women
G3=3rd trimester Malaria uninfected pregnant women

Table 4.7 shows comparison ofmean±SD of the influence of gestational age (by trimester) on the cytokine levelsof malaria infected pregnant women.Group1 represents women in their first trimester while groups 2 and 3 represent women in their second and third trimesters respectively. Values of cytokines were expressed in pg/ml.

The mean±SD (28.97±14.18) of IFNγ for Group 1was higher than 24.10±12.36 and 19.47±11.67 for groups 2 and 3 respectively but showed no significant difference at P>0.05. Also, the mean±SD for group 2 showed no significant difference (P>0.05) when compared to that of group 3

For TNFα, the mean±SD (28.86±16.43) for group 1 was higher than 19.53±11.58 and 20.10±11.18 for groups 2 and 3 women respectively but showed no significant difference on comparison (P>0.05). Again, the mean±SD for group 2 showed no significant difference (P>0.05) when comparedwith that of group 3.

As regards IL-4, the mean±SD (10.11±8.09) for group 1 women was higher but showed no significant difference (P>0.05) when compared with 9.25±7.68 and 9.96±5.95 for groups 2 and 3 women respectively.Similarly, no significant difference (P>0.05) existed between groups 2 and 3 women.

For IL-6, the mean±SD(43.43 ± 35.55)for group 1 was higher than 23.64±20.35 and 37.50±29.82 for groups 2 and 3 respectively but showed no significant difference (P>0.05) on comparison. On the other hand,the mean±SD(23.64±20.35) for group 2 women was lower and showed significant difference (P<0.05) compared with 37.50±29.82 for group 3 subjects.

The mean±SD (26.81±20.15)of IL-10 for group 1 women was lower and showed no significant difference (P>0.05) compared to 36.49±34.78 and 36.76 ±23.50 for groups 2 and 3 subjects respectively. Meanwhile, the values of mean±SD for group 2 women showed no significant difference (P>0.05) with their group 3 counterparts.

TABLE 4.7: COMPARISON OF (MEAN ± SD) OF THE INFLUENCE OF GESTATIONAL AGE (BY TRIMESTER) ON THE CYTOKINE LEVELS OF MALARIA INFECTED PREGNANT WOMEN.

AGE GROUPS	IFN-γ (pg/ml)	TNF-α (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
G1 (n = 21)	28.97 ± 14.18	28.86 ± 16.43	10.11 ± 8.09	43.43 ± 35.55	26.81 ± 20.15
G2 (n = 65)	24.10 ± 12.36	19.53 ± 11.58	9.25 ± 7.68	23.64 ± 20.35	36.49 ± 34.78
G3 (n = 58)	19.467 ± 11.67	20.10 ± 11.18	9.96 ± 5.95	37.50 ± 29.82	36.76 ± 23.50
F(p) Value	25.669 (.000)	12.949 (.000)	1.680 (.141)	12.723 (.000)	6.667 (.000)
G1 vs G2	0.719	0.186	0.998	0.186	0.621
G1 vs G3	0.094	0.244	1.000	0.983	0.445
G2 vs G3	0.276	1.000	0.992	0.042*	1.000

Key
α-level set at 0.05
*(P< 0.05) = Significant
P> 0.05= Not Significant
G1 = Ist trimester Malaria infected pregnant women
G2 = 2nd trimester Malaria infected pregnant women
G3 = 3rd trimester Malaria infected pregnant women

Table 4.8 showsthe comparison of mean±SD of the influene of gravidity on cytokine levels of malaria infected pregnant women. Groups 1, 2 and 3 represented primigravidae, secondigravidae and multigravidae women respectively. Values were expressed in pg/ml.

The mean±SD (21.39±12.15) of IFNγ for group 1 was lower and showed no significant difference (P>0.05) than 21.85±12.70, 24.92±13.14 for groups 2 and 3 women respectively. Similarly, no significant difference (P>0.05) existed between the IFNγ levels of the women in groups 2 and 3.

Alsothe mean±SD (18.81±10.80)of TNFαfor primigravidae was lower and showed no significant difference(P>0.05) to 20.24±12.45 and 23.62±13.75 for secondigravide and multigravidae respectively. Also no significant difference (P>0.05) existed within the groups.

With IL-4, mean±SD (9.39±8.35) for primigravidae was lower and showed no significant difference (P>0.05) to 9.70±6.20, 9.88±6.37 for secondigravidae and multigravidae respectively. Again no significant difference (P>0.05) existed on comparism within secondigravidae and multigravidae.

For IL-6, the mean±SD (31.96±29.76) for primigravidae showed no significant difference (P>0.05) when compared with 30.79±25.85 and 33.01±27.87 for secondigravidae and multigravidae respectively. Also no significant difference (P>0.05) existed when compared with each other.

Themean±SD(28.576±25.142)of IL-10for primigravidae was lower when compared to 34.82±28.49 and 41.11±31.09 for secondigravidae and multigravidae respectively. Comparison within and between the groups showed no statistical significance.

(See appendix IIIc)

Table 4.9 shows comparison of mean±SD of cytokine levels and leucocyte count of infected pregnant women based on parasite count. Values of cytokines were expressed in pg/ml, total leucocyte count and differential leucocyte count in Absolute Values ($\times 10^9$/l).Parasite count

was classified as mild (<999parasite/μl) and moderate (1,000-10,000parasite/μl) parasitaemia.

The mean±SD (21.31±11.74) level for IFNγwas significantly (P<0.05) lower in mild parasitaemia than 29.14±14.48 for moderate parasitaemia.

The mean±SD (20.20±12.30)level for TNFα showed no significant (p>0.05) difference to 24.62±13.19 for moderate parasitaemia.

For IL-4, the mean±SD (9.42±7.08)for mild parasitaemia showed no significant difference (P>0.05) to 10.58±6.99 for moderate parasitaemia.

However, mean±SD (28.00±24.77)of IL-6for mild parasitaemia was significantly (P<0.05) lower than 47.72±33.71 for moderate parasitaemia.

The mean±SD (31.95±26.27)value of IL-10 for mild parasitaemia was lower than 47.49±34.77 for moderate parasitaemia however, it showed no significant difference at P>0.05.

The the mean±SD(7.0±2.06)total leucocyte count for mild parasitaemia was significantly (P<0.05) lower than 8.0±1.68 for moderate parasitaemia.

The mean±SD (2.3±0.79)oflymphocyte count for mild parasitaemia was not different with 2.3±0.69 for moderate parasitaemia.

The mean±SD (3.8±0.95)of neutrophil count blood level for mild parasitaemia was significant (P<0.05) lower than 4.7±0.76 of neutrophil countfor moderate parasitaemia.

Furthermore,mixeddifferential leucocytecount of 0.9±0.42 for mild parasitaemia was higher but showed no significant difference (P>0.05) when compared with 1.0±0.40 for moderate parasitaemia.

TABLE 4.9: COMPARISON OF MEAN ± SD OF CYTOKINE LEVELS AND LEUCOCYTE COUNT OF INFECTED PREGNANT WOMEN BASE ON PARASITE COUNT.

PARAMETERS	MILD PARASITAEMIA (<999parasite/ul) n = 114	MODERATE PARASITAEMIA (1,000-10,000parasite/ul) n = 30	P-Values
IFNγ (pg/ml)	21.31 ± 11.74[a]	29.14 ± 14.48[b]	0.044
TNFα (pg/ml)	20.20 ± 12.29[a]	24.62 ± 13.19[a]	0.359
IL-4 (pg/ml)	9.42 ± 7.08[a]	10.58 ± 6.99[a]	0.851
IL-6 (pg/ml)	28.00 ± 24.77[a]	47.72 ± 33.71[b]	0.024
IL-10 (pg/ml)	31.95 ± 26.27[a]	47.49 ± 34.77[a]	0.120
TOTAL WBC ($x10^9$/L)	7.0 ± 2.06[a]	8.0 ± 1.68[b]	0.035
LYM ($\times 10^9$/l)	2.3 ± 0.79[a]	2.3 ± 0.69[a]	0.059
NEU ($\times 10^9$/l)	3.8 ± 0.95[a]	4.7 ± 0.76[b]	0.042
MXD ($\times 10^9$/l)	0.9 ± 0.42[a]	1.0 ± 0.40[a]	0.293

Key:
α-level set at 0.05
a and b are superscripts used to show significant difference.
Values not sharing the same superscript means there is a significant difference
Values sharing the same superscript mean there is no significant difference

4.2 Definitions of Cut-off Values

In this study, pregnant and non-pregnant women with haemoglobin <110g/L (<11g/dl) and <120g/L (<12.0g/dl) respectively were considered anaemic in accordance with World Health Organization's definition (WHO, 2000). For sTfR, values 25.13- 64nmol/L and >64nmol/L were adopted respectively as indicators for normal and increased erythropoiesis(Monobind, 2013) while serum ferritin <12µg/L wasadopted as indicator ofdecreasediron stores (WHO, 2000).C-reactive protein (C-RP) >10mg/L was adopted as indicator for inflammation (Cook and Skikne, 1989). The results of the parameters of the subjects were presented as follows:

Table 4.10 shows thePercentage distribution of anaemia among pregnant women with malaria and their uninfected counterparts using their haemoglobin levels. For the malaria infected pregnant women, thirty-five percent had normal haemoglobin concentration (Hb>110g/dl)while 64.6% had anaemia. Mild anaemia (Hb 90 – 109.9g/L) showed at 56.94%, moderate anaemia (Hb 70-89.9g/L) at7.64% while none of the subjects had severe anaemia. For the uninfected pregnant subjects, 37.09% had Hb>110g/L while anaemia was recorded at 62.9%. Mild anaemia was 56.45%, moderate anaemia was 3.23% while severe anaemia was 3.23%.

Table 4.11 shows Comparison of sTfR levels in malaria infected pregnant women with those not infected with malaria. For the malaria infected pregnant women, 48.6% had normal erythropoiesis while 50.7% had increased erythropoiesis and 0.7% had marked erythropoiesis. With the uninfected pregnant women, 67.74% had normal iron supply, 29.03% had increased erythropoiesis and 3.23% had marked erythropoiesis.

Table 4.12SF levels among malaria infected pregnant women compared with uninfected pregnant women. For the malaria infected pregnant women a total of 72.22% had normal iron stores, 25.70% decreased iron stores, while 2.08% had increased iron stores. For the uninfected pregnant women, 75.81% had normal iron stores and 24.19%decreased iron stores.

TABLE 4.10: PERCENTAGE DISTRIBUTION OF ANAEMIA AMONG PREGNANT WOMEN INFECTED WITH MALARIA AND THEIR UNINFECTED COUNTERPARTS USING THEIR Hb LEVELS

FUNCTIONAL IRON COMPARTMENT		MALARIA INFECTED PREGNANT WOMEN		MALARIA UNINFECTED PREGNANT WOMEN	
		FREQUENCY (No)	PERCENTAGE (%)	FREQUENCY (No)	PERCENTAGE (%)
Hb(g/L)	>110 (Normal)	51	35.42	23	37.09
	90-109.0 (Mild)	82	56.94	35	56.45
	70-89.9 (Moderate)	11	7.64	2	3.23
	<70 (Severe)	0	0	2	3.23

TABLE 4.11: COMPARISON OF sTfR LEVELS IN MALARIA INFECTED PREGNANT WOMEN WITH THOSE NOT INFECTED WITH MALARIA

TRANSPORT IRON COMPARTMENT		MALARIA INFECTED PREGNANT WOMEN		MALARIA UNINFECTED PREGNANT WOMEN	
		FREQUENCY (No)	PERCENTAGE (%)	FREQUENCY (No)	PERCENTAGE (%)
sTfR (nmol/l)	<64 (normal erythropoiesis)	70	48.6	42	67.74
	65.1 – 114 (increased erythropoiesis)	73	50.7	18	29.03
	>114 (markederythropoiesis)	1	0.7	2	3.23

TABLE 4.12: SF LEVELS AMONG MALARIA INFECTED PREGNANT WOMEN COMPARED WITH THE UNINFECTED PREGNANT WOMEN

STORE IRON COMPARTMENT		MALARIA INFECTED PREGNANT WOMEN		MALARIA UNINFECTED PREGNANT WOMEN	
		FREQUENCY (No)	PERCENTAGE (%)	FREQUENCY (No)	PERCENTAGE (%)
SF (ug/l)	<12 (Decreased)	37	25.70	15	24.19
	12 – 200 (Normal)	104	72.22	47	75.81
	>200 (Increased)	3	2.08	0	0

Table 4.13Comparison of mean±SD of iron parameters ofmalaria infected and uninfected pregnant women.

The mean±SD (102.42±10.98)of Hb concentration for malaria infected pregnant womenshowed no significant (P>0.05) difference when compared with 105.79±14.16 for uninfected pregnant women.

Also for MCV, the mean±SD (89.70±8.34)for uninfected pregnant women showed no significant difference when comparedwith 88.20±11.03 for infected pregnant women (P>0.05).

As for sTfR, the mean±SD (62.78±21.97)for infected pregnant subjects was significantly higher (P<0.05) than 54.07±23.73 for uninfected pregnant subjects.

The mean±SD (43.61±84.99)value of SF for the infected pregnant subjects did not show significant (P>0.05) difference when compared with 31.31±28.18 for uninfected pregnant subjects.

Furthermore,the concentration of C-RP showed that the mean±SD (6.09±4.88)for infected pregnant women was significantly higher (P<0.05) than 4.49±2.74 for uninfected pregnant women when both were compared.

TABLE 4.13: COMPARISON OFMEAN±SD IRON PARAMETERS OFMALARIA INFECTED AND UNINFECTED PREGNANT WOMEN

Parameters	Infected Pregnant (n = 144)	Uninfected Pregnant (n = 62)	P-values
Hb (g/L)	102.42 ± 10.98[a]	105.79 ± 14.16[a]	0.067
MCV (fl)	88.20 ± 11.03[a]	89.70 ± 8.34[a]	0.340
STFRA (nmol/L)	62.78 ± 21.97[a]	54.07 ± 23.73[b]	0.012
SF (ug/L)	43.61 ± 84.99[a]	31.31 ± 28.18[a]	0.267
C-RP (mg/L)	6.09 ± 4.88[a]	4.49 ± 2.74[b]	0.017

Key
α-level set at 0.05
a and b are superscripts used to show significant difference.
Values not sharing the same superscript means there is a significant difference
Values sharing the same superscript means there is no significant difference

Table 4.14Effect of age on iron status of malaria infected pregnant women. The different age groups: 20-24yrs,25 – 29yrs, 30-34yrs, 35-39yrs and 40-44yrs were represented by groups 1 – 5 respectively.

For haemoglobin level, infected pregnant women in group 3 recorded the highest value mean±SD (104.81±12.86) however no significant (P>0.05) difference was shown when compared with 102.43±11.55, 99.73±9.41, 104.50±9.30 and 101.89±7.20 for groups 1, 2, 4 and 5 respectively. Overall different age groups showed no significant (P>0.05) difference when compared within groups.

As for sTfR, different values were recorded as mean±SD for the various groups. The mean±SD (61.28±20.15, 65.70±19.81, 60.26±21.82, 61.21±29.18 and 69.14±25.71) values were obtained for groups 1–5 respectively.However, no significant (P>0.05) difference existed when compared within the groups.

With serum ferritin, group 2 recorded the highest mean±SD (58.79±136.47) while group 4 (35-39yrs) recorded the lowest(25.36±13.23). Overall no significant (P>0.05) different was shown when compared within the groups.

Age groups showed no significant (P>0.05) difference in the iron status of MP infected pregnant women

(See appendix IIId).

Table 4.15Effect of gestational age on iron status of malaria infected pregnant women.

The mean±SD (111.43±11.64) of Hb level for pregnant women in their 1st trimesterwas significantly higher (P<0.05) when compared with 95.66±5.02 for the 2nd trimester but no significant difference (P>0.05) existed when comparedwith 106.74±11.20 for those in their 3rd trimester. Again, mean±SD for those in of 2nd trimester showed significant difference (P<0.05) when compared to those in their 3rd trimester.

For sTfR, lowest value of mean±SD (60.20±21.88) for pregnant women in their 1st trimester showed no significant difference (P>0.05) comparedto 62.22±23.66 and 64.35±20.22 for those in their 2nd and 3rd trimesters respectively. Also mean±SDvalue of sTfR for 2nd trimester showed no significant difference with 3rd trimester at P>0.05.

As for serum ferritin, the mean±SD 53.34±55.61 for pregnant women in 1st trimestershowed no significant difference (P>0.05) when compared with 54.50±118.07 and 27.90±31.02 for those in their 2nd and 3rd trimesters respectively.

The mean±SD (54.50±118.07) of SF for pregnant women in their2nd trimester was significantly higher(P>0.05) than 27.90±31.02 obtained for those in their 3rd trimester.

TABLE 4.15: EFFECT OF GESTATIONAL AGE ON IRON STATUS OF MALARIA INFECTED PREGNANT WOMEN

AGE GROUPS	HB (g/L)	STFRA (nmol/L)	SF (ug/L)
G1 (n = 21)	111.43 ± 11.64	60.20 ± 21.88	53.34 ± 55.61
G2 (n = 65)	95.66 ± 5.02	62.22 ± 23.66	54.50 ± 118.07
G3 (n = 58)	106.74 ± 11.20	64.35 ± 20.22	27.90 ± 31.02
F(p) Value	35.336 (.000)	0.310 (.734)	1.678 (.190)
G1 vs G2	0.001*	0.931	0.998
G1 vs G3	0.261	0.730	0.136
G2 vs G3	0.001*	0.853	0.034*

KEY
α-level set at $p<0.05$
G1 = 1^{ST} TRIMESTER Malaria infected pregnant subjects
G2 = 2^{ND} TRIMESTER Malaria infected pregnant subjects
G3 = 3^{RD} TRIMESTER Malaria infected pregnant subjects
HB = Haemoglobin Concentration
SF = Serum Ferritin
STfRA = Serum Transferrin Receptor Assay

Table 4.16Effect of gravidity on iron status of malaria infected pregnant women.

Group 1 represented primigravidae women while groups 2 and 3 represented secondigravidae and multigravidae women respectively.

For Hb, the mean±SD (103.78±12.13)for primigravidae women was higher and showed no significant difference(P>0.05) with 102.09±11.17 and 101.44±9.84 for secondigravidae and multigravidae subjects respectively. Again the mean±SD values ofHb for secondigravidae showed no significant difference (P>0.05) withthe values obtainedfor multigravidae.

As for sTfR the mean±SD (62.35±18.75)for primigravidae subjects showed no significance (P>0.05) difference with 63.32±22.86 and 62.84±24.27 for secondigravidae and multigravidae subjects respectively. Also values ofmean±SDof sTfR for secondigravidae showed no significant difference (P>0.05) with that for multigravidae subjects

The values of mean±SD (47.98±106.73) of SFblood level for primigravidae showed no significant difference(P>0.05) with 25.92±18.66 and 50.03±86.87 for secondigravidae and multigravidae respectively. Moreover, mean±SD for secondigravidae showed no significant difference (P>0.05) when compared with that for multigravidae.

(See appendix IIIe).

Table 4.17Comparison of cytokines and serum iron of malaria infected pregnant women based on normal and elevatedC-RPlevels as a marker of inflammation.

The mean±SD (24.33±14.93) values ofIFNγ for subjects with elevated C-RPwas higher than 22.70±12.35 for those with normal C-RP but values were not statistically significant.The mean±SD (21.27±12.66)values of TNFαfor subjects with normal C-RP was not significantly higher (P>0.05) than 20.27±12.27 for those with elevated C-RP.Also, the mean±SD (10.59±8.61)values of IL-4for those with elevated C-RPwas higher than 9.51±6.78 for normal C-RP but values were statistically not significant.

For IL-6,the mean±SD (65.37±42.26) for subjects with elevated C-RPwas significantly higher ($P<0.05$) than 26.43±19.96 for normal C-RP.The same applies for IL-10 where mean±SD (47.23±35.60) values for those with elevated C-RP was significantly higher ($P<0.05$)when compared with 33.13±27.14 for subjects with normal C-RP. Also, the mean±SD (116.18±201.46)of serum ferritin for subjects with elevated C-RP was significantly higher ($P<0.05$) than 31.22±27.41 for those with normal C-RP.

With sTfR, the mean±SD (63.45±22.23)for subjects with normal C-RP was higher but showed no significant difference ($P>0.05$) when compared with 58.84±20.42 for those with normal C-RP.

TABLE 4.17: COMPARISON OF CYTOKINES AND SERUM IRON OF MALARIA INFECTED PREGNANT WOMEN BASED ON NORMAL AND ELEVATEDC-RPLEVELS AS MARKER OF INFLAMMATION

Parameters	Normal C-RP (<10mg/L) (n=123)	Elevated C-RP (>10mg/L) (n=21)	P-Values
IFNγ (pg/ml)	22.70 ± 12.35[a]	24.33 ± 14.93[a]	0.590
TNF α (pg/ml)	21.27 ± 12.66[a]	20.27 ± 12.27[a]	0.737
IL-4 (pg/ml)	9.51 ± 6.78[a]	10.59 ± 8.61[a]	0.517
IL-6 (pg/ml)	26.43 ± 19.96[a]	65.37 ± 42.26[b]	0.001
IL-10 (pg/ml)	33.13 ± 27.14[a]	47.23 ± 35.60[b]	0.038
SF (µg/L)	31.22 ± 27.41[a]	116.18 ± 201.46[b]	0.001
STFRA (nmol/L)	63.45 ± 22.23[a]	58.84 ± 20.42[a]	0.376

Key

α-level set at 0.05
a and b are superscripts used to show significant difference.
Values not sharing the same superscript means there is a significant difference
Values sharing the same superscript mean there is no significant difference

Table 4.18Comparison of red blood cell profile of malaria infected pregnant women based onnormal and elevatedlevels of C-RP as a marker of inflammation.

For Hb, the mean±SD (108.24±12.03)was higher in subjects with elevated values of C-RP but showed no significant difference (P>0.05) whencompared with 101.43±10.53 for those with normal values of C-RP.

The mean±SD (3.81±0.56)of total red blood cell count for subjects with normal C-RPshowed no significant (P>0.05) difference when compared with 3.80±0.54 for those with elevated values of C-RP.

Also HCT (PCV) mean±SD (0.31±0.03)for subjects with elevated C-RP was not significant (P>0.05) compared with 0.31±0.02 for subjects with normal values of C-RP.

With MCV, the mean±SD (92.82±6.99)for subject with elevated C-RP was significantly (P<0.05) higher than 87.42±11.41 for those with normal values of C-RP.

As for MCH, the mean±SD (29.44±2.54) for subjects with elevated C-RP showed no significant difference (P>0.05) compared with 28.10±3.09 for those with normal C-RP.

For MCHC, the mean±SD (329.30±7.88)for subjects with normal C-RP shows no significant difference (P>0.05) compared with 317.30±1.56 for those elevated C-RP.

TABLE 4.18: COMPARISON OF RED BLOOD CELL PROFILE OF MALARIA INFECTED PREGNANT WOMEN BASED ON NORMAL AND ELEVATED LEVELS OFC-RP AS A MARKER OF INFLAMMATION.

Red Cell Parameters	Normal C-RP(<10mg/L) (n=123)	Elevated C-RP(>10mg/L) (n=21)	P-Values
HB (g/L)	101.43 ± 10.53[a]	108.24 ± 12.03[b]	0.008
RBC (x10^{12}/L)	3.81 ± 0.56[a]	3.80 ± 0.54[a]	0.955
HCT (L/L)	0.31 ± 0.03[a]	0.31 ± 0.02[a]	0.717
MCV (FL)	87.42 ± 11.41[a]	92.82 ± 6.99[b]	0.038
MCH (pg)	28.10 ± 3.09[a]	29.44 ± 2.54[a]	0.062
MCHC (g/l)	329.30 ± 7.88[a]	317.30 ± 1.56[a]	0.489

Key:
α-level set at 0.05
a and b are superscripts used to show significant difference.
Values not sharing the same superscript means there is a significant difference
Values sharing the same superscript means there is no significant difference

Table 4.19Random blood sugar, creatinine and C-RP levels of malaria infected pregnant women compared with the uninfected pregnant women and control group.

Group 1 and 2 were used to represent malaria infected and uninfected pregnant subjects respectively while groups 5 and 6 represent the malaria infected and uninfected non-pregnant subjects.

For RBS, the mean±SD (57.82±21.55)for group 1 subjects was lower and showed no significant difference($P>0.05$) when compared to 68.97±31.24 for group 2 subjects but showed significant difference ($P<0.05$) when compared with 79.00±18.68 and 91.40±17.63 for women in groups 5 and 6 respectively. The mean±SD for group 2 subjects showed no significant difference($P>0.05$) when compared to that for group 5 subjects but was significantly lower ($P<0.05$) than 91.40±17.63 for group 6 subjects. On the other hand, the mean±SD for group 5 subjects showed no significant difference ($P>0.05$) when compared with that for group 6 subjects.

For creatinine level, the mean±SD 1.32±1.59, 1.09±0.44, 1.24±0.38 and 1.24±0.39 for groups 1, 2, 5, and 6subjects respectively showed no significant difference ($P>0.05$) when compared within and between the groups.

As for C-RP, the mean±SD (6.09±4.88)for group 1 women was significantly higher ($P<0.05$) than 4.49±2.74 for group 2 women, but showed no significant difference ($P>0.05$) when compared with 5.36±1.95 and 5.26±1.97 for those of groups 5 and 6 women respectively. Also the mean±SD for group 2 women showed no significant difference ($P>0.05$) when compared with those for groups 5 and 6 women. Furthermore, there was no significant difference when the mean±SD for group 5 women was compared with that for women in group 6 ($P>0.05$)

TABLE 4.19: RANDOM BLOOD SUGAR, CREATININE AND C-RP LEVELS OF MALARIA INFECTED WOMEN COMPARED WITH THE UNINFECTED PREGNANT WOMEN AND CONTROL GROUP.

GROUPS	RBS (mg/dl)	CREATININE (mg/dl)	C-RP (mg/L)
G1 (n = 144)	57.82±21.55	1.32±1.59	6.09±4.88
G2 (n = 62)	68.97±31.24	1.09±0.44	4.49±2.74
G5 (n = 20)	79.00±18.68	1.24±0.38	5.36±1.95
G6 (n = 20)	91.40±17.63	1.24±0.39	5.26±1.97
F(p) Value	15.291(0.000)	0.485(0.693)	2.281(.080)
G1 vs G2	0.058	0.384	0.017*
G1 vs G5	0.001*	0.954	0.619
G1 vs G6	0.001*	0.962	0.513
G2 vs G5	0.312	0.476	0.414
G2 vs G6	0.001*	0.465	0.531
G5 vs G6	0.153	1.000	0.998

Key
α-level set at 0.05
*(P<0.05) = Significant
P>0.05 = Not Significant
G1 = Malaria Infected Pregnant Subjects
G2 = Malaria Uninfected Pregnant Subjects
G5 = Malaria Infected Non Pregnant Subjects
G6 = Malaria Uninfected Non Pregnant Subjects

Figure 4.1 are correlation scattergramsbetween cytokines (IFNγ, TNFα, IL-4, IL-6 and IL-10) and iron (Hb, MCV, sTfR, and SF) parameters for malaria infected pregnant subjects.

Among the 144 subjects, there was significant strong negative correlation between IFNγ and Hb (r = -0.82, P = 0.030) [figure 4.1a(i)]. IFNγshowed no correlation with MCV (r = -0.13, P = 0.126) [figure 4.1a(ii)]. There was no relationship between IFNγ, and sTfR (r = -0.012, P = 0.886) [figure 4.1a(iii)]. Again, there was no correlation between IFNγ and SF (r = -0.23, P = 0.362) [figure 4.1a(iv)].

In all the subjects, there was negative correlation between TNFα and Hb which was of statistical significance (r = -0.16, P = 0.044) [figure 4.1b(i)]. No relationship was registered between TNFα, and MCV (r = -0.05, P = 0.577) [figure 4.1b(ii)]. No correlation existed between TNFα and SF (r = -0.10, P = 0.241) [figure 4.1b(iv)]. Furthermore, no correlation existed between TNFα and sTfR (r = 0.09, P = 0.282) [figure 4.1b(iii)].

In this study, there was no correlation between IL-4 and Hb (r = 0.039, P = 0.643) [figure 4.1c (i)]. No significant relationshipexistsbetween IL-4 and sTfR(r = 0.14, P = 0.104) [figure 4.1c(iv)].Also, no correlationexisted between IL-4 and MCV (r = -0.033, P = 0.696) [figure 4.1c(ii)]. Again, no correlationexists between IL-4 and SF (r = -0.036, P = 0.671) [figure 4.1c(iii)].

In this study, no significant relationshipexistsbetween IL-6 and Hb (r = 0.07, P = 0.402) [figure 4.1d(i)], between IL-6 and MCV (r = 0.13, p = 0.147) (fig. 4.1dii) and between IL-6 and SF (r = 0.05, P = 0.607) [figure 4.1d(iv)].In addition, there was no relationship between IL-6 and sTfR (r = -0.11, P = 0.190) [figure 4.1d(iii)].

As regards correlation between IL-10 and Hb, no relationship exists between IL-10 and Hb (r = -0.23, P = 0.098) [figure 4.1e(i)]. Againno correlation was registered between IL-10 and SF (r = -0.005, P = 0.949) [figure 4.1e(iv). No correlation was registered between IL-10 and sTfR (r = -0.07, P = 0.411) [figure 4.1e(iii)]. Also,nocorrelation existed between IL-10 and MCV (r = 0.09, P = 0.291) [figure 4.1e(ii)].

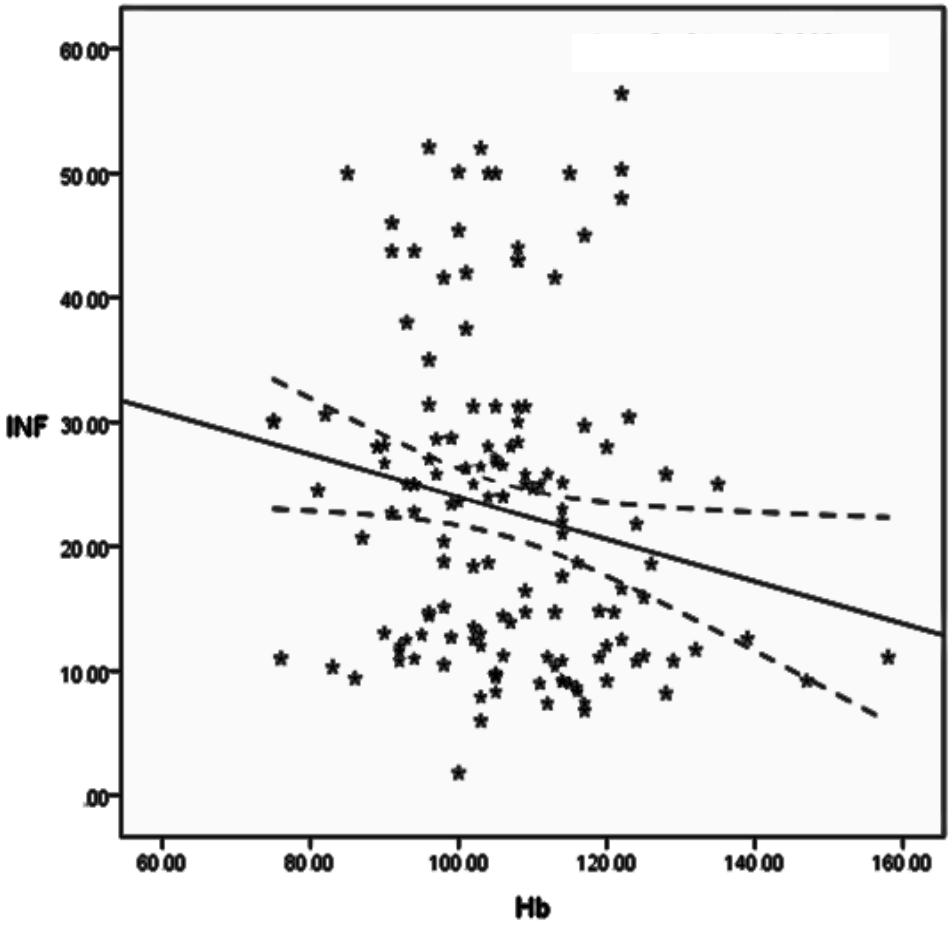
Figure 4.2a shows correlation scatterplot between cytokines (IL-6 and IL-10) and acute phase protein (C-reactive protein).

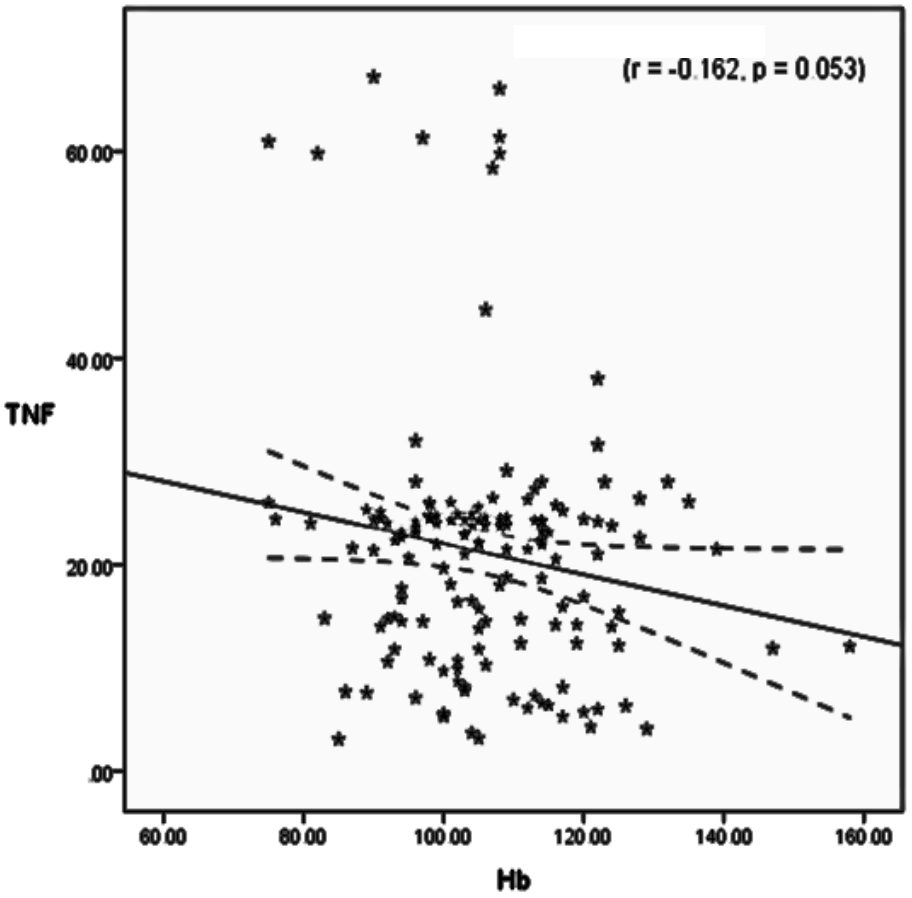
In the study, there were significantly positive correlation between IL-6 and C-reactive protein (r = 0.56, P = 0.001) [figure 4.2ai] and between IL-10 and C-reactive protein (r = 0.79, P = 0.010) [figure 4.2aii].

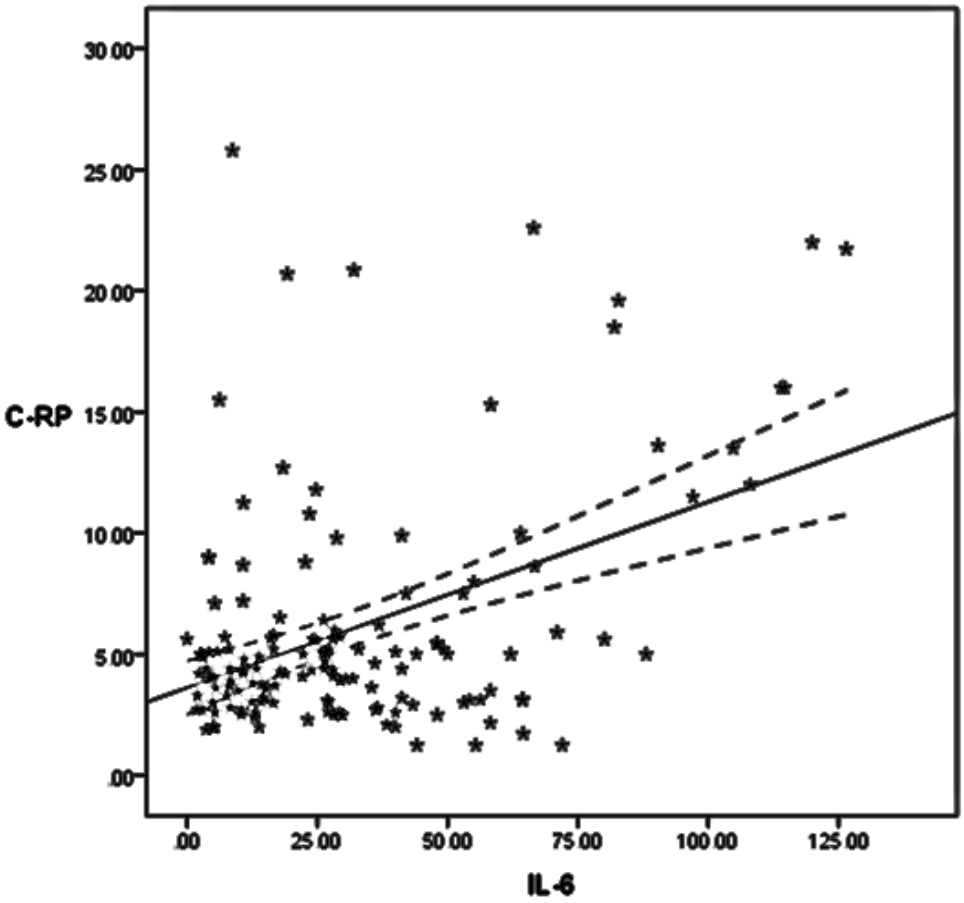
Figure 4.3 showscorrelation scatterplot between iron (SF) and acute phase protein (C-reactive protein).

The level of C-RP showed a positive correlation with levels of serum ferritin in infected pregnant women (r = 0.64, P = 0.000) [figure 4.3]

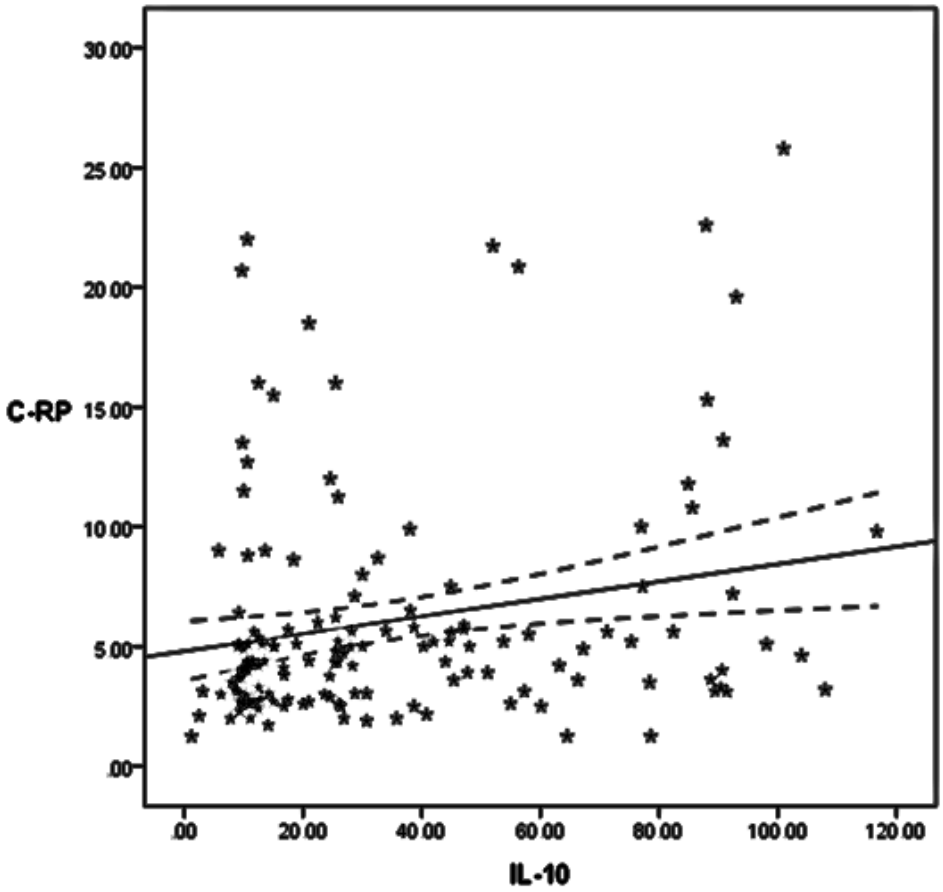
Figure 4.4 shows correlation scatter Diagram between cytokines (IL-6 and IL-10) and malaria parasite count. A strong positive correlation existed between IL-6 and Malaria Parasite density (r = 0.66, p = 0.001) and between IL-10 and Malaria Parasite density (r = 0.71, p = 0.001).


60 00
50 00
40 00
INF 30 00
20 00
10 00
.00
60 00
80 00
100 00
120 00
140 00
160 00
Hb


(r = -0.162, p = 0.053)
60.00
40.00
20.00
.00
TNF
60.00
80.00
100.00
120.00
140.00
160.00
Hb

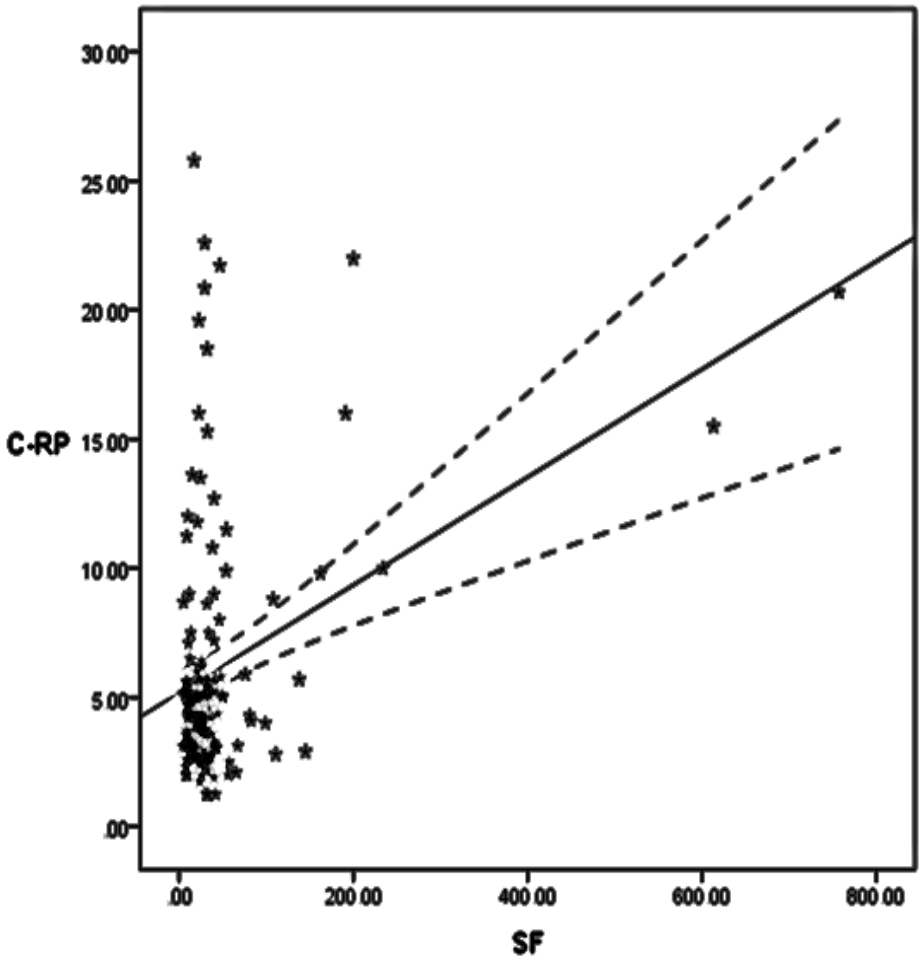


4.2ai: A positive correlation between C-RP and IL-6
($r = 0.56$, $p = 0.001$)



4.2aii: A positive correlation between C-RP and IL-10
($r = 0.79$, $p = 0.010$)

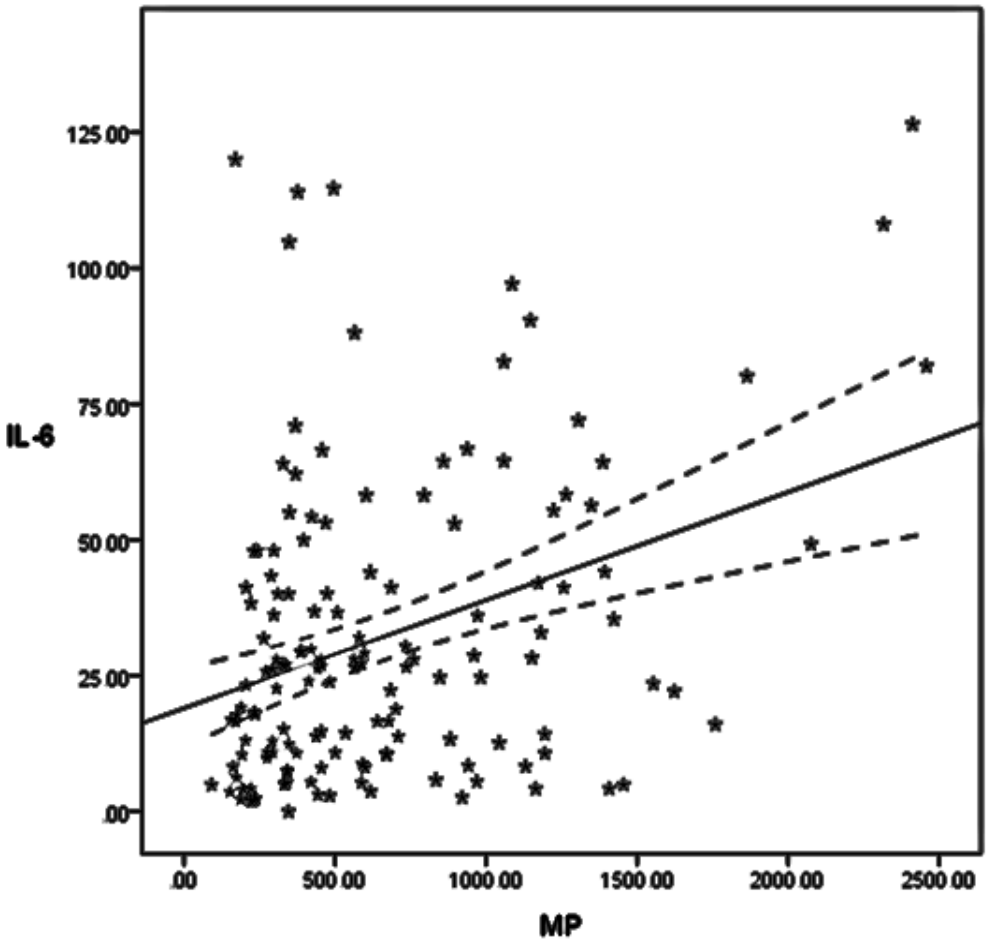
Figure 4.2: Correlation Scattergram between C-RP and Cytokines



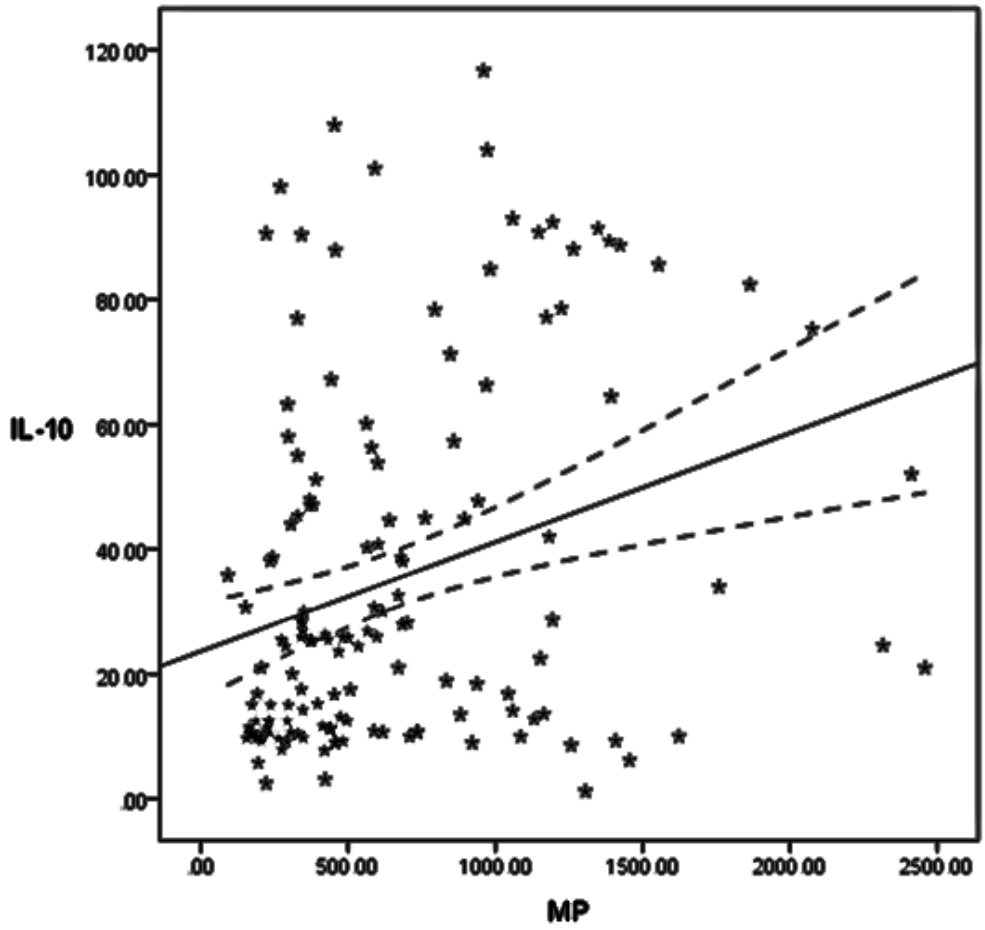
4.3: Apositive correlation between C-RP and SF

(r = 0.64, p = 0.001)

Figure 4.3: Correlation Scattergram between C-RP and Iron



4.4a: A positive correlation between IL-6 and MP
(r = 0.66, p = 0.001)



4.4b: AStrong positive correlation between IL-10 and MP (r = 0.71, p = 0.001)

Figure 4.4: Correlation Scattergram between Cytokines and MP Count

CHAPTER FIVE

5.1 DISCUSSIONS

Malaria is a plasmodial infection that has the propensity to induce immune activation with subsequent release of cytokines and other inflammatory mediators (Inigo and Manuel, 2002; Clark *et al.*,2006). Similarly, non-infectious conditions such as iron replete state can illicit immune stimulation resulting in the expression of cytokines (Van Miert, 1995; Pigeon *et al.*,2001; Ganz, 2003). In such responses,cytokine, iron, nitric oxide and glucocorticoids interact to modulate the systemic acute phase reactions (Gruys *et al.*,1994; Van Miert, 1995). This study apparently focused onmalaria parasitaemia and cytokines as agonists that may contribute to the redistribution of iron in pregnancy and post partum. The resultsgenerated from the study were presented under the following modules: assessment of immune stimulation, iron profiles, metabolic parameters and levels of association.

On immune stimulation, the resultof this study showed that proinflammatory (IFNγ, TNFα, IL-6) and anti-inflammatory(IL-4 and IL-10) cytokines were elevated in malaria infected pregnant women than their uninfected pregnant counterparts. This is consistent with reports; ofNmorsi *et al.*,(2010)who reported that IFNγ and IL-6 were significantly elevated in malaria infected pregnant than the uninfected pregnant women andTorre *et al.*,(2002) whoreported significantly increased levels ofIFNγ, TNFα and IL-10 in malaria infected pregnant women than their control. Also, Bayoumi *et al.*,(2009) and Boston *et al.*,(2012) respectivelyposited significantly increased IL-10 in malaria infected pregnant women than the uninfected pregnant women. In the same direction,Wilson*et al.*,(2010) reported increased levels of IFNγ and TNFα although the expression appeared negligible. This finding shows that malaria contributes by inducing immune cells to secrete cytokines during pregnancy.IFNγ is a critical mediator of immunity to malaria and is generally associated with protective mechanisms

(Ferreira *et al.*,1986; Herrera *et al.*,1992) especially in uncomplicated acute malaria (Torre *et al.*,2002) while IL-10 while IL-10 is a bioactive anti-inflammatory cytokine that inhibits the activity of T_H1 cells, NK cells and macrophages, all of which are required for pathogen clearance (Venugopal, 2007).

On the other hand, the result was at variance withthat of Boston *et al.*,(2012) who reported no statistical differences in the levels of IFNγ and TNFα between malaria infected and uninfected pregnant women. Also, it was not in concert with Bayoumi *et al.*,(2008) who reported that IFNγ, IL-4 and IL-10 were elevated in the uninfected than the malaria infectedperipheral blood. The discordance in the results of this study, with that of Boston *et al.*,(2012), could be due to the differences in sample size. The sample size of 144 malaria infected pregnant women in this study was completely higher than the latter where 42 infected pregnant and 79control uninfected women were recruited.The over-expression of IFNγ and IL-10 which occurred in infected non-pregnant is in agreement with (Torre *et al.*,2002) who reported such expression to be associated with uncomplicated acute malaria.

The finding presented herein agrees with earlier studies that malaria induces immune stimulation. It is believed that constant exposure to malaria parasite induces varying levels of immunity such that asymptomatic or apparently healthy pregnant women may have malaria in their blood stream. The phagocytosis of malaria parasite, haemozoin or toxin by the phagocytic system results in the secretion of cytokines either in the innate or the acquired immune responses. Moreover, the white blood cells, essentially important in combacting malaria parasites, when activated, releases more cytokines probably induced by radicals. Radicals such as Hydrogen peroxide (H_2O_2), Hydroxyl (OH^-)and Super oxide (O_2^-) could be generated via malaria induced oxidative stress. This inturn stimulates the immune cells consequent to the release of more cytokines. Also in this study TNFα, IL-4, IL-6 and IL-10 were elevated in the uninfected pregnant than the uninfected non-pregnant women.

However,IFN-γ was increased but was negligible. The result was in conformity with the report of Vassiliadis *et al.*,(1998) who showedincreased values of IL-10 in uninfected pregnant women than the uninfected non-pregnant.This finding showed that the secretion of more regulatory cytokines such as IL-10 is expected so as to ensure a successful pregnancy. On the other hand, the result was not in agreement with (Sharma *et al.*,2000) who did not detect IL4 throughout pregnancy. The difference could be due to the fact that this study evaluated cytokine levels induced by malaria whereas the later was on assessment of cytokine levels in a gestational age manner and the pregnant women were without malaria parasitaemia.

The finding of this study was that pregnancy induces the production of cytokinesandalters the immune system of the pregnant women. This is in accordance with (Lashley *et al.*,2011). It is reported that normal pregnancy is now considered to be a state of controlled maternal mild inflammation where levels of proinflammatory cytokines, including TNFα, are raised compared to non-pregnant states, in a way similar to what happens during sepsis (Sacks *et al.*,1998). There seems to be the sensitization of maternal circulation via the entry of foetal cell and/or atthe maternal-fetal interphase resulting in the secretion of cytokines. The cytokines diffuse into the extracellular fluid compartments and circulate in the peripheral blood to ensure successful pregnancy (Lashley *et al.*,2011). These proinflammatory cytokines are produced by monocytes and trophoblasts (Haider and Knofler, 2009).Table 4.2:Shows that IL-4 and IL-10 predominated in non-malaria infected placenta.The data supports the idea that successful pregnancy in humans is followed with a bias away from pro-inflammatory cytokine to regulatorycytokine dominance(Nathwani *et al.*,1992).Infection with malaria, the normal cytokine profile wasreversed andpro-inflammatory cytokines (IFNγ and TNFα) wereincreasedas opposed to that of the uninfected placenta. This finding is in agreement with Fried *et al.*,(1998) andSuguitan *et al.*,(2003). The high level of IL-10 in the sera of uninfected

placenta suggested that anti-inflammatory cytokine environment is thought to be maintained in part by the high progesterone level in pregnancy which induces T_H0 to T_H2 conversion (Szekeres- Bartho and Wegmann, 1996). On the contrary, it did not agree with Bayoumi *et al.*,(2008) and Yasnot *et al.*,(2013). The disagreement with Bayoumi *et al.*,(2008) could be that this study was conducted in an area of stable and endemic malaria parasite transmission while that of Yasnot *et al.*,(2013) could be due to the method used in the evaluation; real time PCR.

One striking point in this study was that the level of IL-10 was increased in malaria infected placenta than with malaria uninfected placenta. This result is in conformity with Suguitan*et al.*,(2003). The significant expression of IL-10was due to the fact that the uterine epithelium and placental trophoblasts are known to be sources of IL-10 production during pregnancy. IL-10 characterizes normal human pregnancy and is thought to prevent inflammatory responses that might damage the intergrity of the maternal-foetal placental barrier (Wegmann *et al.*,1993; Fried *et al.*,1998). With placental malaria and despite the placental shift towards T_H1-type cytokines, IL-10 concentrations are elevated compared with uninfected healthy placentas (Suguitan *et al.*,2003). This condition is expected as IL-10 has a major role in controlling inflammatory responses and preventing damage of maternal-foetal placental barrier (Fried *et al.,* 1998 and Suguitan *et al.*,2003).

In a closely related issue, TNFα was notably expressed than IFNγ in malaria uninfected placenta. This suggests that TNFα is assumed to play a role in parturition (Chen *et al.*,1991) which may account to its detection inplacentas uninfected by malaria.

On comparing the concentration of cytokines in the systemic blood and the local maternal-foetal interphase, the proinflammatory cytokines (IFNγ and TNFα) were reduced in the placenta than the peripheral. IL-6 was higher in the placenta but could be considered

negligible when compared with the systemic. Theanti-inflammatory (IL-4 and IL-10)cytokines wereincreased in the placenta than the peripheral blood.Moderate to marked concentrationsof cytokines expressed in the study suggests that the placenta can influence the systemic cytokine profile and this depends on the cytokine of interest (Fried *et al.,*1998).

The malaria parasite densityrecorded in the peripheral blood was higher than that reported by Onyenekwe *et al.,*(2004; Achidi *et al.*, (2007 and Akinboro *et al.,*(2010), but was lower than that of Douamba *et al.,*(2012*).* The differences in the results suggest the differences in malaria transmission in different geographical areas anddifferent methods of evaluation. The parasite density was higher in the blood from the placenta than that of peripheral blood. This is in conformity with Yamada *et al.,*(2007) who reported that the placenta is prone to malaria infection than the peripheral blood and infected erythrocytes accumulate in the intervillous space sometimes in higher density and binds to chondroitin Sulphate-A in the syncytiotrophoblast. Again, the infected erythrocytes in the placenta express a unique variant surface antigen (VSA) which enhances its adhesion. The VSA is not found elsewhere in the body except in the placenta. Moreover, a good number of the pregnant women do not attend antenatal clinics until delivery and are fond of taking antimalarial drugs without prescription. These may reduce the parasite load in the peripheral blood but not necessarily in the placenta.

On the contrary, the elevated level of the malaria parasite in the placenta could raise doubt on the efficacy of the antimalarial interventions and the holistic approach of the pregnant women to taking antimalarial dugs in the course of attending antinatal cares. Granted that this study was not intended to assess the efficacy of antimalarial drugs, did not provide information on the different antimalaial drugs taken, the compliance and duration however, the issue is that the fact that the reduced effectiveness of different antimalarials with different modes of action and pharmokinetic activities may contribute to the elevated parasite density.In addition, that the total leucotyte count was increased in the blood from the placenta than the

peripheralappeared to be a clear indication that the white blood cells would respond adequately and in a large number to the removal of the parasites thus increasing the number of immune cells already found to regulate the normal placental cytokine milieu.

Furthermore, the result of this study showed that maternal age, gestational age and gravidity did not influence cytokine expression. The result agrees with Achidi *et al.,*(2007). Rather the levels of cytokine could be influenced by malaria endemicity, nutritional status and the tendency for normal pregnancy to mount strong measures to regulate the expression of type-1 cytokines for a progressive and successful pregnancy.However, gravidity based differences in antibody production have been proposed to explain the differences in susceptibility to malaria in respect to the level of cytokines expressed between the primigravidae and multigravidae woman (Ricke*et al.,*2000; Iona, 2001).

As regardsthe degree of parasitaemia, the data in this study revealed that both pro and anti-inflammatory cytokineswere moreexpressed in moderate parasitaemia than mild parasitaemia. The finding was at variance with that of Nmorsi *et al.,*(2010*)* where IL-4 and IL-10 were significantly elevated in mild infection than moderate infection. This result suggests that the increased parasitaemia may play a role in cytokine expression even in asymptomatic subjects. Also, the corresponding expression of the anti-inflammatory cytokines is thought to reflect early and effective immune response to malaria. In the same perspective, the total leucocyte count was increased in moderate parasitaemia than mild parasitaemia. This showed that increased leucocyte count may be dependent on parasitaemia where increased parasitaemia may result in the release of more WBC by the myeloid cells.

According to WHO standard, anaemia in pregnancy is present when haemoglobin concentration in the peripheral blood is <110g/L. In this study, anaemia was found to be prevalent in 64% of the malaria infected pregnant subjects. The prevalence of anaemia at first

antenatal visit in this study was lower than 94.07% reported by Nduka *et al.*,(2006). It did not differ from 60.8% reported by Okafor *et al.*,(2012),59.6% repoted by Agan *et al.*,(2010), 62.4% reported by Ogbodo *et al.*,(2009) and 66% reported by Erhabor *et al.*,(2010). Conversely, it was higher than 30% reported by Douamba *et al.*,(2012) and 20.3% reported by Buseri *et al.*,(2008). The difference in the prevalence rates could be related to various levels of transmission of malaria in different locations and possible interactions of other aetiological factors of anaemia in pregnancy.

The prevalence rate of anaemia in pregnant women in this study is high and worrisome. The reasons were not far-fetched. It may be related to several factors such as dirty environment, government's approach to prevention and treatment of malaria and the attitude of most pregnant women to combating malaria. On the environment, the stagnant water in drainages provides enabling habitat for the breeding of mosquitoes. On the part of the government, the inability to curb the incessant and protracted industrial action (strike) in the health sector which shuts down the medical centers could contribute adversely to the pregnant women not having access to antenatal cares.Also the situation where Insecticide Treated Nets (ITNs)are not adequate and evenly distributed contributes to the persistently increased rate of anaemia.As for the subjects, it is possible for some pregnant women to sleep outside the Insecticide Treated Nets thereby exposing themselves to mosquito bites. Moreover, greater number of pregnant women report to antenatal late mostly in the second and third trimester paving way for late administrations of necessary interventions.Furthermore, some pregnant women resort to intake of antimalarial drugs from drug vendors without considering the efficacy of such drugs. This havecontributed to the high rate ofanaemia due to malaria asthe efficacy of these drugs cannot be guaranteed even in the wave of prevailing fake and counterfeit drugs.Granted that the recruited pregnant women had malaria infection, the tendency for susceptibility to other infections was possible. Infections from the

gastrointestinal tract such as hookworm are known to contribute to the anaemia Baidoo *et al.*,(2010). In view of the result of this study, screening of iron status of pregnant women could be reviewed and expanded to include investigation on gastrointestinal tract and genito-urinary tract infections.

Considering the severity of anaemia among the malaria infected pregnant women,56.9% and 7.6% were recorded formild and moderate anaemias respectively. That severe anaemia was not observed coupled with low prevalence of moderate anaemia could be due to the fact that only apparently healthy pregnant women who were on first antenatal bookingwere enrolled while those with clinical malaria were excluded. Also, it could be attributed to wide use of haematinics and prophylactic antimalarials prior to antenatal booking. It is a common practice in Nigeria for women in particular to place themselves on antimalarials and haematinics.This study also revealed 62% prevalence of anaemia among the uninfected pregnant women and by severity, 56.45% mild anaemia, 3.23% moderate while 3.23%had severe anaemia was similar to the result obtained by Erhabor *et al.*,(2010) who reported 48% anaemia. The incidence and severity of anaemia may be used as indicators of the several health conditions of the pregnant women or women of the reproductive age. In addition, some subjects that might be anaemic were undetected and unattended to. Also, other factors, apart from malaria, may contribute to the anaemia.Suchfactors include deficiencies of iron, folic acid, zinc and copper.

On the distribution of iron in the store compartment shown by the concentration of serum ferritin(SF) ofmalaria infected pregnant women, 25.6% had decreased iron store while 2.0% had elevated iron store. But with the malaria uninfected pregnant women, 24% had decreased iron store while there was no elevated iron store.The decrease in the store iron is because iron is consistently moved from the store to match with the increased requirement for iron during pregnancy (Bothwell, 2000).

Pregnancy is a time of considerable maternal adaptation during which iron status is affected. Table 4.13 showed thathaemoglobin concentration wasreduced in the malaria infected than the uninfected pregnant women. The result of this study was in agreement with Buseri *et al.*,(2008) and Brabin *et al.*,(1991) who did not find any statistically significant difference in the mean haemoglobin concentration of the pregnant women with and those without malaria infection. On the other hand, it was at variance with Erhabor *et al.*,(2010) who reported a significantly reduced level of haemoglobin between the infected and uninfected pregnant women. The disagreement could be that this study was on asymptomatic subjects whereas the subjects in the study by Erhabor *et al.*,(2010) included pregnant women with clinical malaria. Malaria contributes to reducinghaemoglobin levels through direct haemolysis, accelerated splenic removal of red blood cells and reduced erythropoiesis. In some areas of malaria endemicity however, other factors that reduces haemoglobin concentration have been identified and these include nutritional and non-nutritional factors (Buseri *et al.*,2008).

The soluble transferrin receptor (sTfR) was significantly higher in the malaria infected pregnant women than the uninfected. It suggests that asymptomatic malaria may also be associated with increased haemolysis of red blood cells.Elevated sTfR concentration is possible in subjects who had asymptomatic or mild malariaand this occurs because of haemolysis of red blood cells (Mockenhaupt,1999; Menedez *et al.*,2000; Northrop-Clewes, 2008). Malariaand other conditionssuch as Glucose-6-phosphate dehydrogenase deficiency, sickle cells anaemia and thalassaemia that are associated with increased haemolysis of red blood cells, maylead to increased erythropoiesis. Increased erythropoiesis inturn, results in increased sTfR. (Singhal *et al.*,1993)

In this study, the concentration of SF was increased in the infected pregnant than the uninfected pregnant women although it did not show any statistical relevance. The presence of the parasite can induce chronic or mild acute phase response even in asymptomatic

subjects resulting in elevated SF concentration. Besides, the inclusion of subjects with marked level of SF (> 200 μg)that could be obtained in some disorders apart from malariagave rise to the elevated values of SF beyond that of uninfected non-pregnant women.Ordinarily, one may suggestexclusionof subjects with marked SF (> 200 μg) but that may lead to loss of valuable diagnostic data.SF could be considerably influenced by malaria or inflammatory processes. Therefore SF could not reflect the exact iron content in the store in such conditions. For the actual iron concentration in the store, it might be ideal to adjust the value of SF using a correction factor. This is in line with the opinion of (Odunukwe *et al.,* 2000) who suggested a correction formula: Serum Ferritin obtained – (0.08μg x malaria density) = Actual ferritin level μg/l. The usefulness of this formular has not been confirmed (Northrop –Clewes, 2008).

C-reactive protein (C-RP) was found to be elevated in the malaria infected pregnant than the uninfected group. This is an indication that infection/inflammation was present in the infected group and C-RP as an acute phase protein may be elicited in suchimmune response. Parasitic infection such as malaria, bacterial and viral infections or some other disorders induce immune response through various mechanisms that suppresseserythropoiesisand impairs nutritional status by reducing daily nutrient absorption and utilization.C-reactive protein (C-RP) rises sharply with the onset of immune response reaching peak concentrations within 24 – 48 hours. Inflammatory processes following tissue injury, infection, malignancy, autoimmune diseases and cardiovascular diseases can result in elevated levels of cardiovascular reactive protein (Fred and Allan, 2006; Venugopal, 2007).

With regards to age, gestational age and gravidity, (table 4.14) this study failed to find any consistency on the effect of age and gravidity oniron parameters. However, iron status was affected by gestational age. The data generated in this study followed the normal physiological pattern in which iron levels decreaseswith increasing gestational age. This is

expected as normal pregnancy progresses. Haemoglobin concentration could be high in the first trimester but reduces as pregnancy progresses to the second but tends to increase in the third trimester above the second trimester. The data clearly shows that the increased haemoglobin level in the first trimester could be as a result of reduced iron requirement since there is usually a temporary ceasation of menstruation in the first trimester. Moreover, the amount of iron transferred to the foetus is still minimal.With the second trimester, there is major expansion in the maternal red cell mass and this continues until the third trimester (Perlas *et al.,*1992). During this period, iron is transported through the placenta to the developing foetus therefore maternal functional and store iron are expected to be reduced. The slight increase in haemoglobin concentration in the third trimester is expected because of less demand for iron by the maternal-foetal placental unit and the foetus. This is necessary to accommodate delivery that would be expected soonest. Another possibility to the increased level of iron in the third trimester could be that the pregnant women may have dabbled into intake of haematinics on their personal volition.

In this study, assessment of iron status made use of values of haemoglobin, soluble transferrin receptor, serum ferritin and cardiovascular reactive protein. This is in conformity with the recommendation of Pippard and Hoffbrand, (2001) and Worwood and May, (2012) that emphasized the choice of parameters to embrace indices that would measure different iron compartments as well as evidence of infection and inflammation. Variations may occur with the result of other studies as a result of the condition of the recruited pregnant women and the inclusion criteria adopted.Whereas this study recruited asymptomatic malaria parasitized pregnant womenwho were not on iron supplements, moststudies recruited the entire pregnant women or anaemic pregnant women and even some pregnant womenon iron supplements. Variations may still occur as a result of the composition of different food or dietary intake and some cultural practices that may be observed in some areas where geophagy is practiced.

Most grain based diets may contain high phytate, and the soil with respect to those who practice geophagy, may contain some salts or Lead (Pb) that may inhibit iron absorption (Papanikolaou *et al.*,2005). Excess intake of diet saturated with copper may also result in increased levels of copper in the serum. Eventually, Iron absorption is negatively affected by elevatedlevels of copper (Tayrab *et al.*,2013). Moreover, iron deficiency or iron deficiency anaemia mayresults from inadequate intake of bioavailable iron promoters. Iron promoters enhance iron absorption. Molecules that promote iron absorption include vitamin A and ascorbic acid.

Soluble transferrin receptor is a good indicator of tissue iron deficiency and values are independent of iron stores. Values of sTfR are not arbitrarily altered in response to inflammation, circardian and diurnal rhythm unlike in SI and TIBC estimations; therefore it was adopted to differentiate between iron deficiency anaemia and anaemia of chronic inflammation or an association of both anaemias (Northrop-Clewes, 2008).

Blood glucose level was reduced in malaria infected pregnant than the uninfected pregnant women. This is because in malaria infection, glucose metabolism is affected by several factors including parasite metabolism, cytokine expression, hormonal changes, fever, drugs, fasting and gastrointestinal disturbances (Davis *et al.*,2003). Blood glucose may be increased probably due to stress-cortosol induced glyconeogenesis. However, the increased glucose levels would be grossly undermined by the increased cytokine production. Cytokines such as TNFα would mount strong counter-regulatory mechanisms that would reduce the concentration of glucose. Another reason may be that glucose is rapidly taken up across the parasite plasma membrane; being facilitated by hexose transporter in the glycolytic pathway (Woofrow *et al.*,1999).

Pregnant women infected with malaria parasite (believed toinduce acute phase response) were divided into two groups based on their cardiovascular reactive protein levels and then compared in terms of their cytokines, biochemical ironparameters and red blood cell profile. (Tables 4.17 and 4.18).In this study, IFNγ, IL-4, IL-6 and IL-10 were higher in malaria infected pregnant women with elevated C-RPthan the group that had normal C-RP and this was similar to the results obtained by (koorts *et al.,* 2011) who posited increased levels of IFNγ, TNFα, IL-4, IL-6, and IL-10 in the pregnant women with elevated C-RPthan the groups that had normal C-RP. TNFα was lower inmalaria infected pregnant women with normalC-RP while IFNγ and IL-4 were higher in malaria infected pregnant women with elevated C-RP. However, they were not significantly expressed. This may have occurred due to the small sample size of those with the elevated C-RP in comparison with size of the group with normal C-RP. But elevated values of IL-6 supports the earlier studies which places the molecule as a major cytokine responsible for inducing the hepatocytes to synthetize C-RP (Castell *et al.*,1989). Conceivably, IL-10, a T-helper cell type-2 cytokine was increased in the group with elevated C-RP. This view is in keeping with previous studies that cytokines can contribute to chronic immune stimulation (Ludwicsek *et al.*,2003; Weiss & Goodnough, 2005).

As regards iron status, the serum ferritin was increased in groups with elevated C-RP than the group with normal C-RP. It is not out of place to believe that serum ferritin as an acute phase protein is increased consequent to chronicimmune response. The group with elevated C-RP levels would be high and obviously correspondsto increased synthesis of ferritin and its retention in the macrophages. Again, the result is expected with the inclusion of 40% and 6% of the subjects with anaemia of infection and anaemia of inflammation respectively. The soluble transferrin receptor was higher and not statistically significant in the group with normal C-RP compared with those with elevated C-RP. This is in conformity with the fact

that sTfR is not affected by inflammation. Also, it would be more reasonable to accept the fact that sTfR is not increased in individuals during acute phase response because of reduced erythropoietic production and suppression of erythropoiesis by the cytokines (Spivak, 2000; Beguin, 2003).

The result of the red blood cell profile in the study was characteristic of mild anaemia found in the study subjects. Haemoglobin concentration was reduced in the group with normal C-RP than those with elevated C-RP while MCV was increased in those with elevated C-RP than the group with normal C-RP. It suggests that asymptomatic malaria is associated with lower than normal haemoglobin concentration and the anaemia typical of anaemia of infection/ inflammatory anaemia. Anaemia of infection/ inflammation often exhibits thenormocytic normochromic picture but on further development, may result in microcytic hypochromic anaemia (Pippard and Hoffbrand, 2001; Roy, 2010; Cullis, 2011).

In this study, the total leucocyte count was higher in the groups with elevated CRP than those with normal CRP. This could be explained by the fact that some cytokines, especially TNFα produced during inflammatory response acts on vascular endothelial cells and macrophages to secrete colony stimulating factors such as Macrophage-Colony Stimulating Factor (M-CSF), Granulocyte-Colony Stimulating Factor(G-CSF) and Granulocyte Macrophage/Monocyte Stimulating Factor (GM-CSF). The colony-stimulating factors are agonists involved in proliferation and development of clonogenic progenitor cells population in the bone marrow resulting in increased production of leukocytes (Venugopal, 2007; Testa and Dexter, 2001).

This study assessed the relationship between cytokines, Iron, malaria parasitaemia and acute phase protein. The data revealed positive association between the cytokines (IFNγ, TNFα, IL-4, IL-6 and IL-10)and malaria parasite density. On determining the strength of associations, it

was discovered thatIL-6 and IL-10 showed strong, positive correlation with malaria parasite count. This study has reinforced the aforementioned reports that malaria induces the secretion of cytokines (Inigo & Manuel, 2002; Clark *et al.*,2006). It is in agreement with Adeoti *et al.*,(2012) who observed a direct relationship between MP density and cytokines and with Rodrigo *et al.*,(2014) who observed a positive correlation between IL-10 and *P. vivax* and *P. falciparum*counts in infected pregnant women. Rodrigo *et al.,(*2014) also observed positive correlation between TNFα and *P.falciparum*count. The strong positive correlations between IL-6 and malaria parasite count and between IL-10 and malaria parasite count in this study agrees with Zeyrek, *et al*. (2006) who observed a positive correlation between IL-10 and the parasite count in pregnant women with *P.vivax*.It suggests that malaria parasite density could be a factor that would determinethe amount of cytokine secretion; as increased parasitaemia leads to increased secretion of cytokine. Cytokines play key roles in the course and outcome of malaria (Warren, 2010). The leukocytes in the innate and adaptive arms of the immune response are activated by malaria parasite or the parasite toxins consequent to the release of cytokines: INFγ, TNFα and to an extent IL-6 are proinflammatory cytokine that have been shown to play protective roles in the cellular immune response to the blood stage of the malaria infections (Venugopal, 2007; Warren, 2010). IL-10 plays significant role in immunoregulation and mediates negative feedback on the production of pro-inflammatory cytokines by antagonizing TH_1 response following stimulation by malaria antigen. Although an immunoregulatory cytokine, little is known about the role of IL-4 in malaria pathogenesis, however it enhances the proliferation of eosinophil and mast cells and induces class switching to IgE (Venugopal, 2007).

On the other hand, this study failed to show a direct relationship between malaria parasite density and iron status. The fact that no association existed between malaria parasitaemia and iron parameters such as MCV and sTfR and where it does exist (between Hb andSF)

itshowed weak associations. This is in agreement with Stoltzfus (1997)Odunukwe *et al.,*(2000) andAkinboro *et al.,*(2010) that reported no association between malaria parasite density and iron parameters. However, an association could exist with iron parameters such as serum ferritin when parasite density is above 1000/µl of blood (Stoltzfus, 1997; Odunukwe *et al.,*2000). This finding was at variance with Okafor *et al.,*(2012) who reported a negative correlation between MP density and Hb. The weak associations recorded between MP density and Iron parameters such as Hb and SF could be that the pregnant women are in asymptomatic conditions; therefore marked changes are not obvious.

Strong, positive correlation which existed between C-RP and malaria parasite count is an indication that increased parasitaemia is consequential to elevated C-RP. The pathway whereby increased parasitaemia leads to elevated C-RP could be explained. C-RP is a positive acute phase protein (APP) and its induction may not be a direct activity of malaria parasite on any otherorgan in the body rather, the parasite induces the hepatic synthesis ofIL-6 and other inflammatory cytokines such as INFγ and IL-10. These cytokines notably IL-6 induces the production of APP by inducing a common transcription factor, NF-IL6 in the liver. NF-IL6 has high degree of sequence homology and bind to the promoter genes encoding various liver proteins. During acute phase response, NF-IL6 increases leading to increased production of APP (Venugopal, 2007).

In addition, this study showed positive correlation between cytokines (IL-6, IL-4, IFNγ, TNFα, IL-10) and acute phase protein (C-RP). The cytokines evaluated and the acute phase protein (C-RP)] tend to move together in the same direction shows that increase in cytokine (IFNγ, TNFα, IL6 and IL-10) levelsmay lead to increased level of C-RP. This result has reinforced earlier studies that cytokines such as IFNγ, TNFα and IL-1β induces the synthesis of acute phase proteins. However, IL-6 appears to be the major inducible cytokine (Castell *et al.,*1989; Le and Vilcek, 1989; Sehgal *et al.,*1989; Heinrich *et al.,*1990).

The determination of the relationship between iron and acute phase protein, in this study showed a positive correlation between serum ferritin and C-RP. Serum ferritin is increased along with increasing values of C-RP. Serum ferritin, as an acute phase protein, increases in inflammation or immune stimulation. This finding is in agreement with (Koortz *et al.*,2011) who reported that IL-10 contributes to reducing bioavailable Iron (SF). IL-10 may contribute to hypoferremia of chronic immune stimulation by regulating the transcription and translation of ferritin and by stimulating the expression of haemoglobin scavenger receptor, CD163 (Ludwiezek *et al.*,2003; Weiss and Goodnough, 2005).

C-RP belongs to acute phase proteins (APP). It binds to phosphocholine on the membranes of microbes. Such interactions may activate the complement system which leads to the elimination of microbes thereby protecting the host from infection. APP does not directly interfere with iron metabolism nor induce anaemia,rather, they may pass through some other pathways. This is easily understood, for example, C-RP interacts with IL-6 to produce hepcidin which mops iron resulting in anaemia. While α-1-antitrypsin may block transferrin mediated iron uptake into the erythroids but not into the reticuloendothelial cells (Graziadei 1994). Moreover, it is posited that α-1-antitrypsin regulates the conversion of pro-hepcidin to hepcidin in a reaction that is mediated by furin, a prohormone convetase (Ganz, 2003). These processes perturb iron homeostasis, limits iron avaliability to the erythrons and subsequent reduction of erythropoiesis.Currently, substantial evidence abounds to the interference on iron homeostasisbeing mediated by hepcidin. Hepcidin is an acute phase protein and a 25-amino acid peptide hormone secreted by hepatocytes which circulate in the plasma. Hepcidin inhibits intestinal iron absorption, iron recycling in macrophages and release of stored iron from hepatocytes, thus decreasing body iron availability (Neimeth *et al.*,2003).

On the determination of the relationship between cytokines and iron, this study revealed negative correlation between most cytokines (IFNγ and TNFα) and haemoglobin. The

cytokines (IFNγ and TNFα) tend to move together in opposite direction with haemoglobin. This suggests that increased levels of the cytokines may interrupt iron homeostasis which in turn compromised iron metabolism specifically at the erythroid precursor cells (erythrons) (Venugopal, 2007; Kawthalkar, 2008; Hoffbrand and Moss, 2011). This results in decreased haemoglobin. It could be remembered that incorporation of iron into the haemoglobin (synthesis of haemoglobin) molecule takes place at the erythrons (Edward, 2001). Cytokines interferes with iron metabolism through various mechanisms that first and foremost effect changes in iron homeostasis. The changes involve interplay of processes that would result inreduction in the release or increased macrophage iron, hypoferremia, relative decline in erythroid activity and shortening of red cell life span. The ideal process of iron homeostasis is that iron enters the body by absorption of dietary sources, bound to transferrin and delivered primarily to the bone marrow for erythropoiesis although much of the iron required for erythropoiesis is recycled from senescent erythrocytes. Iron could be stored in macrophages and in the hepatocytes until it is required. Its removal in the body is carried out only through sloughing of enterocytes and bleeding. Therefore systemic iron balance is maintained by tight regulation of iron absorption and its release from reticuloendothelial and hepatocyte stores (Tolo, 2003) but cytokine dysregulation or up-regulation imposes untold interference in iron homeostasis. For instance, IFNɤ and LPS increase the uptake of non transferrin bound iron (NTBI). Non-transferrin bound iron induces the retention of iron within monocytes. It also down regulates the synthesis of ferroportin. TNFα, IL-1, IL-6 and IL-10 induce synthesis, retention and storage of ferritin within macrophages. Also, TNFα increases macrophage acquisition of erythrocyte iron by increasing expression of C3b receptor (Miller *et al.,* 1994; Rogers, 1996; Ludwiezek *et al.,* 2003; Weiss and Goodnough, 2005).

Also, up-regulated cytokine synthesis induces hypoferremia. Hypoferremia or decreased plasma iron occur through reduction in the hepatic synthesis of transferrin, (the major iron

transporting protein in the plasma) and increased synthesis of iron binding protein such as lactoferrin. Lactoferrin is synthesized and stored in specific granules in neutrophils (Mazza, 2002). It has greater affinity for iron than transferrin and does not transfer its bound iron to erythroid precursors rather it returns the iron to the macrophages of the reticuloendothelial system where it is incorporated into intracellular iron stores. This is because erythroids have receptors only for transferrin bound iron. So iron bound to lactoferrin is returned to macrophages not being used for the synthesis of haemoglobin (Allan, 1995). The release of lactoferrin in lieu of infection is mediated by IL-1 (Hillman and Ault 1995, Ludwiezk *et al.,* 2003), thus resulting indepressederythropoiesis.

In addition over expression of cytokines depresses erythropoiesis. Commonly affected are the erythrocyte progenitors (B-CFU, CFU-E) and the precursor cells. It is reported that IFNγ and TNF produced by cytotoxic T. cells are capable of suppressing growth, affecting mitosis and inducing apoptosis of progenitor cells. Apoptosis is explained as morphological changes associated with programmed cell death. The changes may occur due to decreased cell volume, membrane blebbing, condensation of chromatin and degradation of the DNA into fragments (Elgert, 2009). Moreover, up-regulation of these cytokines may induce the production of nitric oxide synthase, nitric acid and other free radicals by marrow cells. The direct effect of these free radicals may render the erythroid precursors ineffective or contribute to immune mediated cytotoxicity and elimination of haemopoietic cells (Brugnara, 2003; Venugopal, 2007).

Another pathway whereby over expression of cytokine may mediatedepressed erythropoiesis is by programmed death of erythrons through the formation of ceramid. Ceramids are important intermediate in the biosynthesis of sphingolipids. Accumulation of ceramids (sphingolipids) or any toxic metabolite on the cell membrane is injurious resulting in death of the erythrons (Hussain, *et al.,* 2012). Again increased cytokine and endotoxin may alter or

change the erythrocyte membrane and facilitates erythrophagocytosis with subsequent reduction of erythrocyte half life (Fillet *et al.,* 1989; Gasche *et al.,* 2004).On the other hand, spurious orno correlation which existed between most cytokines such as IL-4and iron parameters suggests that the cytokine and iron are independent of each other. In summary, elevated synthesis of acute phase proteins and/or upregulated cytokine secretion may pertub iron homeostasis which eventually compromises its metabolism. This study thushighlights the inter-relationship of cytokines, iron and malaria parasitaemia.

5.2 CONCLUSION

This study has demonstrated that pregnancy is accompanied by the production of different cytokine away from non-pregnant state. Again, the placenta either with past or present malaria infection showed alteration of cytokine profile. Moderate to marked cytokine concentration in the placenta can influence the systemic circulationbutits measurement in the pheripheral may not be reflective of the placental environment. The most common biomarkers of inflammation are cytokines and acute phase proteins. This study has also demonstrated that peripheral parasitaemia does not equally represent the level in the placenta as more parasites are attracted to the placenta bed. Correspondingly, more leukocytes migrate to the local materno-foetal interphase against the number in the peripheral blood probably to militate (against) the elevated malaria parasite density in the placenta. Furthermore, age, gestational age and gravidity have no influence in determining the level of cytokines rather parasitaemia does and to a large extent, more cytokines are secreted in moderate than mild parasitaemia. Iron status was not affected by age and gravidity but was affected by gestational age. The data generated in this study followed the normal physiological pattern in which iron concentration decreases with increasing gestational age. Malaria induces the redistribution of iron. So,in the assessment of iron status in malaria endemic areas, it would be vital to expand antenatal screening tests to include assessment of malaria parasitaemia and several iron parameters. It is also important to assess infections especially theintestinal and urogenital tract. Pregnant women with anaemia of infection in which iron supplementation might not be of immense benefit (WHO, 2004)could be treated for infection before iron administration. A remarkable finding in this study is that some pregnant women had marked values of iron store.Such conditioncould be borne in mind in administration of Iron intervention.Essentially, this study aimed at assessing the relationship between cytokines, iron, acute phase proteins and malaria parasiteaemia revealed strong positive correlation betweenmalaria parasitaemia,most cytokinesand acute phase proteins. Parasitaemia is

consequential to elevated levels of cytokines.It showed that apart from direct haemolysis of red cells which reduces haemoglobin concentration, malaria parasitaemia indirectly interruptiron metabolismbyelevated levels of cytokines and acute phase proteins.So, differences in levels of cytokineand/or acute phase proteins may be related to normal conditions but over-expressioncould be delecterious.Over-expression of cytokines and acute phase proteins may perturb iron homeostasis and compromise iron metabolism and significantly impairsthe immune response.This appears to be the hallmark for most adverse conditions in pregnant and post partum women.Findings of this study may serve as background information for the proper management of malaria infected pregnant women.

5.3 RECOMMENDATIONS

1. Greater number of pregnant women habour malaria more in the placenta than the peripheral blood. Obtertricians and Gynaecologist's could commenceantenatal care and interventionsearly to checkmate its effect in time.

2. Evaluation of levels of iron at various compartments and at different trimesters would be better than relying solely on Hb concentration. This would afford the clinicians a better understanding of iron for the management of pregnant women.

3. Anaemia due to malaria could be regarded as anaemia of infection. It could be ideal to expand assessment of other infections especially the gastrointestinal and urogenital tracts.

4. Due to the possible occurance of infection/inflammatory anaemias, selective prophylactic iron supplementation should be a better approach for the management of pregnant and post-partum women.

5. The number of HIV infected subjects that were excluded because they were not part of the study should not be treated with levity and calls for a more concerted effort to prevent HIV infection in the environment.

5.4 LIMITATIONS

This study was not done without some limitations.

1. The scope of this study did not include genetic factors, screening of bacterial isolates and gastrointestinal parasitic infections that may contribute to other sources of immune stimulation.

2. The study did not take note of the dietary intake of the subjects so as to detemine the influence of dietary iron.

3. The huge amount of moneyexpended at procuring the cytokine kits and efforts invested to sustain the kits and samples on storage to avoid deterioration and contamination, in a society where frequent power outage is an acceptable phenomenon, did not pave way for inclusion of more cytokines in the study.

CONTRIBUTION TO KNOWLEGDE

The malaria parasite density is higher in the placenta than the peripheral blood of pregnant women. It may induce up-regulated levels of cytokines and acute phase proteins. These may compromise iron metabolism resulting in most adverse conditions in pregnant and post-partum women.

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APPENDIX IA

ABIA STATE UNIVERSITY TEACHING HOSPITAL

P.M.B. 7004 ABA

Chairman Board of Management
DR. KINGSLEY N. ENWEREMADU
M.B.B.S. MNIBE

Director of Administration/Secretary to the Board
DAME ROSELINE C. SHIRIMA (JP)
DIP.(INS) Bsc. (Bus Admin), MBA (MGT), AIHSAN, CIPA.
Tel: 082-232777

Chief Medical Director
DR. UWA U. IWEHA
BM; BCH, (U. NIG.) FRCS (Ed); FICS.
Tel: 082-232776

Chairman Medical Advisory Committee
DR. CHUKWUEMEKA ONYEARUGHA
M.B.B.S. (Ibadan) FMC Paed.

Your Ref: _____

Our Ref: _____

Date: 28th January, 2015.

ETHICS & RESEARCH COMMIITTEE APPROVAL

Okamgba Okezie Caleb
Department of Medical Laboratory Sciences,
College of Health Science and Technology,
Nnamdi Azikiwe University, Nnewi Campus,
Anambra State.

Dear Sir,

I would like to notify you of the decision of the ABSUTH Ethics and Research Committee on your proposal.

FULL TITLE OF PROPOSAL	Cytokine Expression and iron Status of Pregnant and Post Partum Women with Malaria Parasitemia in Aba, South Eastern Nigeria.
PRINCIPAL INVESTIGATOR	Okamgba Okezie Caleb

The Ethics and research Committee has approved of the conduct of the above research at ABSUTH. The research methodology does not pose a major ethical risk to the participants. But we would like you to obtain an informed consent from the participants as well as ensure confidentiality of their records.

Thank you.

Yours sincerely,

DR. CHIGBU B. MD, FICS
Chairman, Ethics and Research Committee

APPENDIX IB

LIVING WORD MISSION HOSPITAL LTD

Medical arm of Living Word Ministries Inc.

082290950, 082290951

5-7, Umuocham Road Abayi P. O. Box 1453, Aba, ; 119, Ikot Ekpene Road Ogbor Hill Aba, Tel: 082-848061
E-mail:livingwordmissionhospital@yahoo.com

26th March, 2015.

Okamgba Okezie Caleb
Department of Medical Laboratory Sciences
College of Health Science and Technology
Nnamdi Azikiwe University, Nnewi Campus,
Anambra State.

Dear Sir,

Research Approval

I wish to notify you of the approval of the management committee of Living Word Mission Hospital for the Research on Cytokine Expression and Iron Status of Pregnant and Post Partum Women with Malaria Parasitemia in Aba, South Eastern Nigeria.

The research methodology does not pose any health risk to our patient though we insist you obtain informed consent from our patients for such research. We also hope that utmost confidentiality of the patient will be maintained.

Wishing you the best.

Yours Faithfully,

Dr J.O. Ehiemere
Medical Director

APPENDIX II

QUESTIONNAIRE AND CONFIDENTIALITY FORM

DEPARTMENT OF MEDICAL LABORATORY SCIENCE

NNAMDI AZIKIWE UNIVERSITY, NNEWI CAMPUS

Date: …….

The content of the questionnaire and confidentiality form will be read and explained both in English and Igbo languages for proper interpretation and understanding of the documentation. To this level, the questionnaire and confidentiality form uses the laboratory code and numbers instead of subject's names. Residential address and correspondences of the subjects are not required.

Also, unlinked anonymous testing will be used in the study. After sample collection, all information that could identify the source of the blood will be eliminated from the sample containers.

PLEASE GIVE THE INFORMATION REQUIRED CORRECTLY

LAB NO: …….. PATIENT'S CODE: …….

SECTION A: ANTHROPOMETRIC DATA

AGE ……

MARITAL STATUS: SINGLE/ MARRIED

LEVEL OF EDUCATION: PRIMARY SCHOOL

SECONDARY SCHOOL

POST SCONDARY SCHOOL

ARE YOU RESIDENT IN ABA? YES/NO

HOW LONG HAVE YOU BEEN RESIDING IN ABA? .....

SPOUSE OCCUPATION:…….

SECTION B: MEDICAL HISTORY

ARE YOU HYPERTENSIVE? YES/ NO

HAVE YOU SUFFERED ANY HEART DISEASE BEFORE? YES/NO

ARE YOU DIABETIC? YES/NO

HAVE YOU SUFFERED ANY KIDNEY DISEASE BEFORE? YES/NO

HAVE YOU BEEN TREATED FOR URINARY TRACT INFECTION IN

THE PAST ONE YEAR? YES/NO

ARE YOU SUFFERING FOR ANY LIVER DISEASE?

HAVE YOU RECEIVED ANY BLOOD TRANSFUSION YES/NO

WHEN LAST WERE YOU TESTED FOR HIV?

CURRENTLY, WHAT IS THE RESULT OF YOUR HIV TEST?

POSITIVE/NEGATIVE

DO YOU HAVE TUBERCULOSIS? YES/NO

DO YOU SUFFER FROM ULCER /PILE? YES/NO

WHEN LAST DID YOU TAKE ANTI-HELMITHIC DRUGS?

DO YOU HAVE CANCER? YES/NO

HAVE YOU HAD CEASERIA SECTION BEFORE? YES/NO

HAVE YOU HAD PRE-TERM BIRTH BEFORE? YES/NO

HAVE YOU EVER HAD LOW BIRTH WIEGHT BABY BEFORE?

SECTION C: STUDY SURROGATE DATA

DO YOU SLEEP UNDER MOSQUITO NET? YES/NO

DO YOU APPLY MOSQUITO INSECTICIDE IN YOUR APARTMENT? YES/NO

HOW OFTEN ARE YOU BITTEN BY MOSQUITO? ……..

WHEN LAST WERE YOU TREATED FOR MALARIA? ……

CURRENTLY, ARE YOU ON ANTIMALARIA DRUGS? YES/NO

WHICH TYPE ARE YOU TAKING? …….

IS THIS YOUR FIRST ANC ENROLMENT IN THIS PREGNANCY? YES/NO

CAN YOU REMEMBER YOUR LAST MENSTRAL PERIOD? YES/NO

IF YES, INDICATE THE LAST MENSTRUAL PERIOD ……

WHAT IS THE GESTATIONAL AGE OF THIS PREGNANCY?

DO YOU HAVE A SCAN RESULT? YES/NO

IS THIS YOUR FIRST PREGNANCY? YES/NO

HOW MANY CHILDREN DO YOU HAVE? …….

HAVE YOU EXPRIENCED ANY SPOTTING OR DROPPING OF BLOOD IN THE COURSE OF THIS PREGNANCY? ……..

ARE YOU ON IRON DRUGS? YES/NO

WHEN LAST DID YOU TAKE IRON DRUGS/SUPPLEMENT? ……..

DID YOU ATTEND ANY TRADITIONAL MATERNAL BIRTH HOME OR EMBARKED ON MEDICATION ELSEWHERE BEFORE COMING TO ANTENATAL CARE AT ABSUTH? ……

APPENDIX IIIa

TABLE 4.4: COMPARISON OF THE EFFECT OF AGE ON CYTOKINE LEVELS OF MALARIA INFECTED PREGNANT WOMEN

AGE GROUPS	IFN-γ (pg/ml)	TNF-α (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
G1 (n = 14)	21.00 ± 10.25	19.49 ± 7.99	6.94 ± 4.38	32.64 ± 29.43	17.55 ± 7.73
G2 (n = 47)	22.83 ± 13.80	19.19 ± 10.69	8.46 ± 5.53	29.60 ± 23.40	34.26 ± 26.99
G3 (n = 60)	22.94 ± 12.63	21.38 ± 13.89	10.94 ± 6.56	33.36 ± 29.83	40.74 ± 31.46
G4 (n = 14)	26.28 ± 13.86	23.16 ± 12.89	10.77 ± 13.03	26.33 ± 24.15	30.89 ± 29.82
G5 (n = 9)	21.37 ± 10.33	28.84 ± 16.37	9.96 ± 7.33	45.12 ± 39.70	37.10 ± 31.65
F(p) Value	0.351 (.843)	1.295 (.275)	1.469 (.215)	0.760 (.553)	2.024 (.094)
G1 vs G2	0.982	1.000	0.818	0.996	0.003*
G1 vs G3	0.972	0.959	0.067	1.000	0.000*
G1 vs G4	0.791	0.852	0.831	0.971	0.508
G1 vs G5	1.000	0.530	0.796	0.923	0.421
G2 vs G3	1.000	0.887	0.218	0.949	0.782
G2 vs G4	0.923	0.829	0.964	0.991	0.995
G2 vs G5	0.996	0.477	0.975	0.785	0.999
G3 vs G4	0.920	0.990	1.000	0.880	0.804
G3 vs G5	0.993	0.699	0.995	0.907	0.997
G4 vs G5	0.865	0.899	1.000	0.710	0.989

Key
α-level set at 0.05
*(P< 0.05) = Significant
P> 0.05= Not Significant
G1 = Age group 20 – 24 years
G2 = Age group 25 – 29 years
G3 = Age group 30 – 34 years
G4 = Age group 35 – 39 years
G5 = Age group 40 – 44 years

APPENDIX IIIb

TABLE 4.5: COMPARISON OF THE EFFECT OF AGE ON CYTOKINE LEVELS OF MALARIA UNINFECTED PREGNANT WOMEN

AGE GROUPS	IFN-γ (pg/ml)	TNF-α (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
G1 (n = 7)	4.67 ± 3.41	7.39 ± 2.12	9.30 ± 1.87	11.49 ± 2.94	13.79 ± 6.19
G2 (n = 15)	5.92 ± 2.76	9.75 ± 2.36	5.90 ± 3.00	7.18 ± 5.27	15.88 ± 8.14
G3 (n = 29)	5.70 ± 3.11	10.75 ±3.39	7.79± 4.37	10.10 ±11.07	15.61 ± 5.84
G4 (n = 8)	7.40 ± 3.54	10.33 ±2.53	5.65 ± 3.95	4.44 ± 3.87	11.19 ± 1.95
G5 (n = 3)	8.33 ± 2.12	9.90 ± 3.61	6.63 ± 5.35	7.40 ± 4.59	12.77 ± 3.35
F(p) Value	1.234 (.307)	1.884 (.126)	1.452 (.229)	1.049 (.390)	1.058 (.386)
G1 vs G2	0.909	0.190	0.032*	0.144	0.961
G1 vs G3	0.945	0.035*	0.631	0.975	0.950
G1 vs G4	0.570	0.164	0.210	0.011*	0.818
G1 vs G5	0.339	0.790	0.899	0.660	0.997
G2 vs G3	0.999	0.787	0.454	0.761	1.000
G2 vs G4	0.838	0.983	1.000	0.622	0.256
G2 vs G5	0.526	1.000	0.999	1.000	0.807
G3 vs G4	0.733	0.995	0.683	0.173	0.013*
G3 vs G5	0.448	0.992	0.994	0.919	0.718
G4 vs G5	0.981	1.000	0.998	0.846	0.923

Key
α-level set at 0.05
*(P< 0.05) = Significant
P> 0.05= Not Significant
G1 = Age group 20 – 24 years
G2 = Age group 25 – 29 years
G3 = Age group 30 – 34 years
G4 = Age group 35 – 39 years
G5 = Age group 40 – 44 years

APPENDIX IIIc

TABLE 4.8: COMPARISON OF THE EFFECT OF GRAVIDITY ON CYTOKINE LEVELS OF MALARIA INFECTED PREGNANT WOMEN

GROUPS	IFNγ (pg/ml)	TNFα (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
G1 (n=51)	21.39±12.15	18.81±10.80	9.39±8.35	31.96±29.76	28.58±25.14
G2 (n=34)	21.85±12.70	20.24±12.45	9.70±6.20	30.79±25.85	34.82±28.49
G3 (n=21)	24.92±13.14	23.62±13.75	9.88±6.37	33.01±27.87	41.11±31.09
F(p) Value	1.224(0.297)	2.150(0.120)	0.066(0.937)	0.068(0.934)	2.652(0.074)
G1 vs G2	0.985	0.849	0.980	0.980	0.557
G1 vs G3	0.313	0.105	0.938	0.980	0.055
G2 vs G3	0.512	0.448	0.990	0.921	0.584

KEY

α-level set at 0.05
*(P<0.05) = Significant
P>0.05 = Not significant
G1 =PRIMIGRAVIDAE
G2=SECONDIGRAVIDAE
G3=MULTIGRAVIDAE

APPENDIX IIId

TABLE 4.14: COMPARISON OF THE EFFECT OF AGE ON IRON STATUS OF MALARIA INFECTED PREGNANT WOMEN

AGE GROUPS	HB (g/L)	sTfRA (nmol/L)	SF (μg/L)
G1(n = 14)	102.43 ± 11.55	61.279±20.15	35.471±46.26
G2(n = 47)	99.73 ± 9.41	65.701±19.81	58.792±136.47
G3(n = 60)	104.81 ± 12.86	60.255±21.82	38.203±40.90
G4(n = 14)	104.50 ± 9.30	61.207±29.18	25.364±13.23
G5(n = 9)	101.89 ± 7.20	69.144±25.71	41.467±60.37
F(p) Value	0.417(.796)	0.623 (.647)	0.624 (.646)
G1 vs G2	0.924	0.949	0.856
G1 vs G3	0.961	1.000	1.000
G1 vs G4	0.984	1.000	0.931
G1 vs G5	1.000	0.933	0.999
G2 vs G3	0.147	0.661	0.854
G2 vs G4	0.450	0.982	0.472
G2 vs G5	0.927	0.995	0.972
G3 vs G4	1.000	1.000	0.268
G3 vs G5	0.862	0.856	1.000
G4 vs G5	0.940	0.957	0.927

KEY

α-level set at 0.05

*(P<0.05) = Significant

P>0.05 = Not Significant

G1 = Age group 20 – 24 years
G2 = Age group 25 – 29 years
G3 = Age group 30 – 34 years
G4 = Age group 35 – 39 years
G5 = Age group 40 – 44 years

APPENDIX IIIe

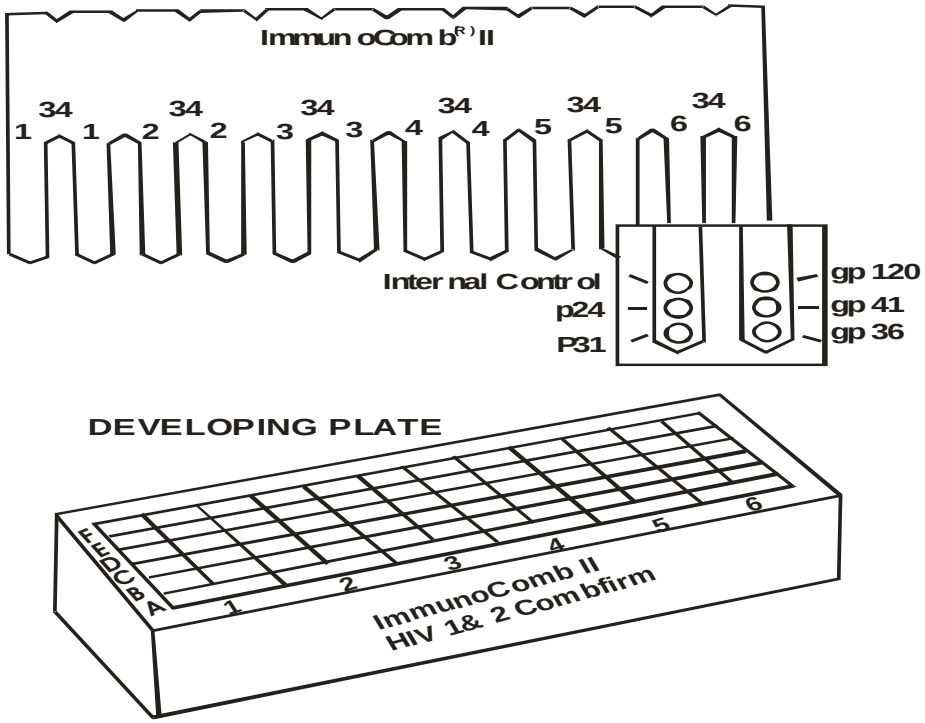
TABLE 4.16: EFFECT OF GRAVIDITY ON IRON STATUS OF MALARIA INFECTED PREGNANT WOMEN

AGE GROUPS	HB (g/L)	sTfRA (nmol/L)	SF (μg/L)
G1 (n = 51)	103.78 ± 12.13	62.35 ±18.75	47.98±106.73
G2 (n = 34)	102.09 ± 11.17	63.32 ± 22.86	25.92±18.66
G3 (n = 59)	101.44 ± 9.84	62.84 ±24.27	50.03± 86.87
F(p) Value	.641 (.528)	.020 (.980)	.973 (.381)
G1 vs G2	0.786	0.977	0.326
G1 vs G3	0.515	0.992	0.998
G2 vs G3	0.957	0.995	0.108

Key
α-level set at 0.05
*(P<0.05) = Significant
P>0.05 = Not Significant
G1 = PRIMIGRAVIDAE
G2 =SECONDIGRAVIDAE
G3 =MULTIGRAVIDAE

Appendix IV

Showing the Immunocomb confirmatory kit for HIV I & II



Appendix V

Diagram showing the working mechanism of the Immunocomb kit for HIV I & II